Journal of Advances in VetBio Science and Techniques

E-ISSN: 2548 - 1150 - Period: Tri-annual

VET BIO

J Adv VetBio Sci Tech – Volume 7(2) – August 2022

JOURNAL OF ADVANCES IN VETBIO SCIENCE AND TECHNIQUES

e-ISSN 2548-1150 | **Period** Tri-annual | **Founded:** 2016 | *J adv* ***VetBio*** *Sci Tech*

Year 2022 | **Volume 7** | **Issue 2**

EDITORIAL ARCHIVE

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VetBio 2022, 7(2)

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JOURNAL OF ADVANCES IN VETBIO SCIENCE AND TECHNIQUES

e-ISSN 2548-1150 | **Period** Tri-annual | **Founded:** 2016

Investigation of the effectiveness of ultrasonography in determining pregnancy and the number of fetuses on the 35th day of pregnancy in Awassi sheep

Research Article

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ABSTRACT

In this study, it was aimed to determine the effectiveness of transabdominal ultrasonographic imaging on the 35th day of pregnancy in determining the pregnancy status and number of fetuses in sheep. 69 Awassi sheep were used in the study. Sheep were mated after oestrus synchronization during the breeding season. On the 35th and 50th days after mating, a pregnancy examination was performed twice transabdominally in each animal by ultrasonography. Sheep with single/multiple offspring were noted according to examination performing on day 35 and lambing record. According to ultrasonography results, early foetal death rate was determined as 6.1%. Sensitivity, specificity, positive predictive value, negative predictive value ratios for pregnancy examination findings and single/multiple offspring data by transabdominal ultrasonography on day 35 were determined as 91.30%, 100%, 100%, 83.3% and 38.46%, 81.25%, 76.92%, 44.82%, respectively. While the consistency of transabdominal ultrasonographic imaging for pregnancy on day 35 and 50 were high agreement (Kappa=0.864, p<0,001), consistency of single/multiple pregnancy findings on day 35 and at birth were low (Kappa=0,170, p>0.05). The rates of transabdominal ultrasonographic examination on day 35 for correct diagnosis of pregnancy status and the number of fetuses were found as 93.93% and 54.76%, respectively. Finally, it can be concluded that transabdominal ultrasonographic examination on day 35 for early pregnancy diagnose in sheep is highly effective in determination of pregnant sheep, and recurrent examination on days 35 and 50 may be useful for detecting of early foetal deaths. In order to determine the number of fetuses, repeated examinations should be performed in the following days of pregnancy.

Keywords: Early fetal death, early pregnancy diagnosis, fetal number, sheep, transabdominal ultrasound

INTRODUCTION

Sheep show a seasonal reproductive activity, so losing the opportunity to breed could mean losing a whole productive year. Diagnosing pregnancy at an early stage is of great significance in sheep production (Ganaie et al., 2009). If pregnancy cannot be detected early, economic losses may occur in milk and lamb production due to longer lambing intervals (Ishwar, 1995). The widespread use of controlled breeding techniques, in-season/off-season synchronization, and artificial insemination increases the need for accurate and practical tests to diagnose pregnancy at an early stage (Ganaie et al., 2009). Reproductive and production losses in the form of abortion, stillbirth, or weak lambs at birth can be reduced by dividing the herd into pregnant and non-pregnant groups. Accurately knowing the time of pregnancy can be helpful for ceasing to milk lactating females at the appropriate time and for close follow-up of late-pregnant females.

How to cite this article
Tekin, TC., Köse AM. (2022). Investigation of the effectiveness of ultrasonography in determining pregnancy and the number of fetuses on the 35th day of pregnancy in Awassi sheep. *Journal of Advances in VetBio Science and Techniques, 7*(2), 143-152.
https://doi.org/10.31797/vetbio.1091526

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Article info
Submission: 22-03-2022
Accepted: 26-06-2022
Online First: 04-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

Another benefit to early pregnancy detecting is the determination of the number of fetuses in each sheep, an essential information for sheep herds. Because this allows nutritional management to target the different needs of each sheep group. This way, the herd's management can be planned, and reproductive losses can be minimized (Crilly et al., 2017; Mali et al., 2022).

Numerous methods have been used to diagnose pregnancy in sheep. These methods include some less practical ones like not returning to oestrus, abdominal palpation, palpation of the caudal uterine artery, laparotomy, peritonoscopy, vaginal biopsy, and vaginal smear, and some rather practical methods like radiography, hormonal tests, pregnancy protein tests, and ultrasonography (Karen et al., 2001; Singh et al., 2004). In small ruminants, ultrasonography is a safe, fast, accurate, cost-effective, and practical method that can be used to detect pregnancies at an early stage (Crilly et al., 2017). It also provides other advantages like determining the number of fetuses, fetal age and sex, fetal deaths, and monitoring fetal development (Gürler and Kaymaz, 2011, Alkan et al., 2020).

Early detection of pregnancy by ultrasonography gives breeders, clinicians, and researchers the necessary information to improve prenatal care, improve the condition of postnatal lambs, and increases production efficiency in small ruminants (Jyothi et al., 2020). Ultrasonography can be used transrectally or transabdominally with high sensitivity. Although, in practice, transabdominal scanning is less painful for animals, easier and faster for practitioners, and provides a very wide field of view, so it is preferred more (Crilly et al., 2017). For the transrectal approach, the ideal time is 25-30 days; this method also requires more technical equipment and may cause rectal damage and embryonic death (Erdem et al., 2008; Gürler and Kaymaz, 2011). Lone et al. (2016) report that real-time transabdominal ultrasound systems in sheep are reliable for determining pregnancy and numbers of fetuses 50 days after mating. The sensitivity of ultrasound increases as pregnancy progresses. Jones et al. (2016) found that the transabdominal method had a sensitivity of 40% on the 21st day of pregnancy and 100% on the 39th day. In most cases, sensitivity increases from the 40th day of pregnancy, when the uterus becomes intra-abdominal. Hence, the ideal time for diagnosing pregnancy by the transabdominal method is the 40-75th days (Fthenakis et al., 2012; Mali et al., 2022; Lone et al., 2016).

In the present study, we aimed to evaluate the level of agreement between transabdominal ultrasonographic examination on the 35th day of pregnancy and on the 50th day of pregnancy, the latter being more accurate, for diagnosing early pregnancy in sheep. We also tried to examine the level of agreement between single/multiple fetuses detected at the early stage (35th day) and lambing records.

MATERIAL and METHOD

The animal used in the study consisted of 69 Awassi sheep (45-50 kg b.w.) and 10 Awassi ram (60-70 kg b.w.), healthy and fertile, between the ages of 3-5 and in an animal breeder in Altınozu district of Hatay province. While the animals were taken to herding in fields with dry grass and pasture in the morning, were supplemented with a ration prepared with a mixture of barley grain and straw in the evening. For the drinking water to the animals, clean and fresh water was supplied.

In the study oestrus synchronization was performed to the sheep during the breeding season. Cylindrical polyurethane sponge (Esponjavet, HIPRA) containing 60 mg of Medroxyprogesterone acetate (MPA) was applied intravaginally to all sheep. Sponges were kept in the vagina for 10 days. 125 µg d-

cloprostenol (Gestavet Prost, HIPRA) and 400 IU Pregnant Mare Serum Gonadotropin (PMSG; Oviser 500, HIPRA) were administered intramuscularly to all sheep on the day of sponge removal. At 24 hours following sponge removal, the rams joined the flock and kept with the sheep for 2 hours in the morning and evening, twice a day. The oestrus of the sheep was observed for three days and sheep in oestrus were naturally mated. Rams were left from herd following the mating.

The first examinations for pregnancy were performed 35 days after mating and the second examinations 50 days after mating. All pregnancy examinations were performed transabdominally (tUSG) using a 5 MHz convex probe real-time ultrasound device (Falco, Pie Medical, Netherlands). For the examination area, we chose the hairless region above the udder, ventral to the right fossa paralumbalis when the animals stand and on ventrodorsal recumbency (Dinç et al., 1994). Pregnancy results were accepted as positive when fluid-filled uterus, placentome, fetus with movement, and heartbeat were observed all together (Figure 1). And, sheep with lack of these appearances on tUSG were accepted as non-pregnant. Besides, the diagnosis of fetal losses were also made by determining pregnancy at the first diagnosis and then non-pregnancy at the next diagnosis by tUSG. (Ridler et al., 2015). We recorded animals with single/multiple offspring on examination on the 35th day. All ultrasonographic examinations were performed by the same experienced veterinarian. We also recorded the animals that gave birth to single/multiple lambs.

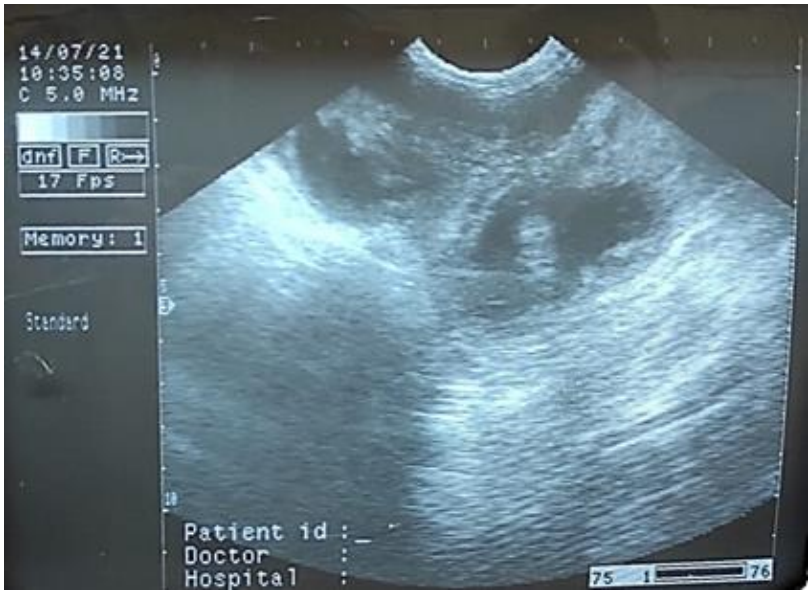

Figure 1. Ultrasonographic image of pregnancy viewed by using a 5 mHz transabdominal probe on day 35 in a sheep.

The fertility parameters were calculated according to the formulae below.

Oestrus rate: (Number of sheep in oestrus/Number of sheep undergoing oestrus synchronization) x 100

Conception rate: (Number of pregnant sheep/Number of mating sheep) x 100

Pregnancy rate: (Number of pregnant sheep/Number of sheep in the group) x 100

Lambing Rate: (Number of lambing sheep/Number of pregnant sheep) x 100

In the study, ultrasonographic examination findings on the 50th day were accepted as reference for the confirmation of the pregnancy determined on the 35th day transabdominal ultrasonographic imaging. And, lambing records were accepted as reference for confirmation of single/multiple pregnancy data determined on 35th day transabdominal

ultrasonographic imaging. To analyze the validity and reliability of pregnancy examination findings and single/multiple offspring data by transabdominal ultrasonography on the 35th day, we determined the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy rate (Kastelic, 2006; Kaya et al., 2017).

We performed a Kappa analysis to determine the level of agreement between transabdominal ultrasonography findings on the 35th and 50th days after mating, and the level of agreement between single/multiple offspring data on the 35th day and the lambing records (excluding the 4 sheep with false negative results). The level of agreement for the Kappa coefficients were classified as minimal (≤ 0.20), weak (0.21-0.40), moderate (0.41-0.60), strong (0.61-0.80), or perfect (0.81-1.00) (Altman, 1991). We used the SPSS 23.0 software for statistical analysis. For all statistical analyses, $p<0.05$ was considered significant.

RESULTS

We observed oestrus was in 58 sheep, with an oestrus rate of 84.05%. According to transabdominal ultrasonography examinations, the conception and pregnancy rates were 77.5% and 65.21% on the 35th day and 79.3% and 66.7% on the 50th day, respectively (Table 1). The lambing rate for the whole sample was 100%.

Table 1. Fertility parameters

Oestrus rate	*Conception rate*		*Pregnancy rate*		*Lambing rate*	*Early fetal death rate*
	tUSG on Day 35	**tUSG on Day 50**	**tUSG on Day 35**	**tUSG on Day 50**		
84.05% (58/69)	77.5% (45/58)	79.3% (46/58)	65.2% (45/69)	66.7% (46/69)	100% (46/46)	6.1% (3/49)

Repeated ultrasonographic examinations (on the 35th and 50th days after mating) revealed early fetal death in 3 animals. The early fetal death rate was 6.1% (3/49). These 3 animals were excluded from the Kappa analysis for the transabdominal pregnancy examination findings on the 35th and 50th days.

The transabdominal pregnancy examination findings on the 35th and 50th days were found to be perfectly fit (Kappa=0.864, $p<0.001$) (Table 2). Table 2 shows the validity and reliability of transabdominal ultrasonography findings on the 35th day.

Table 2. Compatibility of tUSG results on days 35 and 50 and validity and reliability of tUSG results on day 35

Pregnancy Status	*tUSG on Day 35 and 50*		*Total*
	available	**unavailable**	
Positive	42 (TP)	0 (FP)	42
Negative	4 (FN)	20 (TN)	24
Total	46	20	66
Sensitivity	TP/(TP+FN)x100		91.30%
Specificity	TN/(FP+TN)x100		100%
Positive Predictive Value	TP/(TP+FP)x100		100%
Negative Predictive Value	TN/(FN+TN)x100		83.3%
Accuracy ratio	(TP+TN)/(TP+FP+FN+TN)x100		93.3%

Kappa=0.864; Compatibility between the two conditions = EXCELLENT True Positive (TP): Pregnant according to ultrasound examination both on day 30 and on day 50, False Positive (FP): Pregnant according to ultrasound examination on day 35, but not on day 50, False Negative (FN): Pregnant according to ultrasound examination on day 50, but not on day 35, True Negative (TN): Not pregnant according to ultrasound examination both on day 35 and on day 50.

The single/multiple offspring findings on the 35th day had minimal agreement with the lambing records (Kappa=0.170, p>0.05) (Table 3). Table 3 shows the validity and reliability of day 35 single/multiple pregnancy findings.

Table 3. Compatibility of single/multiple pregnancy findings of tUSG on day 35 with lambing records and validity and reliability of day 35 single/multiple pregnancy findings

Offspring status	*tUSG on Day 35 and lambing records*		*Total*
	single	**multiple**	
Single	10 (TS)	3 (FS)	13
Multiple	16 (FM)	13 (TM)	29
Total	26	16	42
Sensitivity	TS/(TS+FM)x100		38.46%
Specificity	TM/(FS+TM)x100		81.25%
Positive Predictive Value	TS/(TS+FS)x100		76.92%
Negative Predictive Value	TM/(FM+TM)x100		44.82%
Accuracy ratio	(TS+TM)/(TS+FS+FM+TM)x100		54.76%

Kappa=0,170; Compatibility between the two conditions = POOR. True Single (TS): Single according to both ultrasound examination on day 35 and lambing records, False Single (FS): Single according to ultrasound examination on day 35, but multiple in lambing records, False Multiple (FM): Multiple according to ultrasound examination on day 35, but single in lambing records, True Multiple (TM): Multiple according to both ultrasound examination on day 35 and in lambing records.

DISCUSSION

In sheep, progesterone or prostaglandins have been used most commonly for oestrus synchronization. Gonadotropins (eCG and GnRH) are often used to increase the efficiency of P4-based protocols (Hameed et al., 2021). In small ruminants, oestrus occurs 24-48 hours after removing the intravaginal sponges for oestrus synchronization (Uçar and Özyurtlu, 2015). In sheep, the conception rates for progesterone applications are reported to be around 70-80% (Gordon, 1997). Synchronization studies using different doses of MAP+eCG report pregnancy rates between 20% and 100% in sheep (Hameed et al., 2021). In the present study, consistent with the literature, the oestrus rate was 84.05%, the conception and pregnancy rates were 77.5% and 65.21% on the 35th day and 79.3% and 66.7% on the 50th day, respectively (Table 1). Awassi ewes bred during June and July reported 60.7% pregnancy rate, 90% lambing rate and 1.08 fecundity (Talafha and Ababneh, 2011).

The method for diagnosing pregnancy in small ruminants depends on equipment availability, costs, number of days since mating, desired accuracy, and the examiner's experience (Singh et al., 2004). B-mode real-time diagnostic ultrasonography is a useful management tool for reproduction and has been used successfully to detect pregnancy (Karadaev, 2015; Mali et al., 2022). In small ruminants, early diagnosis of pregnancy is possible by detecting fetal heartbeat and uterine characteristics, which indicate implantation and fetal viability (Garcia et al., 1993). Due to the initial position of the uterus and the proximity of the ultrasound probe to the uterine wall, the transrectal method is suitable for early screening before the 30th day (Amer, 2008). As the pregnancy progresses and the uterus moves towards the abdominal wall and into the limits of transabdominal ultrasound imaging, the transabdominal approach becomes more suitable for diagnosing pregnancy (Jones et al., 2016; Kandiel et al., 2015).

In sheep, placentomes develop from the 33rd day of pregnancy (Crilly et al., 2017) and placentome units become viewable transabdominally or transrectally from the

32nd-33rd days of pregnancy. This initially presents as irregular shapes on the uterine wall and then matures into hollow hemispheric structures after the 39th-40th days (da Silva et al., 2018; Jones and Reed, 2017). The transabdominal approach can be used to detect fetal heartbeat between the 27th-30th days in both goats and sheep (Amer, 2008; Amer, 2010; Karen et al., 2009). The limb buds extending from the fetal body become observable from the 35th day (Jones et al., 2016). In the current study, pregnancy findings were evaluated as positive when fluid-filled uterus, placentome, fetal movement, and heartbeat were observed. In our research, we detected pregnant sheep with an accuracy of 93.93% by the transabdominal approach on the 35th day (Table 2). According to the transabdominal ultrasonography findings on the 50th day, the number of false positives was 0 and the number of false negatives was 4. The transabdominal examination findings on the 50th had a perfect agreement (Kappa=0.864, Table 2). Goel and Agrawal (1992) stated that the ultrasound scanner (B-mode) has been used successfully in sheep and the abdominal (5 MHZ) or rectal (7 MHZ) probe is ideal for sheep and goats. Garcia et al. (1993) suggested that while early pregnancy status could be determined at any time after the 30th day using the transabdominal technique with linear-array 5 MHz transducer, these procedures are more accurate when performed between the 40th-80th days. Goel and Agrawal (1992) found that images of uterine fluids, placentomes, and fetuses were evidence of pregnancy with 90% accuracy on the 45th-50th days in sheep. Çelik et al. (2019) determined pregnant sheep with an accuracy of 84% on the 50th day by using ultrasonography device with 3.5 MHz linear transabdominal probe. Küplülü et al. (2002) detected pregnant sheep with an accuracy of 89.4% on the 32nd day after insemination by using 5 MHz transabdominal rectal probe.

Ganaie et al. (2009) reported that using a real-time ultrasound scanner equipped with a 3.5MHz sector array transducer had an accuracy of 68% on the 15th-30th days, which later increased to 100% on the 61st-75th days and remained constant until lambing. Aziz and Lazim (2012) used a real-time ultrasound scanner equipped with a 3.5 MHz convex probe to detect pregnancy in Awassi sheep and reported accuracy rates of 53%, 80%, and 100% on the 21st-24th, 28th-32nd, and 40th-43rd days after insemination, respectively. Navarrete et al. (2021) reported that with transabdominal ultrasonography at 3.5 MHz, pregnancy can be detected with 100% accuracy at day 31 and the embryonic vesicle depth can be used to predict fetal age. Anwar et al. (2008) stated that on the 42nd day of pregnancy in Balkhi sheep, 3.5 MHz transabdominal probe ultrasound with 100% accuracy. Georgel et al (2021) stated that they detected pregnancy with an accuracy of 45% on the 25th day and the accuracy increased to 100% on the 31st day at a transducer frequency of 3.5 MHz transabdominal ultrasound.

Roberts et al (2019) pregnant sheep on the 30th day using a 3.5-5 MHz probe transabdominal ultrasound detected that sensitivity, specificity, positive predictive value, negative predictive value and accuracy ratio determined as 98.6%, 100%, 100%, 96.36%, 98.98%, respectively. In our study sensitivity, specificity, positive predictive value, negative predictive value and accuracy ratios for pregnancy examination findings determined as 91.30%, 100%, 100%, 83.3%, 93.93%, respectively (Table 2). According to literature data, as the penetration depth of the probes increases, the accuracy of the pregnancy diagnosis increases in the early stages. We diagnosed pregnancies with rate of accuracy 93.93% by using 5 MHz probe transabdominal approach on the 35th day. This may be associated with using a convex probe, which improves scanning penetration and allows for

deeper scanning into the abdominal cavity. Goel and Agrawal (1992) highlighted that sector scanners provide a much wider field of view, allowing to visualize the entire uterus. Besides, the accuracy rates are quite variable among studies; these rates seemingly depend on many factors like the sheep's breed, age, type of probe, examination day and region, and operator's experience (Erdem and Sarıbay, 2015).

In sheep, the period until the 34th day of pregnancy is defined as the embryonal period, and the period from the end of the embryonal period to lambing is called the fetal period (Fthenakis et al., 2012). Embryonic or fetal deaths in sheep cause great economic loss. According to research, embryonic and fetal death rates are nearly 30% (Dixon et al., 2007; Fthenakis et al., 2012; Sarıbay and Erdem, 2007). There is limited information on early fetal deaths in sheep, with rates ranging between 3.5-12% (Jones et al., 2016). Researchers show that most embryonic losses occur before the 18th day, with 9.4% of losses from the 18th day to lambing, and 1-5% of late embryonic or fetal losses from the 30th day to lambing (Dixon et al., 2007).

The diagnosis of a fetal loss is made by detecting non-viable fetuses on transabdominal ultrasonography or by determining pregnancy at the first diagnosis and then non-pregnancy at the next diagnosis (Ridler et al., 2015). Studies on embryonic and fetal losses emphasize that plasma progesterone concentrations are similar among animals with fetal loss and continuing pregnancy, they are insufficient to determine losses, and therefore ultrasound examination is necessary (Ridler et al., 2017; Sarıbay and Erdem, 2007). Ridler et al. (2015) performed transabdominal ultrasonography examinations twice per animal and detected 6.8% fetal loss in the second examinations. In our research, repeated transabdominal ultrasonography on the 35th and 50th days revealed that 3 sheep that were pregnant at the first examination were non-pregnant at the second examination, indicating a fetal death rate of 6.1%. We believe that making two examinations per sheep can help determine fetal death rates and allows to make the necessary planning without losing time.

Erdem et al. (2008) reported that, despite finding all sheep to be pregnant after transrectal examination, they could detect single pregnancies at a rate of only 64% in transabdominal examinations on the 34th day. Alkan et al. (2020) reported that singleton pregnancy determination by using transabdominal ultrasonography with 5 MHz convex transducer was detected with percentage 62.06%, 84.21%, 81.81%, 73.80% and 70.27% on days 40, 45, 50, 55, 60, respectively. Karen et al. (2006) investigated 61 single offspring pregnancies and 39 multiple offspring pregnancies and correctly diagnosed 21 of the 39 multiple pregnancies by transabdominal ultrasonography on the 43rd-56th days. The authors found that the remaining 18 sheep were falsely diagnosed with single offspring. They reported that transabdominal ultrasonography had a specificity of 78.6% for diagnosing of single offspring pregnancies. Alkan et al. (2020) stated that the accuracy of the diagnosis of twin pregnancies by transabdominal ultrasonography was higher on day 60 and its specificity was 91.66%. Fridlund et al. (2013) assessed accuracy and affecting factors for pregnancy screening by transabdominal ultrasonography and found that the accuracy (percentage of fetuses scanned/number of lambs born) decreased as the number of fetuses increased. The authors demonstrated that for the number of fetuses diagnosed, the sheep's breed, age, gestational age, and operator's experience significantly impacted the accuracy. They reported accuracy rates of 71.8%, 91.6%, and 89.3% on days <40, 40-80, and 81-100, respectively. They found that the number of fetuses was often overestimated during screening.

Karen et al. (2006) reported that the rate of accurate detection of multiple pregnancy was 53.8% on days 43-56 and 60% on days 76-87. In the present study, the lambing records showed that 26 sheep had single offspring and 16 had multiple offspring. According to the transabdominal findings on the 35th day, there were 16 false diagnoses for multiple offspring (Table 3). As stated by Allabban and Erdem (2020), the reason for false diagnosis of multiple offspring may be that the existing fetus may have been counted twice, or premature fetal death or fetal death may have occurred after the examination. Because, we performed no further examination for the number of offspring until lambing.

Gonzalez-Bulnes et al. (2010) stated that embryos can be viewed from the 28th day with transabdominal ultrasonography, however they are recommended to postpone the examination to the 35th day. In addition, same authors highlighted that pregnancy diagnosis is recommended to be performed after days 40–55 as efficiency in counting the number of conceptuses in multiple pregnancies reaches 100%. Compared to the lambing records, the transabdominal examinations on the 35th day had an accuracy rate of 76.9% for single offspring and 44.8% for multiple offspring (Table 3). We observed that the results obtained for diagnosing single offspring were similar to the literature, and the rate of detecting single offspring by the transabdominal approach on the 35th day was sufficient. However, the accuracy rate for diagnosing multiple offspring by transabdominal ultrasonography may decrease in the earlier days of pregnancy. By knowing the intervals of pregnancy in sheep, more practical and rapid examinations can be done transabdominal in field conditions (Erdem et al., 2020). High accuracy rates can be obtained by performing transabdominal examinations on the 30th day for diagnosing pregnancy and after the 44th day for the number of fetuses (Erdem et al., 2008). We diagnosed pregnancies with a high rate of accuracy (93.93%) by the transabdominal approach on the 35th day under field conditions, but the accuracy rate for diagnosing single/multiple offspring was only 54.76%.

CONCLUSION

In conclusion, we believe that performing transabdominal ultrasonographic examinations on the 35th day is sufficient for diagnosing early pregnancy in sheep. If the number of fetuses needs to be determined, transabdominal ultrasonograhy should be performed in the following days of pregnancy rather than day 35. Besides, it should be kept in mind that the accuracy rates for diagnosing pregnancy and determining the number of fetuses by transabdominal examination depend on many factors like the sheep's breed, age, probe type, day and region of examination, and the operator's experience. Moreover, performing two transabdominal examinations per sheep on the 35th and 50th days could help detect early fetal losses at a significant rate. This allows to investigate the causes and to take measures on time, contributing to higher efficiency in production.

ACKNOWLEDGMENT

This study was funded by Hatay Mustafa Kemal University Coordinatorship of Scientific Research Projects, Project number of 21.D.008. This study was carried out by using some of the data from Tufan Can Tekin's PhD thesis project. The authors wish to thank Dr. Pınar Ambarcıoğlu from ‘Department of Biostatistics, Faculty of Veterinary Medicine University of Hatay Mustafa Kemal’ for helping in the statistical analysis in this study.

Ethical approval: Hatay Mustafa Kemal University Rectorate Animal Experiments Local Ethics Committee, 20/10/2021 No: 2021/06-12

Conflict of interest: The authors declared that there is no conflict of interest.

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Methods of estimating lactation milk yield by using control day yields in Awassi sheep

Research Article

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Article info
Submission: 13-01-2022
Accepted: 25-07-2022
Online First: 04-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

The objective of this study was to determine the effect of various milk control methods on prediction accuracy of lactation milk yield for Awassi sheep. Different control methods (Sweden, Vogel, Holland I, Holland II, Trapeze I, Trapeze II and State Production Farm methods) and control periods (14th, 21st, 28th, 42nd and 56th day) used to determine lactation milk yield were compared. The data of the research was created within the scope of the "National Project of Small Ruminant Animal Breeding in the Hands of the People" in 2018 and a total of 3173 sheep records belonging to 31 enterprises were used. The actual lactation yield in the study was determined as 255.57±0.85 kg and the lactation length as 170.62±0.19 day. Correlation coefficients between the actual lactation yield and lactation yield calculated according to different control methods were found to be high and significant for all control periods and control methods (P<0.05, P<0.01, P<0.001). It gave the similar results to the actual lactation yield in the Vogel method calculated according to different control methods in a period of 28th days, in the Trapeze I method in a period of 28th and 42nd days, in the Trapeze II method in all periods (P>0.05). In the other control periods, all methods differed significantly from the actual lactation yield (P<0.05, P<0.01, P<0.001). As a result, it has been concluded that results close to the actual lactation yield will be obtained by using one of the Vogel or Trapeze I-II methods calculated by using the milk yields on the control day of Awassi sheep raised under Şanlıurfa conditions.

Keywords: Awassi, control methods, control periods, lactation milk yield, test day.

INTRODUCTION

Success in animal breeding studies is possible by estimating the breeding value accurately and without deviation. It is necessary to determine the lactation milk yield (LMY) of sheep in order to calculate the breeding values and to make an effective selection. There is a significant variation between individuals in terms of LMY. Yield controls allow determination of LMY. Thanks to the yield controls, accurate and reliable information can be obtained about the quality and quantity of the production (Thomas et al., 2014). Estimating a sheep's breeding value is both costly and time consuming. The determination of the actual lactation yield (ALY) is possible by measuring and recording the milk obtained every day and every milking during the LL. This process is not both economical and practical for enterprises. For this purpose, different calculation methods have been developed to determine the LMY, giving results close to the ALY. Research in recent years have been concentrated on methods of estimating LMY from control day yields (Angeles-Hernandez et al., 2013, Mc Gill et al.,2014).

How to cite this article
Mundan, D., Demir, R., Paydaş E. (2022). Methods of estimating lactation milk yield by using control day yields in Awassi sheep. *Journal of Advances in VetBio Science and Techniques, 7*(2), 153-160. https://doi.org/10.31797/vetbio.1056967

The first studies to develop methods that take control periods into account in the calculation of LMY began in Denmark. Here in 1895 the first official "Yield Control Society" was established. Thanks to these studies, positive results have been obtained in increasing milk production.

The first five countries in the world with the larger sheep milk production are China, Turkey, Syria, Greece and Romania. Awassi sheep has a 3.5% portion in the world sheep population. Ewe's milk production in Turkey is about 1.4 million ton, according to data from 2018. Average LMY per sheep milked is 77 kg (FAO,2019; Skapetas and Kalaitzidou,2017; Talafha and Ababneh,2011; TUIK,2021).

For the calculation of these yields, milk production is generally monitored at average intervals of 30 days. The yield obtained from an animal on each test-day is called the 'test day' yield (Tonhati,2004). Using test-day yields instead of increasing yield records is an alternative to analyze problems. The main advantage of this approach is the possibility of reducing the number of test-day, thus reducing the generation interval (Ptak and Schaeffer,1993). Different methods have been developed to calculate LMY reported by ICAR. One of these methods is the test interval method (TIM). Different control methods used in the calculation of LMY (Sweden, Vogel, Holland I, Holland II, Trapeze I, Trapeze II, SPF etc.) and control periods (7th, 14th, 21st, 28th, 42nd, 56th day or monthly, bimonthly such as quarterly) has been developed. Average daily milk yield (ADMY) is calculated with the help of control day yields. This value is multiplied by the LL and LMY is obtained. Thus, with the correct calculation of the LMY, the control costs of the enterprises will decrease (Everett and Carter,1968).

Some authors have focused on finding mathematical models that describe the biological processes of milk production by mammary gland cells (Elvira et al.,2013; Pollott,2000). There are also researchers who recommend a statistical approach by modeling the shape of the lactation curve such as random regression test-day models (Brito et al.,2017; Mucha et al.,2014).

Awassi sheep is known as a very popular and proliferative breed in the non-European region due to its ability to absorb the environmental deviation. This study was carried out in order to reveal the most appropriate control method and control period to be used in estimating the LMY of Awassi sheep raised in Şanlıurfa.

MATERIAL and METHOD

Location

Şanlıurfa is located at 37 49 '12 "- 40 10' 00" east longitude and 36 41 '28 "- 37 57' 50" north latitudes in the Southeastern Anatolia Region of Turkey. It has elevation of 546.85 meters above sea level.

Data

The animal material of the study consists of sheep that are included in the "Small Ruminant Animal Breeding National Project in the Hands of the People". The data used in this study consisted of 3173 Awassi sheep and 3263 lambs raised in 31 family farms. The study was conducted in the 2018 breeding season. This study has been prepared from the project (TAGEM / 63IVE2017-03) supported by TAGEM.

Milk Yield Controls

Milk yield controls were done by hand milking method. Milk yield measurements were started on the 7^{th} day of lactation. Milking was continued until the daily milk yield decreased to 50 ml. The amount of milk milked was accepted as the daily milk amount of the sheep.

The data for the lactation curves at 14^{th}, 21^{st}, 28^{th}, 42^{nd} and 56^{th} days were calculated by interpolation and extrapolation methods.

The lambs were separated from their mothers one day before the control day and were not breastfed on the control day. The lambs were separated from their mothers at least 12 hours before the control time in order not to adversely affect the reliability of milk control. The lambs were separated from their mothers from 20:00 on the control day. The lambs were placed in a different section than the sheep for 24 hours. The amount of milk was measured with a measuring cylinder sensitive to 5 ml. The maximum daily milk yield is the highest daily yield obtained during control days. ADMY was obtained by dividing the total amount of milk obtained in the controls by the number of controls.

Control methods

Daily milk yield of sheep was determined by milking twice a day, in the morning and in the evening. ALY were obtained by collecting daily milk yields. Control period taking into consideration four widely used in international Sweden, Vogel, Netherlands I and Trapeze II methods, both of the Netherlands II, Trapeze II methods and LMY with State Production Farm (SPF) method developed in Turkey calculated and compared results. In the calculation of LMY were used at 14^{th}, 21^{st}, 28^{th}, 42^{nd} and 56^{th} days intervals as control periods.

These control methods:

The Swedish method

The yield determined on the control day is accepted as the ADMY within that period. The quantity of milk in the period is calculated by multiplying the yield on the control day and the number of days in the control interval. The LMY is calculated by collecting the controls made separately for each period. The calculation formula for the Swedish method is as follows:

$$LMY = a \cdot \sum_{i}^{n} k_i - \left(\frac{a}{2} - A\right).k_1$$

The Vogel method

The sum of the yields determined on the control days is multiplied by the control interval and the LMY is calculated. It can be applied in cases where the date of birth of the animal is unknown. It is a method with low sensitivity and is preferred for control yields with a low number. LMY calculation formula for Vogel method is as follows:

$$LMY = a \cdot \sum_{i}^{n} k_i$$

The Netherlands I method

The Dutch I method is a practical way to use when control intervals are unequal. The yields determined on the control day are collected and divided by the number of controls milking. The value obtained is considered as the daily milk yield. The calculated daily milk yield is multiplied by the LL and LMY is obtained. As the time between birth and first control gets longer, the error increases. The LMY calculation formula for the Netherlands I-II method is as follows:

$$LMY = \left(\sum_{i}^{n} k_i / \sum n\right). LL$$

The Netherlands II method

$$LMY = \left(\sum_{i}^{n} k_i / \sum n\right). LL$$

The Trapeze I method

The Trapeze method is similar to the Swedish method in terms of calculation method. The efficiency of the period is calculated by multiplying the average of the data detected at the beginning and end of each control period by the number of days in that period. In the Trapeze I method, milk yield between the last control day and drying is not taken into account.

The LMY calculation formula for the Trapeze I method is as follows:

$$LMY = (A\text{-}1).k_1 + \frac{a}{2}(k_1 + k_n + 2\,(k_2 + k_3 + \ldots\ldots\ldots + k_{n-1}))$$

The Trapeze II method

The LMY calculation formula according to ICAR is as follows:

$$LMY = (A\text{-}1).k_1 + \frac{a}{2}(k_1 + k_n + 2\,(k_2 + k_3 + \ldots\ldots\ldots + k_{n-1})) + Bk_n$$

The SPF method

If the control number (n) is single in the SPF method:

$$LMY = (A\text{-}1).k_1 + a.(k_1 + k_2 + k_3 + k_4 + \ldots\ldots\ldots + k_{n-1}) + Bk_n$$

If the control number (n) is double in the SPF method:

$$LMY = (A\text{-}1).k_1 + a.(k_1 + k_2 + k_3 + k_4 + \ldots\ldots\ldots + (k_{n-1} + k_n)/2) + Bk_n$$

Description of the abbreviations used in the formula of the above methods:

LMY: lactation milk yield (kg),

LL: lactation length (day),

ADMY: average daily milk yield (g),

A: time between birth and first control (day),

B: time between the last control and the end of lactation (day),

C: time between the last control day and the dry period (day),

n: control number,

a: control period (day),

$\mathbf{k_i}$: milk yield determined in any control (kg),

$\mathbf{k_1}$:milk yield determined at the first control (kg),

$\mathbf{k_n}$: milk yield determined in the final control (kg).

The relationship between LMY and control day yield was determined by calculating the ratio of the total monthly milk yields on the control day to the milk yield on each control day.

Statistical analysis

Control efficiencies were calculated by formulating the data with the help of Microsoft Office 2010 Excel programme. Statistical analyses were performed using statistical programme MINITAB (MINITAB,2005). Data were obtained by repeated measurements on sheep and analyzed using Least Squares mean method.

The absolute differences (| D | * 100 / ALY) were determined by calculating the arithmetic mean of the differences between ALY and LMY (D = (ALY-LMY) / N) and the standard deviation (SD) of the differences.

Correlation analysis was done relationships between control method and control period. The relationship between LMY calculated according to control methods was determined by a correlation analysis. Duncan was used for intergroup comparisons, and t test was used for intergroup comparisons in interaction tables.

RESULTS

This study was carried out with 3173 sheep from Awassi sheep breed raised in Şanlıurfa. After the birth of the lambs, a control milk yield was done according to the Trapeze II method by ICAR. It was determined that the studied sheep had an average of 265.68±0.39 kg milk for the 170.62±0.19 days of LL. The ALY in the study was determined as 255.57±0.85 kg and the LL as 183.41±0.57 days.

Monthly test-day milk yield means varied according to the phase of lactation, with the highest production being recorded for the second month of lactation, followed by a gradual decrease through to the tenth month. The increase in milk yield was 7.80% from the first to the second month, corresponding to a

milk yield of 0.55 kg. The observed decline after the 2nd test day was calculated to be 7.09%. A minimal decline in percentage of production was observed from the second to the third month (4.48%), while the largest decrease (9.82%) occurred between the eighth and ninth month of test. These results support the recommendation from ICAR for using milk yield adjusted for 270th day of lactation.

In absolute terms, the average monthly decline in milk yield was 0.42 kg from the lactation peak. The largest decrease (0.52 kg) was observed from the fourth to the fifth test-day, while the smallest decrease (0.30 kg) occurred between the ninth and tenth test-day. The percentage and absolute total losses in milk yield from the second to the tenth test-day were 44.46% and 3.39 kg, respectively.

The distribution of LMY calculated in this study according to the number of sheep is given in figure 1. According to this figure, the highest number of sheep (980 sheep) gave milk between 261-270 kg, while the least number of sheep (6 sheep) gave 210-220 kg milk.

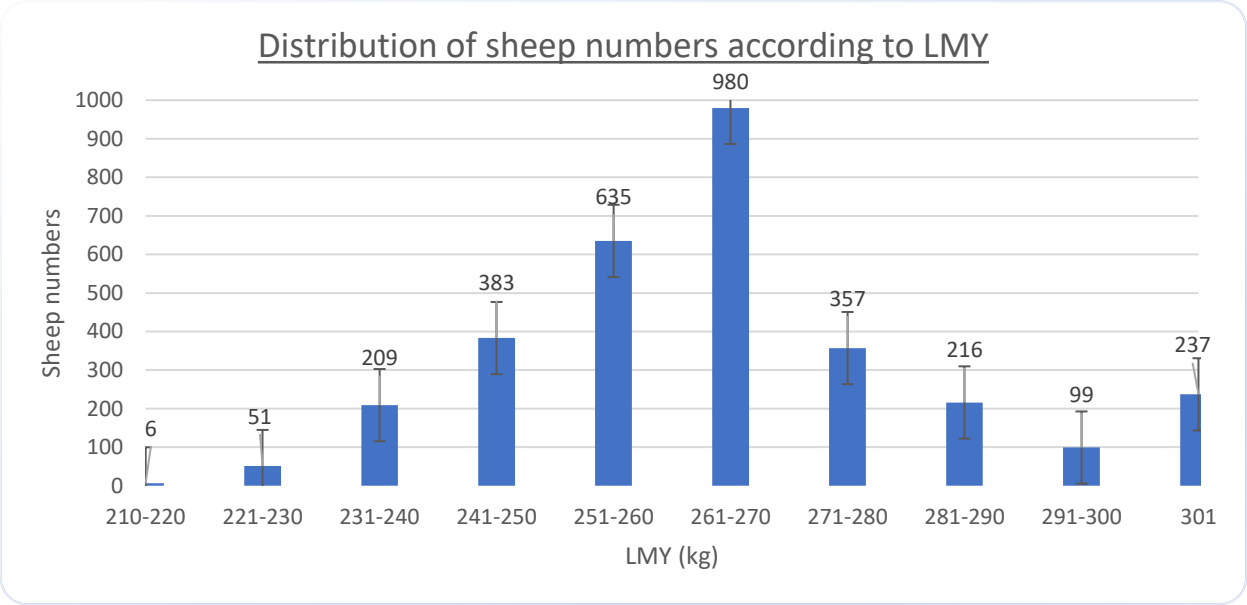

Figure 1. Distribution of sheep numbers according to LMY.

In this study, it was determined that the estimation error increased significantly ($P<0.01$) as the time between inspections increased. The effect of the calculation method on the estimation error was found to be significant ($P<0.01$) and it was determined that the Trapeze II method predicted the yield more accurately.

Correlation values between ALY and estimated yields were found to be very high and significant ($P<0.01$). Therefore, if the aim is to rank sheep according to their yield level, any combination of calculations examined in the study can be used. However, if it is desired to estimate the yield closest to the ALY, the inspection interval should be 28th day. In this case, the Dutch or Trapeze method can be used. If the inspection interval is 56th day, the yield should be estimated with the Trapeze method.

Arithmetic means of LMY calculated according to different control methods, mean of differences (D), SD values and % absolute difference values are given in Table 1.

According to Table 1, the lowest D value was observed in the 28th day control period of the Vogel method, the 28th to 42nd day control period of the Trapeze I method and the whole period of the Trapeze II method. These differences are statistically insignificant ($P>0.05$). LMY in other control periods differed significantly from ALY ($P<0.05$, $P<0.01$, $P<0.001$).

Table 1. Arithmetic means ($\overline{X}$) of LMY calculated according to control methods, mean of differences (D) and % absolute difference values.

CONTROL PERİOD (Day)		CONTROL METHOD						
		SWEDISH	VOGEL	HOLLAND I	HOLLAND II	TRAPEZE I	TRAPEZE II	SPF
14	$\overline{X}$ D±SD MF% P	243.01 12.56±0.015 * *	230.59 24.98±0.03 10 ***	244.51 11.06±0.02 4 *	292.25 -36.68±0.05 14 ***	238.35 17.22±0.03 7 **	253.77 1.80±0.01 1 -	235.11 20.46±0.07 8 **
21	$\overline{X}$ D±SD MF% P	241.45 14.12±0.036 ** **	245.67 9.90±0.04 4 *	247.55 8.02±0.02 3 **	263.10 -7.53±0.05 3 -	248.77 6.80±0.01 3 -	257.72 -2.15±0.05 1 -	269.24 -13.67±0.07 5 *
28	$\overline{X}$ D±SD MF% P	267.74 -12.17±0.025 * *	261.79 -6.22±0.03 2 -	267.83 -12.23±0.09 5 *	265.36 -9.79±0.03 4 *	259.53 -3.96±0.04 2 -	261.68 -6.11±0.03 2 -	267.99 -12.42±0.07 5 *
42	$\overline{X}$ D±SD MF% P	264.05 -8.48±0.04 3 -	264.58 -9.01±0.09 4 *	264.11 -8.53±0.02 3 -	281.33 -25.76±0.04 10 ***	249.99 5.58±0.01 2 -	258.36 -2.79±0.05 1 -	237.87 17.70±0.01 7 **
56	$\overline{X}$ D±SD MF% P	267.34 -11.77±0.03 5 *	263.02 -7.45±0.01 3 -	245.77 9.80±0.05 4 *	266.63 -11.06±0.03 4 *	239.01 16.56±0.03 6 **	261.82 -6.25±0.07 2 -	263.83 -8.26±0.02 3 -

*: P<0.05; **: P<0.01; ***: P<0.001; -: No significant; SD: Standart Deviation.

The results obtained in the present study indicate that adoption of test-day yields as selection criteria might contribute to greater genetic gain in LMY. The results show that test day milk yields are most closely related to total milk yield.

The results of the correlation coefficients between control methods and control periods are given in Table 2.

Table 2. Correlation coefficients between control methods and control periods.

Control Method	Control Period (day)				
	14	21	28	42	56
SWEDISH	0.93**	0.92**	0.95**	0.94**	0.93*
VOGEL	0.91**	0.90*	0.98***	0.91*	0.90*
HOLLAND I	0.95***	0.92**	0.95**	0.94**	0.92**
HOLLAND II	0.91**	0.89*	0.95**	0.96**	0.91*
TRAPEZE I	0.93***	0.92**	0.98***	0.97***	0.92**
TRAPEZE II	0.97***	0.96***	0.97***	0.98***	0.99***
SPF	0.97***	0.93**	0.95**	0.93**	0.90*

*: P<0.05; **: P<0.01; ***: P<0.001.

According to this table, the correlation coefficients between control methods and control periods were found to be positive and highly significant in all control methods and periods of Trapeze II (P<0.001).

Estimates of permanent environmental correlations were high, suggesting that both traits are affected in part by the same environmental factors.

DISCUSSION

In this study performed in Awassi sheep, the ALY was calculated as 255.57±0.85 kg and the lactation length as 170.62±0.19 days. Awassi sheep can produce an average of 60-80 liters milk with a LL of 150 days under different methods of production while an improved

Awassi sheep can yield 504 liters milk within 214 days of LL under a well-managed production system (Talafha and Ababneh,2011). Mavrogenis (1996) calculated the lactation milk yield between 109-133 liters in the first 90-day period in Awassi sheep. Reiad et al. (2010) calculated the average LMY of Awassi sheep in a flock obtained as 243.3 ± 3.96 kg at the Agricultural Scientific Research Centre in Salamieh / Syria. The LMY calculated in this study was found to be lower than the values of 359.3 and 345.1 kg in the Eastern Friesian and Lacaune breeds, respectively. LL was found to be similar with 188.6th and 180.3rd days in East Friesian and Lacaune breeds, respectively (Thomas et al.,2014).

The study by Panayotov et al. (2018) was carried out with 50 sheep from the Lacaune breed imported from France. During the testing of the milk yield was used the Method AC by ICAR. LMY were calculated by measuring the milk yields of each animal studied on five separate test days, once a month during the LL. It was established that the studied sheep had an average 213.29 L for the 150 days LL. The highest was the milk yield on the first test-day an average 2.279 L, with maximum deviation 3.310 L. A 77% of the ewes had a milk yield over 180 L, a relatively smaller part (6.5%) had a milk yield under 150 L, while 14.5% of the ewes had a milk yield over 270 L, and the maximum value was 298.38 L. Panayotov et al. (2018) reported in their study that 31.3% of sheep gave 180-210 kg of milk, while 6.3% of sheep gave less than 150 kg of milk.

Jawasreh and Khasawneh (2007) defined a study for the evaluation of genetic traits of milk yield in Awassi sheep at an agriculture research center in Amman, Jordon. The observed mean for total milk yield (kg) was 1,532.68 ± 660.88, which is similar to those reported by Tonhati et al. (2000). Basdagianni et al. (2018) evaluated some reference LL and finalized an appropriate LL for the Chios sheep breed. They used 24 474 dams from 130 herds for test days milk recording for a total of 260042 between 2003 and 2014.

Gootwine and Pollott (2000) had done a study in which he analyzed Awassi sheep's LMY for estimation of milk production factors and lactation curve parameters. The experimental flock was milked two times a day after lambing and reared under intensive management conditions. As a result of this study, the researchers reported that they achieved an average of 506 liters of total milk yield in a 214th day LL. The highest milk yield reached on the 45th day of lactation was 3.44 liter, and the maximum daily milk yield was 3.9 liters. Milk yield increased by 62 g / day from the first day of lactation to mid-lactation and decreased as 16.5 g / day from mid-lactation to the end of lactation.

Pollott and Gootwine (2000) reported that Awassi ewes peaked on the 27th day (3.34 liters) and showed a decrease in milk production of 15.3 g / day on the 150th day, giving a total of 543 liters of milk on the 280th day.

CONCLUSION

Enterprises should take 3 points into account when deciding for the control method and control period. It should be known how much loss the error made at the decision stage causes the enterprises. It should take account of the environmental conditions that changing over time. Milk should be taken exactly in all control days.

As a result, it has been concluded that results close to the actual lactation yield will be obtained by using one of the Vogel or Trapeze I-II methods calculated by using the milk yields on the control day of Awassi sheep raised under Şanlıurfa conditions.

ACKNOWLEDGMENT

This study was funded by TAGEM / 63IVE2017-03

Ethical approval: Permission was obtained from the Ministry of Agriculture and Forestry to publish the data of this study.

Conflict of interest: The authors declare that there is no conflict of interest for this article.

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The effect of Anti-Mullerian hormone on yield of oocytes recovered by ovum pick-up (opu) in heifers

ABSTRACT

The aim of this study was to determine the relationship between the oocyte yield by the ovum pick-up (OPU) technique with the Anti-Mullerian Hormone (AMH) concentrations of the donors. Ten healthy Holstein heifers aged 12 to 15 months were included in the study. AMH measurements were performed with Bovine VIDAS® Anti-Mullerian Hormone kits (Biomeriux, Marcy l'Etoile, France) using the Mini Vidas device. A total of 67 OPU sessions were performed on a random day of the cycle. Oocytes were classified according to their quality, and viability evaluation of oocytes was made according to the cell layer number and cumulus integrity in the cumulus-oocyte complex (COC), the homogeneity of their cytoplasm. The average oocyte yield in OPU sessions per animal was range from 4–8. There was an significant negative correlation between the collected oocyte numbers and plasma AMH levels. In conclusion, it was observed that AMH concentration did not affect the number of viable and the quality oocytes collected in weekly OPU administration in animals. It was thought that OPU applications performed without knowing the day of the cycle did not provide the expected correlation with AMH data.

Keywords: AMH, heifer, oocyte, ovum pick-up

INTRODUCTION

Assisted reproductive technologies, such as in vitro embryo production, (IVEP) have been widely practiced in the recent years for the purpose of genetic improvement (Guerreiro et al., 2004). In 2019 IVEP accounted for 72.68% (1.031.567) of the total embryo production (1.419.336) in cattle breeding worldwide. The vast majority of embryos (1.010.680) were produced from OPU (Ovum Pick-Up) while few (20.887) were obtained through slaughterhouses (Viana, 2020).

The success of oocytes obtained by the OPU technique for IVEP is associated with physiological characteristics, such as the number of antral follicles in the ovary and oocyte developmental competence. Recent studies have shown that Anti-Mullerian hormone (AMH) can be used as a biomarker to identify factors including selection of donors with superior production traits for IVEP, estimation of ovary reserves, future fertility characteristics, milk production level, and longevity in herd (Guerreiro et al., 2014; Viana, 2020).

Antral Follicle Count (AFC) is a reliable phenotypic biomarker positively associated with fertility, ovarian reserve, ovarian function, superovulation response, in vitro blastocyst production, transferable embryo production/count, and offspring birth weight (Ireland et al., 2008; Sabuncu et al., 2019).

How to cite this article
Çizmeci, SA., Dinç, DA., Bucak, MN., Çiftçi, MF., Yeşilkaya, ÖF., Ağır V. (2022). The effect of Anti-Mullerian hormone on yield of oocytes recovered by ovum pick-up (opu) in heifers. *Journal of Advances in VetBio Science and Techniques, 7*(2), 161-168.
https://doi.org/10.31797/vetbio.1106019

Research Article

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Article info
Submission: 19-04-2022
Accepted: 24-07-2022
Online First: 03-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

Circulating AMH concentration is reported to have a positive correlation with total AFC in mice, human, Bos taurus, and Bos indicus cattle (Guerreiro et al., 2014). AMH production begins with the onset of follicular wave in the ovary and peaks in the primordial, primary, and secondary follicles. AMH production decreases after the selection of the dominant follicle and is not secreted from atretic follicles (Lussier et al., 1987). Studies have shown that the circulating AMH is highly associated with AFC. It has been suggested that determining the level of AMH can be an endocrine marker to define the best donors in IVEP, regardless of the genotype and age of the animal (Broer et al., 2011; Baruselli et al., 2018; Maculan et al., 2018).

Possible functions of AMH in the ovary include inhibition of follicular activation and growth, FSH-stimulated growth, granulosa cell growth, and aromatase (Poole et al., 2016). In the absence of AMH, the follicles develop faster and the ovarian follicular reserves deplete sooner (Umer et al., 2019). Since the AMH concentration in cows remains almost unchanged after puberty, heifers who reach puberty with a low ovarian reserve are at risk for faster depletion of their ovarian reserves and a shorter reproductive life (Pankhurst, 2017; Alward ve Bohlen, 2020).

It is reported that in cattle, circulating AMH concentration can be used as a possible marker for veterinarians to predict AFC in ovaries as well as to predict superovulation response and IVEP performance (Mossa et al., 2012; Ribeiro et al., 2014). A recent study investigating the possibility of producing embryos from young female calves has found that AMH concentration was a very useful marker to predict IVEP performance (Batista et al., 2014). It is reported that the determination of circulating AMH concentration in very young calves can be an important biomarker in selecting the best donors for in vitro embryo production because ultrasound examination of ovaries in this age group is difficult and impractical (Armstrong et al., 1992). Given the assumption that AMH concentration is not affected by factors, such as period of cycle, age, or nutrition, and remained almost constant throughout the life of the cow, the present study aimed to determine the relationship between the oocyte yield collected by the OPU technique with the AMH concentrations of the donors.

MATERIAL and METHOD

Ten healthy Holstein heifers aged 12 to 15 months were included in the study. Heifers were selected all their genomic features were examined and among with high AMH levels (>300 pg/mL). Before OPU applications, blood samples were taken from the jugular vein into heparinized tubes for once. Blood samples were centrifuged at 5000 rpm for 10 min and serum samples were stored at -20°C until the time of measurement. AMH measurements were performed with Bovine VIDAS® Anti-Mullerian Hormone kits (Biomeriux, Marcy l'Etoile, France) using the Mini Vidas device (Biomeriux, Marcy l'Etoile, France).

A total of 67 OPU sessions were performed on a random day of the cycle at intervals of minimum one and maximum two weeks without any hormone administration. The OPU was done using Esaote MyLab TwiceVet ultrasonography device and a compatible intravaginal OPU probe, catheter and aspiration device combination (Esaote 5001) were used. For OPU, animals had lower epidural anesthesia (4–6 cc of local anesthetic, Adokain, Sanovel, İstanbul, Turkey). After cleaning the rectum, the perineal region was cleansed. Rectal palpation of the ovary was performed through the rectum Special convex vaginal probe (4.0–9.0 MHz probe and catheter with 20 G needle at the tip) was inserted into the vagina and directed to the right fornix of the vagina for aspiration from the right ovary and fixed. The

right ovary was manually brought into the probe's scanning surface through the rectum and the ovary was fully visualized. The ovaries were examined before aspiration and follicle numbers were recorded. The ovaries were manually rotated from the rectum, and each follicle was positioned sequentially on the needle drilling line and all follicles with a diameter of 3-8 mm were aspirated. The follicle fluid was aspirated using vacuum (80–90 mm/Hg) with the aspiration pump installed in the system immediately after the performance of puncture of each follicle. After the aspiration process was completed, the needle apparatus of the probe was removed and washed, allowing the oocytes that might remain in the system to be taken into the commercial OPU medium (IVF Bioscience, Denmark). The same procedure was repeated for the left ovary.

The collected OPU fluid was transferred to the petri dishes and the oocytes were screened (1.6X magnification) in stereomicroscope (Leica S8 APO, Wetzlar, Germany). The retrieved oocytes were taken into the washing solution (BO-WASH, IVF Bioscience, Denmark). At this stage, oocytes according to their quality were classified as A, B, C, and D quality under an inverted microscope (Leica DM IL, Germany). Collected COCs were evaluated according to:

A Quality: It has > 5 compact cell layers and homogeneous cytoplasm (Image 1),

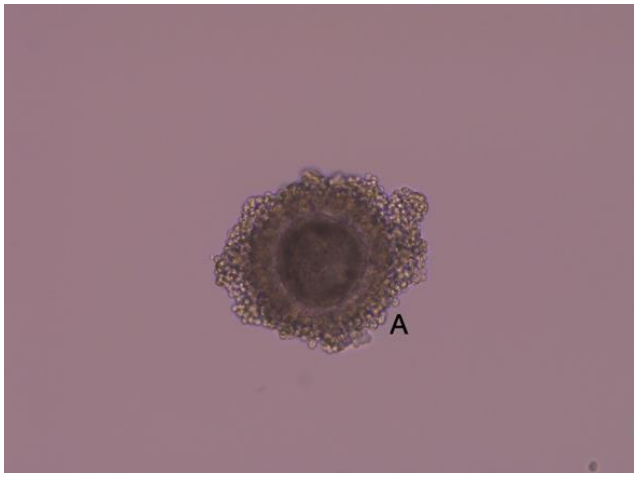

Image 1. A quality oocyte. A: A quality oocyte

B Quality: It has 3-5 layers of compact cells and few inhomogeneous areas in the cytoplasm or >5 layers of compact cells and dense inhomogeneous areas (Image 2),

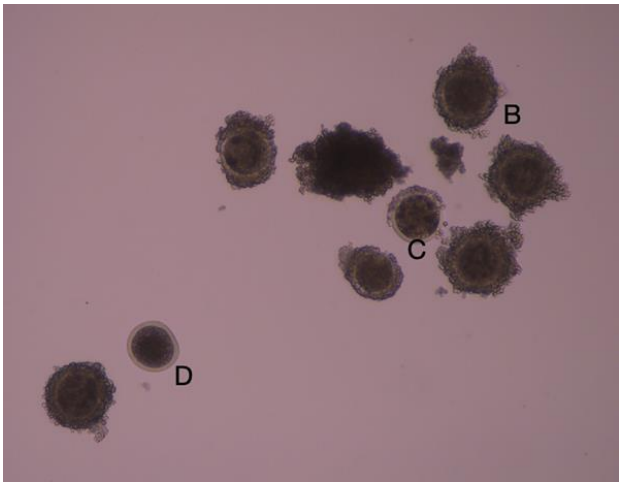

Image 2. B, C and D quality oocytes. B: B quality oocyte, C: quality oocyte, D: D quality oocyte.

C Quality: It has several cell layers (>3) and few inhomogeneous areas in the cytoplasm or no regional cumulus, and has a little homogeneous area (Image 2),

D Quality: It has completely bare, small, granular, inhomogeneous cytoplasm (Image 2) (Petyim et al., 2003).

Viability evaluation of oocytes was made according to the cell layer number and cumulus integrity in the COC, the homogeneity of their cytoplasm, and the absence of degenerated areas. Non-cumulus cells and degenerated oocytes were considered as quality D (dead). In bovine oocytes, A quality oocyte has compact cumulus layers and a translucent ooplasm. B quality oocyte has less compact cumulus layers and dark ooplasm, and C quality oocyte has expanded cumulus cells and dark ooplasm D quality oocyte hasn't cumulus layer and has dark ooplasm. SPSS-Statistics-22 package program (IBM, Richmond, USA) was used in the statistical analysis of the results. A correlation test was performed between blood AMH results and collected oocytes counts and qualities ($P<0,05$). The data were presented as the mean ± SD.

RESULTS

The data on the ratio of quality grades of oocytes, AMH values, and average antral follicle counts after 67 OPU applications in 10 Holstein heifers are given in Table 1 and 2. The average oocyte yield in OPU sessions per animal was range from 4–8. The mean number of follicles aspirated per OPU was 14.44, the number of oocytes collected per OPU was 6.1, and the mean recovery rate was 49.81%. The averages of oocyte quality were determined as A quality 22.37%, B quality 23.45%, C quality 28.61%, and D quality 25.68% in total OPU applications.

Table 1. Oocyte qualities, total oocytes counts, AMH concentration, and correlation

Animals	A quality (%)	B quality (%)	C quality (%)	D quality (%)	Total oocytes count	AMH value pg/ml	Average number of antral follicles (3-8 mm)
1	34.38	28.13	21.88	15.63	34.38	839.11	17.43
2	15.79	23.68	39.47	21.05	15.79	818.92	16.17
3	19.57	21.74	32.61	26.09	19.57	696.55	15.40
4	19.44	27.78	22.22	30.56	19.44	683.26	17.67
5	12.50	28.57	23.21	35.71	12.50	604.14	12.43
6	28.00	12.00	28.00	32.00	28.00	460.97	13.17
7	10.42	22.92	33.33	33.33	10.42	418.53	11.14
8	31.71	26.83	24.39	17.07	31.71	396.98	14.71
9	25.00	17.86	32.14	25.00	25.00	382.72	13.50
10	26.92	25.00	28.85	19.23	26.92	332.31	12.25
Average%	22.37	23.45	28.61	25.58	22.37		
Correlation	-0.233	0.053	-0.086	-0.092	-0.122		
MEAN	1.63	1.49	1.78	6.84	38.89	563.35	14.62
SD	0.08	0.06	0.06	0.07	9.99	180.61	2.25
SEM	0.03	0.02	0.02	0.02	3.33	60.20	0.75

Table 2. Aspirated follicle counts, collected oocyte counts, recovery rate, viable and degenerated oocyte counts.

Parameters	Results
Total 3 mm follicle	488
Total 3-8 mm follicle	928
Total ≥8 mm follicle	157
Number of aspirated follicles	803
Number of oocytes collected	400
Average recovery rate (%)	49.81
Average AMH (pg / ml)	563.35
Viable oocyte rate (%)	75
Degenerate oocyte rate (%)	25
Average number of antral follicles (3-8 mm) per OPU	14.44
Average collected oocytes per OPU	6.1
Total oocytes per OPU section	57.43

DISCUSSION

Among the existing reproductive technologies, IVEP is an important tool for reproduction of superior quality genetic material (Lohuis, 1995; Camargo et al., 2005). However, oocyte and antral follicle population competence is one of the most important limiting factors encountered during in vitro embryo production (Taneja et al., 2000; Pontes et al., 2011; Bó et al., 2012). AFC has minimal lifetime variability for an animal; similarly, AMH also varies little during the estrous cycle (Sabuncu et al., 2019). Therefore, AMH concentration and AFC are positively correlated with each other and the ovarian reserve (Burns et al., 2005; Ireland et al., 2007).

AMH controls the number of follicles and the selection of dominant follicles during the follicular wave. It prevents premature depletion of ovarian follicular reserve by regulating the selection of 4–6 small antral follicles from the pool in each follicular wave and their entry into the cycle (Umer et al., 2019). If the antral follicle pool of the donor animal is large and 50% of the oocytes in this antral follicle pool are viable, all oocytes in the selected preovulatory follicles may be viable (Pankhurst, 2017). This indicates that cows with a larger antral follicle population have much higher AMH concentrations than cows with a smaller antral follicle population. Therefore, AMH measurements are considered an independent indicator of the follicle reserve in cows (Ireland et al., 2008; Monniaux et al., 2012; Baruselli et al., 2018).

It has been reported that a positive correlation was observed between plasma AMH concentration and follicle number, total COC, viable COC number, and IVEP in the study of 59 Holstein cow performing OPU. In addition, greater number of COCs and higher viability rates of the collected COCs have been reported in cows with high AMH concentrations compared to those with low AMH levels (Guerreiro et al., 2014).

The measurement of circulating AMH concentrations helps to predict superovulation response for in vivo embryo production and IVEP in cattle (Batista et al., 2014; Gamarra et al., 2015; Vernunft et al., 2014). Heifers with low AMH concentrations were found to have lower pregnancy rates, greater probability of removing from a herd after birth of first calf, and shorter herd life than those with higher AMH concentrations (Ireland ve Mossa, 2018). In a study conducted with 1200 cows, AMH levels ranged from 10 to 3.198 pg/ml, and the mean concentration was 320.3 ± 251.1 pg/ml. When these animals were grouped by AMH level as the top 20% and the lowest 20%, the average of the intermediate group, which constitutes 60% of the animals, was reported as 263 pg/ml (141 to 450 pg/ml) (Ribeiro et al., 2014). However, studies show that normal ranges of AMH are between 0.01–400 pg/ml, and very few animals reached levels above 400 pg/ml (Rico et al., 2009; Souza et al., 2015). The average plasma AMH concentrations of the animals used in our study were found to be 563.35 pg/ml. It was considered that the absence of statistical differences between numbers and quality of oocyte obtained from animals could be due to the high concentration of AMH in all animals (Table 1). It was thought that the fact that the animals used as donors in the study were selected from a facility where genomic selection was performed may have minimized the individual differences.

The viability of collected oocytes had been reported to 77.7% and 96.7% after estrus stimulation (Machatkova et al., 2004; Beck, 2014). In the present study, it was determined that 22.25% of the oocytes obtained were quality A, 24% quality B, and 28.75% quality C. It was determined that 75% of the oocytes obtained were alive. In cattle, it has been

reported that the time interval between individual OPU applications has a molecular effect on the quality of oocytes and embryos (Hanstedt et al., 2009; Wrenzycki, 2018). It is believed that an increase in blood flow to individual follicles is associated with follicular growth rates, while the decrease is associated with follicular atresia. It was observed that the timing of OPU sessions has an impact on the quality of viable oocytes obtained and 75% viability rates were at an acceptable level. Given that AMH concentration is correlated with AFC, high AMH concentration provides insight about the abundance of AFC (Acosta et al., 2003; Acosta, 2007).

Due to the fact that OPU applications were performed on random days and at 1-2 week intervals, no individual difference was detected in the number of oocytes collected in repeated OPU applications. It was thought that the lack of statistical difference between the number of oocytes collected and AMH concentrations in the present study may be related to the selection of animals with high AMH levels during donor selection or the AMH level being above the average values. It is known that AMH concentration can be used as an important biomarker in biotechnology studies. During the selection of animals to be used in biotechnology studies, it is reported that AMH concentration is a parameter that should be evaluated in addition to breed, yield, or genomic characteristics.

CONCLUSION

In conclusion, it was observed that AMH concentration did not affect the number of viable and the quality oocytes collected in weekly OPU administration in animals. It was considered that AMH, which was reported to be used to determine the duration of ovarian reserve and uses of donors, may be effective in the number of embryos per OPU. As a result of the presented study, it was thought that OPU applications performed without knowing the day of the cycle did not provide the expected correlation with AMH data.

ACKNOWLEDGMENT

We want to acknowledge TAGEM 18/AR-GE 59 project for funding of this research.

Ethical approval: The study was conducted with the approval of Selcuk University Veterinary Faculty Experimental Animals Production and Research Center Ethics Committee, Turkey (SÜVDAMEK) (2019/28).

Conflict of interest: The authors declare that they have no competing interests.

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Ratlarda intravenöz kontrast madde uygulamasının göz içi basıncı, göz yaşı miktarı ve oksidatif stres üzerine etkileri

The effects of intravenous contrast substance administration on intraocular pressure, tear amount and oxidative stress in rats

ÖZET

Endirekt radyografi, anjiyografi, intravenöz ürografi ve bilgisayarlı tomografi gibi radyolojik prosedürlerde sıklıkla kullanılmakta olan iyotlu radyo konrast ajanlar genellikle güvenli olmalarına rağmen ciddi yan etkilere sebep olabilmektedir. Bu çalışmada intravenöz iyonik yüksek ozmolar kontrast madde uygulamasının göz içi basıncı, göz yaşı miktarı ve göz dokusu oksidan ve antioksidan parametreler üzerindeki etkisinin araştırılması amaçlandı. Çalışma grupları Grup 1 (Kontrol) ve Grup 2 (Ürografin) olmak üzere 2 gruptan oluştu ve toplamda 16 adet wistar albino ırkı dişi rat kullanıldı. Denemenin ilk günü kontrol grubuna intravenöz olarak 6 ml/kg dozda serum fizyolojik, grup 2'ye ise aynı dozda kontrast madde uygulaması yapıldı. İntravenöz uygulamalardan sonraki 1, 6, 12, 24 ve 48. saatlerde göz içi basıncı ve gözyaşı miktarları ölçüldü. Denemenin 48. saatinde ölçümler yapıldıktan sonra bütün ratlar ötanazi edildi ve göz dokuları çıkarıldı. Göz dokusunda oksidatif hasar ve antioksidan aktivite durumunu ortaya koyabilmek için malondialdehit ve redükte glutatyon düzeyleri ile katalaz ve glutatyon peroksidaz enzim aktivitelerine spektrofotometrik olarak bakıldı. Göz yaşı miktarı ölçümlerinde schimer tear test (STT-1) stripi, göz içi basıncı ölçümlerinde ise tonometre olarak rebound tonometre Tonovet® kullanıldı. Ürografin uygulamasından sonraki 1, 6, 12, 24 ve 48. saatlerde yapılan ölçümlerde kontrol ve ürografin grupları arasında göz içi basıncı ve gözyaşı miktarları açısından istatistiki olarak anlamlı bir farklılık saptanmadı. Aynı uygulamanın göz dokusunda malondialdehit düzeyini ($P<0,005$) anlamlı şekilde arttırdığı görüldü. Göz dokusu redükte glutatyon düzeyi ile katalaz ve glutatyon peroksidaz enzim aktiviteleri açısından gruplar arasında anlamlı bir farklılık tespit edilemedi. İntarvenöz kontrast madde uygulamasının göz dokusunda oksidatif strese neden olduğu ve bunun da uzun sürede oküler etkisinin olabileceği değerlendirildi.

Anahtar Kelimeler: Kontrast madde, göz, oksidatif stres.

ABSTRACT

Although iodinated radiocontrast agents, which are frequently used in radiological procedures such as indirect radiography, angiography, intravenous urography and computed tomography, are generally safe, they can cause serious side effects. In this study, it was aimed to investigate the effect of intravenous ionic high osmolar contrast agent administration on intraocular pressure, tear amount and oxidant and antioxidant parameters of eye tissue. Study groups consisted of 2 groups, Group 1 (Control) and Group 2 (Urographin), and a total of 16 Wistar albino female rats were used. On the first day of the experiment, 6 ml/kg of physiological saline was administered intravenously to the control group, and the same dose of contrast agent was administered to group 2. Intraocular pressure and tear amounts were measured at 1, 6, 12, 24 and 48 hours after intravenous administration. After measurements were made at the 48th hour of the experiment, all rats were euthanized and their eye tissues were removed. In order to reveal the oxidative damage and antioxidant activity in the eye tissue, malondialdehyde and reduced glutathione levels, catalase and glutathione peroxidase enzyme activities were measured spectrophotometrically. Schimer tear test (STT-1) strip was used for tear amount measurements, and rebound tonometer Tonovet® was used as tonometer for intraocular pressure measurements.

How to cite this article
Cellat, M., İşler CT. (2022). The effects of intravenous contrast substance administration on intraocular pressure, tear amount and oxidative stress in rats. *Journal of Advances in VetBio Science and Techniques, 7*(2), 169-178. https://doi.org/10.31797/vetbio.1087898

Research Article

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Article info
Submission: 15-03-2022
Accepted: 24-07-2022
Online First: 04-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

No statistically significant difference was found between the control and urographin groups in terms of intraocular pressure and tear amounts in the measurements performed at 1,6,12,24 and 48th hours after urographin administration. It was observed that the same application significantly increased the malondialdehyde level (P<0.005) in the eye tissue. There was no significant difference between the groups in terms of reduced glutathione level and catalase and glutathione peroxidase enzyme activities in eye tissue. It was evaluated that intravenous contrast agent administration causes oxidative stress in the eye tissue and this may have a long-term ocular effect.

Keywords: Contrast agent, eye, oxidative stress.

GİRİŞ

Radyografik kontrast maddeler, radyografi ve bilgisayarlı tomografi gibi X-ışını tabanlı görüntüleme tekniklerinde iç organ ve yapıların görünürlüğünü artırmak için kullanılan tıbbi ajanlardır. İyot bazlı kontrast maddeler genellikle iyonik veya iyonik olmayan ve monomerik ve dimerik olarak sınıflandırılır ve genellikle damarları, dokuları, organları ve idrar yolunu görselleştirmek için kullanılır. Normal ve patolojik alanları ayırt etmede yardımcı olurlar. Radyografik kontrast maddenin yan etkileri, kaşıntı gibi hafif bir rahatsızlıktan hayati tehlike arz eden bir acil duruma kadar değişebilir (Lightfoot vd., 2009). Radyokontrast medya (RCM) radyolojik prosedürlerde sıklıkla kullanılır. Ancak RCM'nin yan etkisi bu maddenin kullanımını sınırlar (Yeşildağ vd., 2009). Kontrast maddenin en önemli yan etkileri aşırı duyarlılık reaksiyonları, tiroid fonksiyon bozukluğu, kontrast kaynaklı nefropati (Thomson ve Varma, 2010) ve hepatotoksisitedir (Yeşildağ vd., 2009). Ayrıca iyotlu kontrast madde uygulamasından sonra submandibular tükürük bezlerinin ağrısız bilateral genişlemesi ile karakterize olan sialadenitis komplikasyon olarak gelişebilir (Nazzaro vd., 2013). RCM'nin neden olduğu hepatotoksisitenin patogenezi tam olarak açıklanamamakla birlikte oksidatif stresin etkili olabileceği bildirilmektedir (Yeşildağ vd., 2009). İyot, yüksek kontrast yoğunluğuna sahip kontrast maddelerde kullanılan önemli bir elementtir. Tipik radyolojik prosedürde kullanılan bir kontrast madde dozu, vücutta serbest iyodür olarak serbest bırakılabilen yaklaşık 13500 µg serbest iyodür ve 15 ile 60 gr arasında değişen bağlı iyot içerir (Molen vd., 2004). Bu aslında önerilen günlük iyodür alımının (150μg) 90 ila birkaç yüz bin katı akut iyodür yüküdür (Trumbo vd., 2001). Plazma proteinlerine düşük oranda bağlanma kapasitesine sahip olan iyotlu kontrast maddeler böbrek fonksiyonları normal olan insanlarda yaklaşık 90-120 dakika arasında vücuttan elimine edilebilirken böbrek fonksiyon bozukluğu olan insanlarda bu süre haftalara uzayabilmektedir (Aydın vd., 2020). Enjekte edilen iyodürün %98'i böbrekler tarafından elimine edilir ve tükürük, ter ve gözyaşı bezleri gibi diğer organlardan sadece %2'si atılır. Normalde, enjekte edilen kontrast madde dozu, sialadenitise neden olmak için yeterince yüksek bir iyodür konsantrasyonu sağlamaz, ancak kontrast maddenin böbreklerden atılımının bozulması, iyodür serbest kalmasına neden olur ve tükürük bezlerinin inorganik iyodür konsantre etmesine olanak tanır (Nazzaro vd., 2013). Kontrast maddenin renal kan akışını azalttığı ve renal arter vazokonstriksiyonu yoluyla iskemik reperfüzyon hasarına neden olduğu gösterilmiştir (Zhao vd, 2016). Artan oksidatif stres ve azalan nitrik oksit üretimi kontrast madde nefrotoksisitesinin patogenezinde önemli rol oynamaktadır (Agmon vd., 1994; Myers vd., 2006). Oksidatif stres, oksidanların üretimi ile hücrelerin antioksidan savunma potansiyeli ve ayrıca hasar onarım mekanizmalarının fazla oksidanları destekleme yeteneği arasındaki bir dengesizlik olarak tanımlanır. Bu dengesiz durum doku hasarına neden olabilir veya tüm hücre

bileşenlerinde toksik türlerin üretilmesine neden olabilir (Jensen 2003; Sies 2018). Sağlıklı gözlerde, kontrollü oksidan üretimi, sinyal yollarının koordinasyonunda hayati önem taşır (Sies vd., 2017). Farklı kuru göz hayvan modellerinde konjonktiva ve gözyaşı filminde reaktif oksijen türleri (ROS), oksidatif stres belirteçleri ve inflamatuar hücrelerde artış olduğu vurgulanmaktadır (Choi vd., 2016; Pinazo Duran vd., 2014; Yarosz ve Chang, 2018).

Kuru göz hastalığı, günlük aktivitelerini ciddi şekilde engelleyebilecek hastalık semptomları ile hastaların görme ve yaşam kalitesi üzerinde büyük etkisi olan, çok faktörlü, sıkıntılı bir durumdur (Baudouin vd., 2018). Artmış oksidatif hasarın farelerde gözyaşı bezinin fonksiyonel olarak azalmasına ve kuru göz hastalığına neden olduğu bildirilmektedir (Draper vd., 1998; Rios vd., 2005). Çok sayıda araştırma, oksidatif stresin katarakt, retrolental fibroplazi, glokom, yaşa bağlı makula dejenerasyonu, diyabetik retinopati, otoimmün ve inflamatuar üveit, endotelyal korneal distrofi ve granüler korneal distrofi tip 2, retina ışık hasarı, prematüre retinopatisi ve kanser gibi çeşitli oküler durum ve hastalıkların patogenezinde rol oynadığını göstermiştir (Dogru vd., 2018; Reuter vd., 2010; Tangvarasittichai ve Tangvarasittichai, 2018; Ung vd., 2017).

Gözün optik özelliklerini korumak ve iç dokulara biyomekanik destek sağlamak için göz içi basıncı (GİB) gereklidir. Başlangıç çizgisinden sapmalar, GİB değişikliğinin büyüklüğüne, yönüne ve süresine bağlı olarak çeşitli görme sorunlarına neden olabilir. Oküler hipotansiyon retina dekolmanlarına neden olabilirken (Fine vd., 2007), oküler hipertansiyon retina ve optik sinirde glokomatöz dejenerasyona neden olabilir (Morrison vd., 2011). Aşırı oksidatif stres GİB yükselmesine katkıda bulunabilir ve bunun tersi de geçerlidir. Kontrolsüz oksidatif stres ve antioksidan savunma eksikliği, yüksek GİB'de mitokondriyal yetmezliğin ve hücre kaybının başlıca nedenleri olabilir. Spesifik olarak, ön kamaradaki ROS, aköz hümör çıkışını bozabilir ve ardından GİB yükselmesine neden olabilir (Fahy vd., 2016; Knox vd., 2007; Pease vd., 2000; Salinas Navarro vd., 2010). Hem oküler hipertansiyonu hem de birincil açık açılı glokomu olan hastalarda reaktif oksijen türlerinde artış ve antioksidan kapasitede azalma olduğu görülmüştür (Jabbehdari vd., 2021).

Bu çalışmada intravenöz kontrast madde uygulamasının göz içi basıncı, göz yaşı miktarı ve göz dokusu oksidan ve antioksidan parametreler üzerindeki etkisinin araştırılması amaçlandı.

MATERYAL VE METHOD

Hayvan materyali

Çalışmada; HMKÜ Deney Hayvanları Merkezi'nden temin edilen 180-250 g ağırlığında 32 adet wistar albino ırkı dişi rat kullanıldı. Deneysel uygulamalar laboratuvar hayvanlarının bakım ve kullanım şartlarına (12 saat aydınlık-12 saat karanlık ve 21±1 oC) uygun olarak yürütüldü. Ratlara deneysel uygulamalar süresince standart ticari yem (pelet yem) ve musluk suyu ad-libitum olarak sağlandı.

Deney grupları

Bu çalışmada her grupta 8 rat olmak üzere 2 grupta toplam 16 adet wistar albino ırkı dişi rat kullanıldı. Çalışma grupları Grup 1 (Kontrol grubu) ve Grup 2 (Urografin) olmak üzere toplam 2 gruptan oluştu. Çalışma başlamadan önceki 48 saatlik zaman diliminde ratlar sudan mahrum bırakıldı. Ratlarda kontrast maddenin kullanım dozu Özbek ve ark. (Özbek vd., 2015)'nın makalesi esas alınarak planlandı. Gözyaşı miktarı ölçümünde schimer tear test (STT-1) stripi kullanıldı. Strip alt göz kapağı medial kantusuna yerleştirildi, bir dakika beklendi ve süre sonunda stripteki ıslanan kısım okunarak gözyaşı miktarı belirlendi. Göz içi

basıncı ölçümü ise tonometre ile yapıldı. Tonometre olarak rebound tonometre Tonovet® (Icare Finland Oy, Vantaa, Finland) kullanıldı.

Grup 1 (Kontrol Grubu): Bu gruptaki ratlara denemenin ilk günü 6 ml/kg dozda ve tek doz olmak üzere serum fizyolojik kuyruk veninden intravenöz olarak uygulandı. İntravenöz serum fizyolojik uygulamasından sonraki 1,6,12,24 ve 48. saatlerde göz içi basıncı ve gözyaşı miktarları ölçüldü.

Grup 2 (Urografin): Bu gruptaki ratlara denemenin ilk günü 6 ml/kg dozda ve tek doz olmak üzere iyonik yüksek ozmolar kontrast madde (Urografin %76, 50 ml, meglumin/sodyum diatrizoat, Bayer, Germany) kuyruk veninden intravenöz olarak uygulandı. İntravenöz urografin uygulamasından sonraki 1,6,12,24 ve 48. saatlerde göz içi basıncı ve göz yaşı miktarları aynı şekilde ölçüldü.

Denemenin 48. saatinde göz içi basıncı ve göz yaşı miktarı ölçümleri yapıldıktan sonra ratlar anestezi (ketamin (60 mg/Kg İM) + ksilazin (10 mg/Kg İM) altında dekapitasyon yöntemi kullanılarak sakrifiye edildi ve göz dokuları çıkarıldı. Alınan göz doku örnekleri serum fizyolojik ile yıkandı ve biyokimyasal analizler yapılıncaya kadar derin dondurucuda (-80 °C) saklandı.

Göz dokusu MDA ve GSH düzeyi ile CAT ve GSH-Px enzim aktivite analizleri

Derin dondurucudan (- 80°C) çıkarılan dokularda oksidatif hasar ve antioksidan aktivite durumunu ortaya koyabilmek için spektrofotometrik olarak malondialdehit (MDA) ve redükte glutatyon (GSH) düzeyleri ile katalaz (CAT) ve glutatyon peroksidaz (GSH-Px) enzim aktiviteleri incelendi. Lipid peroksidasyonu seviyesi, tiyobarbitürik asit reaktif maddeler konsantrasyonuna göre ölçüldü ve elde edilen MDA miktarı, lipid peroksidasyonunun bir indeksi olarak kullanıldı. MDA seviyesi 532 nm'de protein gramı başına nanomol cinsinden ifade edildi (Placer vd., 1966). GSH düzeyi, Sedlak ve Lindsay tarafından tanımlanan yöntem kullanılarak ölçüldü (Sedlak ve Lindsay, 1968). GSH seviyesi 412 nm'de protein gramı başına nanomol olarak ifade edildi. Glutatyon peroksidaz (GSH-Px, EC 1.11.1.9) aktivitesi, Lawrence ve Burk tarafından tanımlanan metoda göre belirlendi (Lawrance ve Burk, 1976). 340 nm'de GSH-Px enzim aktivitesi, gram protein başına uluslararası birimler olarak ifade edildi. Katalaz (CAT, EC 1.11.1.6) aktivitesi, 240 nm'de hidrojen peroksit (H202) ayrışımının ölçülmesi ile belirlendi ve kg/protein olarak ifade edildi (Aebi, 1983). Protein analizleri için Lowry ve ark.'nın metodu kullanıldı (Lowry vd., 1951).

İstatistiksel analizler

İstatistiksel analiz SPSS programı (22.0 software, IBM) kullanılarak yapıldı. Çalışmadan elde edilen tüm parametreler ortalama±standart sapma (SD) olarak sunuldu. Veri dağılımının normalliği Shapiro-Wilk testi ve varyansın homojenliği Levene testi ile değerlendirildi. Grup içi istatistiksel değerlendirmede tek yönlü varyans analizi (ANOVA) ve posthoc Tukey testi kullanıldı. Gruplar arası değerlendirme ise bağımsız t testi ile analiz edildi. $p<0.05$ Değeri istatistiksel önem derecesi olarak kabul edildi.

BULGULAR

Makroskobik bulgular

Çalışmada kullanılan hayvanların çalışma öncesinde genel sağlık ve göz hastalıkları yönünden yapılan kontrollerde herhangi bir patolojik bulguya rastlanılmadı. İlk uygulamalar sırasında schimer stripleri yerleştirilirken deney hayvanları bu uygulamalara karşı savunma refleksleri gösterdi ve uygulamalarda zorluklar yaşandı. Daha sonra muayeneye adapte oldular ve uygulamalar kolaylıkla gerçekleştirildi. STT miktarının ürografin grubunda makroskobik olarak arttığı gözlendi ise de istatistiksel olarak anlamlı farklılık saptanmadı.

Göz içi basıncı bulguları

Tonometre ile yapılan göz içi basıncı ölçümlerinde her iki gruptaki ratların sağ ve sol gözleri arasında önemli bir farklılık bulunamadı. Ayrıca kontrol grubu ile ürogrofin grupları arasında göz içi basıncı açısından istatistiki açıdan anlamlı bir farklılık gözlenmedi. Göz içi basıncı ile ilgili bulgular Tablo 1'de sunuldu.

Tablo 1. Göz içi basıncı bulguları

	Sağ göz		Sol göz	
Saatler	Kontrol	Ürografin	Kontrol	Ürografin
1.saat	11.06±0.42	11.13±0.38	11.11±0.29	11.31±0.38
6.saat	11.01±0.33	11.26±0.30	11.09±0.29	11.43±0.30
12.saat	11.07±0.41	11.29±0.18	11.34±0.36	11.34±0.28
24.saat	10.90±0.21	11.16±0.25	10.94±0.35	11.14±0.30
48.saat	10.93±0.31	11.04±0.34	10.96±0.32	11.17±0.13

Göz yaşı miktarı bulguları

Schimer tear test ile yapılan gözyaşı miktarı ölçümlerinde göz içi basıncında olduğu gibi her iki grubun sağ ve sol gözleri arasında farklılık saptanmadı. Yine kontrol ve ürografin grupları göz yaşı miktarları açısından kıyaslandıklarında istatistiki açıdan anlamlı bir farklılık tespit edilemedi. Gözyaşı miktarı ile ilgili bulgular Tablo 2'de sunuldu.

Tablo 2. Gözyaşı miktarı bulguları

	Sağ göz		Sol göz	
Saatler	Kontrol	Ürografin	Kontrol	Ürografin
1.saat	2.07±1.24	2.43±0.98	2.07±1.24	2.43±0.98
6.saat	2.86±0.90	2.71±0.76	2.86±0.90	2.71±0.76
12.saat	2.71±1.11	2.86±0.90	2.71±1.11	3.00±1.15
24.saat	1.57±0.79	2.57±0.98	1.57±0.79	2.57±0.98
48.saat	2.86±0.69	2.86±0.69	2.86±0.69	2.86±0.69

Göz dokusu MDA ve GSH düzeyi ile CAT ve GSH-Px enzim aktivite bulguları

Ürogrofin grubunun göz dokusu MDA düzeyi kontrol grubu ile kıyaslandığında ürografin grubunda MDA düzeyinin istatistiki açıdan anlamlı derecede arttığı belirlendi (P<0,005). Ürografin grubunda göz dokusu GSH düzeyi ile GSH-Px ve CAT enzim aktivitelerinin azaldığı fakat kontrol grubu ile kıyaslandığında istatistiki açıdan anlamlı bir farkın olmadığı tespit edildi. Göz dokusu MDA ve GSH düzeyi ile CAT ve GSH-Px enzim aktivite bulguları Tablo 3'de sunuldu.

Tablo 3. Göz dokusu MDA ve GSH düzeyi ile CAT ve GSH-Px enzim aktivite bulguları

Parametre	Kontrol	Urografin
MDA (nmol/g prot)	10.93±1.07	12.59±1.07*
GSH (nmol/g prot)	2.61±0.75	2.18±0.39
GSH-Px (IU/gr prot)	25.32±2.80	23.82±2.44
CAT (k/g prot)	30.74±2.94	29.67±1.80

*; gruplar arası istatistiksel farkı gösterir (p<0.05). MDA, malondialdehit; GSH, redükte glutatyon; CAT, katalaz; GSH-Px, glutatyon peroksidaz.

TARTIŞMA

Urografin, aktif bileşen sodyum diatrizoat meglumindiatrizoat içeren iyonik bir radyo-kontrast ajandır (Kepner vd., 2012). Radyokontrast ajanlar sıklıkla endirekt radyografi, anjiyografi, intravenöz ürografi ve bilgisayarlı tomografi gibi radyolojik prosedürlerde kullanılır (Başarslan vd., 2013; Sütçüoğlu vd., 2019). Klinik uygulamalarda yaygın olarak kullanılmakta olan iyotlu radyo kontrast ajanlar genellikle güvenli olmalarına rağmen ciddi yan etkilere ve ilaç reaksiyonlarına da sebep olabilmektedir (Yi Wei vd., 2016). Bu yan etkiler kontrast maddenin uygulanmasından hemen sonra ortaya çıkabildiği gibi geç dönemde ciddi reaksiyonlar şeklinde de ortaya çıkabilmektedir (Cintaş vd., 2019). Radyolojide, intravenöz kontrast uygulamasına karşı şiddetli akut reaksiyonların genel insidansı, uygulanan ajanın sınıfına bağlıdır (Kepner vd., 2012). Kontrast ajanların aşırı duyarlılık reaksiyonları, mast hücrelerinin aktivasyonu, pıhtılaşma, kinin ve kompleman mekanizmaları, enzimlerin inhibisyonu ve trombosit agregasyonu ile hem Ig E hem de Ig E aracılı olmayan anafilaksiyi içerir (Thomson ve Varma, 2010). Anjiyografide ürografin (sodyum amidotrizoat) kullanılmasının oküler myastenia gravise neden olabileceği bildirilmektedir (Modi vd., 2016). Sodyum-meglumin diatrizoatın damar içi uygulamasının iki taraflı görme bulanıklığı, tek taraflı orbital ödem ve iki taraflı yoğun konjonktival tıkanıklık gibi oküler yan etkiler gösterebilmektedir (Sharma vd., 1990). Kontrast madde uygulamalarının aşırı duyarlılık reaksiyonları ve allerji (Persson, 2005), nefrotoksisite (Sheriff vd., 2018) ve hepatotoksisite (Yeşildağ vd., 2009) gibi yan etkilere sebep olabileceği ifade edilmektedir. Radyokontrast maddelerin karaciğer ve böbrek gibi dokularda meydana getirdiği toksisitelerin temel mekanizmalarında oksidatif stresin önemli role sahip olduğu vurgulanmaktadır (Akyol vd., 2014; Yeşildağ vd., 2009).

İntravenöz kontrast madde uygulamasının böbrek dokusu MDA (Özbek vd., 2015; Sheriff vd., 2018) düzeyini anlamlı derecede arttırdığı, GSH düzeyini ise azalttığı bildirilmektedir (Sheriff vd., 2018). Başka bir araştırmada ise aynı uygulamanın karaciğer dokusu MDA düzeylerinde artışa, GSH düzeyi ve CAT enzim aktivitesinde azalmaya neden olduğu gösterilmiştir (Başarslan vd., 2012). Serbest oksijen radikallerine bağlı lipid peroksidasyonu, hücre zarı hasarının ve hücre yıkımının en önemli nedenidir. Lipid peroksidasyon derecesi, MDA seviyeleri ölçülerek belirlenebilir (Özbek vd., 2015). Bu çalışmada ürografin grubundaki ratların göz dokusu MDA düzeylerinin kontrol grubuna göre istatistiki açıdan anlamlı derecede arttığı görüldü. Aynı grubun GSH düzeyi ve CAT ve GSH-Px enzim aktivitelerinde kontrol grubuna göre azalmaların olduğu fakat bu azalmaların istatistiki açıdan anlamlı olmadığı belirlendi. Bu çalışmanın MDA sonuçları incelendiğinde intravenöz kontrast madde uygulamasının göz dokusunda oksidatif strese sebep olabileceği değerlendirildi.

GİB, aköz hümör üretim hızı ve gözden çıkma hızı ile belirlenir. Gözü şişirmek ve kürenin şeklini ve optik özelliklerini korumak için GİB gereklidir (Goel vd., 2010). Aköz hümörün çıkış yoluna artan direncin GİB yükselmesine neden olabileceği bildirilmektedir (Johnson, 2006). Bazı araştırıcılar GİB'nin geceleri gündüze göre daha yüksek olduğunu bildirmiş, bunun da GİB sirkadiyen ritminin varlığından kaynaklanabileceğini öne sürmüşlerdir (Lozano vd., 2015; Valderrama vd., 2008). Glokom araştırmalarında bu durum için kabul edilen risk faktörü olan göz içi basıncının doğru ve tekrarlanabilir ölçümlerini gerektirir (Mermoud vd., 1994). Albino sıçanlar, glokom gibi göz rahatsızlıklarının araştırılmasında ve ayrıca kimyasalların ve ilaçların klinik olmayan toksisitesine ilişkin genel çalışmalarda yaygın olarak

kullanılmaktadır (Morita vd., 2020). Son on yılda, kontakt yöntemlerle yapılan tonometri doğru ve tekrarlanabilir olsa bile GİB değerlendirmenin en popüler yöntemi rebound tonometrisidir. Geri tepme tonometrisinin tercih edildiği önemli nokta, cihazın laboratuvar hayvanları da dahil olmak üzere yeni evcil hayvanlarda kullanım kolaylığıdır. Bunlarda, GİB rebound tonometri ile ölçüldüğünde kornea anestezisinin kullanılması zorunlu değildir (Rodrigues vd., 2021; Yakan vd., 2021). Sıçanlarda topikal anestezinin ortalama GİB üzerinde önemli değişikliğe yol açmadığı bildirilmiştir (Kim vd., 2013). Anestezik ve preanestetik ajanların GİB ve gözyaşı üretiminde azalmaya neden olduğu da ifade edilmektedir (Ghaffari vd., 2010). İntravenöz kontrast madde uygulamasının göz içi basıncına etkisi ile ilgili literatür bilgisi bulunmamaktadır. Bu çalışmada intravenöz ürografin uygulamasından sonraki 1,6,12,24 ve 48. saatlerde rebound tonometre kullanılarak ratların sağ ve sol gözlerinde GİB değerleri ölçüldü. Yapılan GİB ölçümlerinde sağ ve sol gözler arasında herhangi bir farklılık saptanmadı. Kontrol ve ürografin grubu ratlarda 1,6,12,24 ve 48. saatlerde yapılan GİB ölçümlerinde gruplar arasında herhangi bir istatistiki fark belirlenemedi. Çalışmanın GİB sonuçları incelendiğinde intravenöz kontrast madde uygulamasının ratların sağ ve sol gözlerinde akut olarak GİB değerleri üzerinde değişikliğe sebep olmadığı tespit edildi.

Gözyaşının nicel ve nitel değerlendirmeleri göz muayenesi için kritik öneme sahiptir (Gilger ve Stoppini, 2011). Tedavide kullanılan ilaçların gözyaşı akışı üzerindeki etkilerini anlamak önemlidir. Anestezik ve preanestetik ajanların gözyaşı üretiminde azalmaya neden olduğu bildirilmektedir (Ghaffari vd., 2010; Kanda vd., 2019). Birtakım ilaçlar gözyaşı üretimini azaltarak keratokonjonktivit siccaya neden olabilir (Margadant vd., 2003). Xylazin, detomidin, butorfanol uygulanması STT değerini düşürür. Romifidin ise gözyaşı üretimini etkilemez (Leonardi vd., 2020). Oftalmik hastalık öyküsü olmayan köpeklerde intravenöz medetomidin ve medetomidin-butorfanol ile intravenöz sedasyonun Schirmer gözyaşı testi (STT) ile yapılan ölçümlerde gözyaşı miktarında azalmaya neden olduğu fakat bu azalmanın geçici olduğu bildirilmiştir (Sanchez ve Mellor, 2006). Kedilerde asepromazin veya ksilazin ile yapılan sedasyonda her iki grupta da ortalama gözyaşı üretiminin istatistiksel olarak anlamlı şekilde düştüğü bildirilmektedir (Ghaffari vd., 2010). Bu çalışmada schimer tear test (STT-1) stripi kullanılarak yapılan gözyaşı miktarı ölçümlerinde kontrol ve ürografin gruplarındaki ratların sağ ve sol gözleri arasında farklılık saptanmadı. 1,6,12,24 ve 48. Saatlerde yapılan gözyaşı miktarı ölçümlerinde kontrol ve ürografin grupları arasında istatistiki olarak bir fark tespit edilemedi.

SONUÇ

Bu çalışmada intravenöz iyonik yüksek ozmolar kontrast madde uygulamasının göz içi basıncı, göz yaşı miktarı ve göz dokusu oksidan ve antioksidan parametreler üzerindeki olası etkileri araştırıldı. Bu konu ile ilgili olarak daha önce kaydedilmiş bir literatür bilgisi bulunmamaktadır. Çalışma bulguları incelendiğinde intravenöz ürografin uygulamasının akut olarak göz dokusunda MDA düzeyinde artışa ve oksidatif strese neden olduğu görüldü. Ayrıca bu uygulamanın gözyaşı miktarı ve göz içi basıncında herhangi bir değişikliğe neden olmadığı tespit edildi. Oksidatif stresin farelerde gözyaşı bezlerinin fonksiyonunu bozduğu ile ilgili literatür bilgisi bulunmaktadır. Ayrıca intravenöz olarak verilen iyotlu kontrast maddenin %98'inin böbrekler tarafından elimine edildiği ve geri kalan %2'lik kısmın ise tükürük, ter ve gözyaşı bezleri

tarafından atıldığı ile ilgili literatür bilgileri vardır. Böbrek yetmezliği olanlarda kontrast maddenin atılımında tükürük, ter ve gözyaşı bezlerinin daha fazla etkileneceği değerlendirilmektedir. Tek doz veya tekrarlanan dozlarda ürografin uygulamalarının daha uzun sürede göz içi basıncı ve gözyaşı miktarında değişimlere sebep olabileceği ve bu nedenle değişik doz ve tekrarların uygulandığı yeni araştırmaların yapılması gerektiği değerlendirildi. Ayrıca deneysel böbrek hasarı oluşturulmuş deney hayvanlarında intravenöz kontrast maddenin göz üzerine etkileri ile ilgili araştırmaların yapılması önem arz etmektedir.

AÇIKLAMALAR

Etik beyan: Bu çalışma, Hatay Mustafa Kemal Üniversitesi Hayvan Deneyleri Yerel Etik Kurulunun 31.12.2021 tarihli toplantısında almış olduğu 2021/07-02 numaralı izin kararı ile yapıldı.

Çıkar çatışması: Yazarlar bu çalışma için herhangi bir çıkar çatışması olmadığını beyan ederler.

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Investigation of milk origin in sheep and goat cheese with Real-Time PCR

Research Article

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Article info
Submission: 15-01-2022
Accepted: 12-07-2022
Online First: 04-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

Milk and dairy products are very important in life, because they contain important nutrients for human health. Recently, some producers have used cow's milk instead of goat and sheep's milk for producing goat and sheep cheese and sell these cheeses as sheep and goat cheese. In this study, it was aimed to determine the milk type of white cheese and tulum cheese, which are sold under the names of sheep cheese and goat cheese in the markets and supermarkets of Afyonkarahisar and Antalya. In this study, the Real-Time PCR analysis method was utilised to detect fraud in products. For the analysis, 60 goat cheese samples and 60 sheep cheese samples in total were collected from the aforementioned provinces. As a result of the analysis, among all goat cheese samples which were obtained from both provinces, only 20% were produced from goat milk, 38.33% were produced from goat-cow's milk mixtures, and 41.67% were produced using cow's milk only. In the sheep cheese samples from both provinces, only 18.33% were produced from sheep's milk, 50.00% were produced from sheep's-cow's milk mixtures, and 31.67% were produced from cow's milk only. Consequently, regular inspections and controls are required to detect counterfeit cheeses and we recommend that consumers be made aware of these cheats.

Keywords: Cheese, adulteration, imitation, Real-Time PCR

INTRODUCTION

Cheese is an important dairy product which has high nutritional value, is a concentrated form of milk and contains more nutrients than milk per volume (Walther et al., 2008). It is a 99% digestible food for all age groups (Kosikowski, 1982). There are more than 4000 varieties of cheese all around the world, and Turkey produces around 100 varieties of it (Steele and Unlu, 1992; Coskun, 2005). The highest percentage of cheese is produced by using cow's milk, while cheese can also be made using goat and sheep's milk. Sheep's and goat milk is mostly used in local and special cheese production (Guney and Kaymakci, 1997).

Quality and safe food consumption is one of the most fundamental rights and freedoms for human safety. Nowadays, with the development of technology, adulterations applied on foods are increasing, and it is very difficult to detect counterfeit products. Food authenticity and safety problems like imitation and adulteration in the agriculture and food industry are a problem not only in Turkey but also in other countries (Ertas and Topal, 2009). Reasons for practicing imitation and adulteration activities in the food sector may include the purpose of functional food production and similar purposes, while these processes may also reduce the health risks and increase the shelf life of food products (Ertas and Topal, 2009).

How to cite this article
Sadıgzade Zengin, Z., Kara R. (2022). Investigation of milk origin in sheep and goat cheese with Real-Time. *Journal of Advances in VetBio Science and Techniques, 7*(2), 179-184.
https://doi.org/10.31797/vetbio.1057572

Recently, food safety, quality and composition have become an important issue for consumers, and for the consumers of today, the authenticity of food has an important place. Therefore, especially in animal products, identification of animal species has gained increasing importance (Dalmasso et al., 2012). Consumers are expected to believe the vendors and what is written on the labels because the authenticity of some products is not directly observable (Mayer et al., 2012). In this study, it was aimed to determine the origins of milk used in cheese sold as goat-sheep cheese by Real-Time PCR.

MATERIAL and METHOD

Cheese Samples

In total, 120 goat cheese and sheep cheese samples were collected from markets and supermarkets in the Afyonkarahisar and Antalya provinces of Turkey between November 2018 and March 2019. Each cheese sample was bought at quantities of about 250-500 g, and 60 goat cheese samples (32 white cheeses / 28 tulum cheeses) and 60 sheep cheese samples (26 white cheeses / 34 tulum cheeses) were gathered. Until the analysis, the cheese samples were stored at -18 ± 2 °C.

DNA Isolation

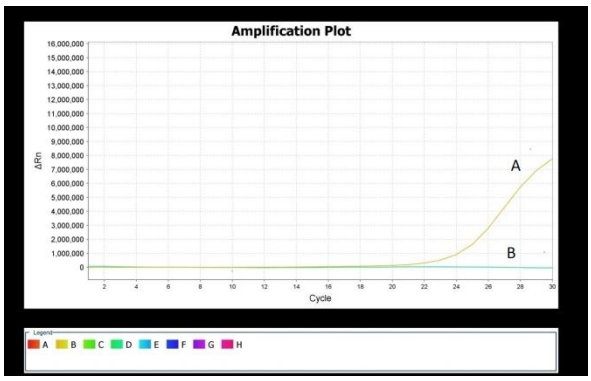
DNA isolation was performed in the cheese samples in accordance with the instructions of the manufacturer of the kit that was used (Genomic DNA Isolation kit, SNP ure Genomic DNA Extraction Kit 1806/001). The method which was applied in the analysis was the spin column procedure. 100 mg of the cheese sample, 300 µl of solution B3 and 25 µl of Proteinase K were added together for the analysis.

The mixture was mixed and incubated at 70 °C for 30 minutes. After the incubation, the product was centrifuged at 13,000 rpm for 2 minutes. At the isolation stage, approximately 400 µl was taken from the middle phase of the 3 phases that formed and transferred to a 1.5-ml tube. 250 µl of 95% alcohol was then added to the tube and mixed gently, and the contents of the tube were transferred to a new spin column. The samples were centrifuged at 11,000 rpm for 1 min. After centrifugation, the Eppendorf tube was replaced, and 500 µl of Solution WB was added to the column. This process was repeated twice, the columns were then placed in clean new tubes, and 100 µl of the elution solution (preheated) was added to these columns and centrifuged. As a result, DNA samples were obtained and kept at -20 °C.

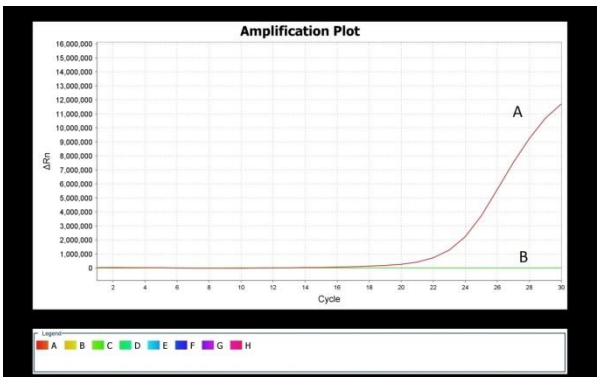
Real-Time PCR Process

Real-Time PCR was performed in the extracted DNA sample in accordance with the instructions of the manufacturer of the kit that was used (SNP Detection Real-Time PCR Kits goat milk-401R-10-01, sheep milk-402R-10-01, cow milk-403R-10-01 in Turkey). Twenty µl master mix and 5 µl (nearly 10-100 ng) of the extracted of DNA sample were added to the strips and gently mixed separately for each sample. The analysis of the prepared mixture was conducted with the program set (95°C - 5 min. Taq Activation; 95 °C - 15 sec. / 60 °C - 1min. = 30 cycles) in the Real-Time PCR (Applied Via 7, Thermo Fisher Scientific).

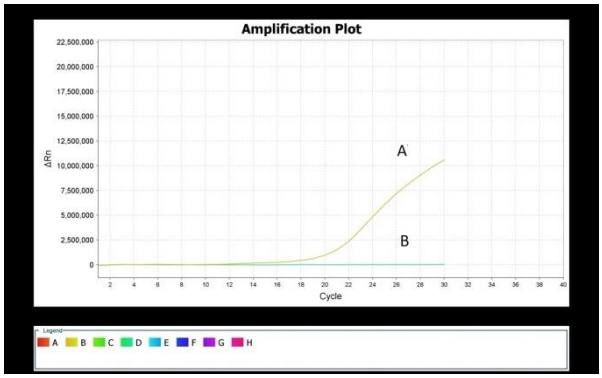
The results were evaluated by analysing the internal control peaks with the VIC-TAMRA dye and sample peaks with the FAM-TAMRA dye. Positive and negative controls were used for each species (Sheep-Goat-Cow) in the analyses, and these control peak images and internal control peak images are shown in Figure 1.



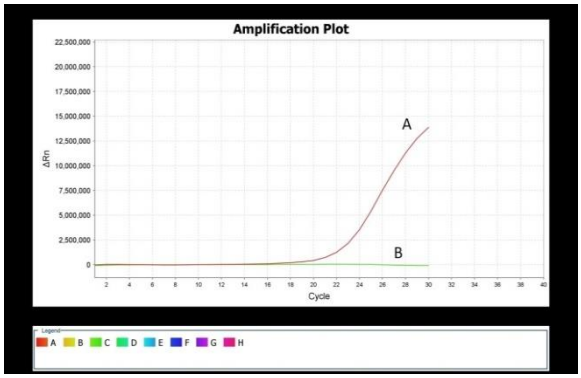
The sorts of goat positive (A) and negative (B) control peaks used goat species analysis



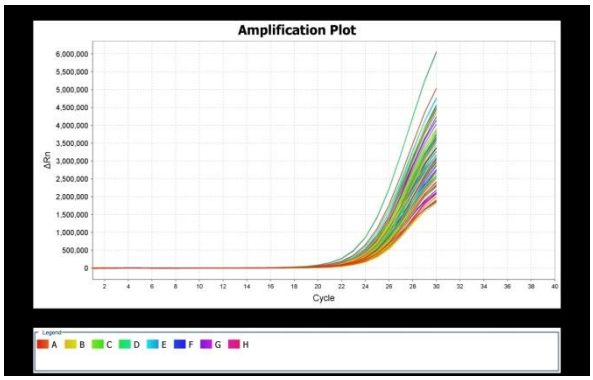
The sorts of cow positive (A) and negative (B) control peaks used goat species analysis



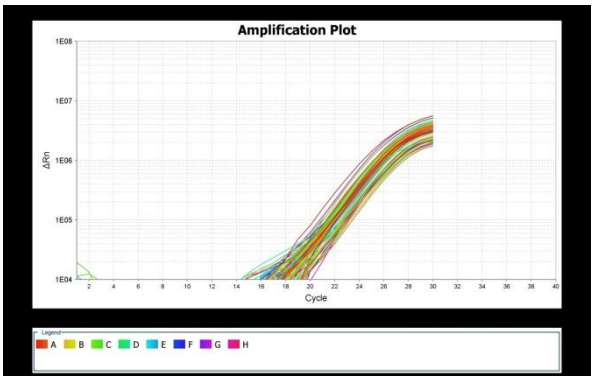
The sorts of sheep positive (A) and negative (B) control peaks used sheep species analysis



The sorts of cow positive (A) and negative (B) control peaks used sheep species analysis



İntegral control peaks in goat cheese samples



İntegral control peaks in sheep cheese samples

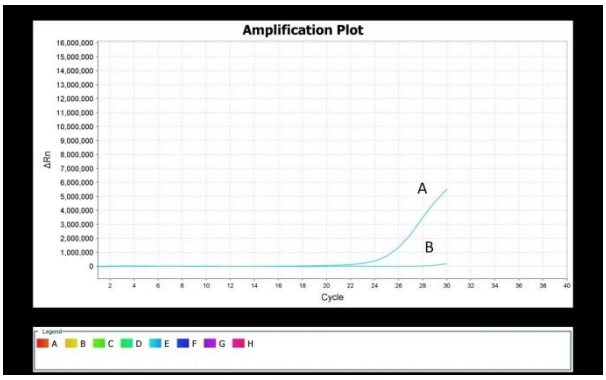
Figure 1. Positive, negative control and internal control peaks of species

RESULTS

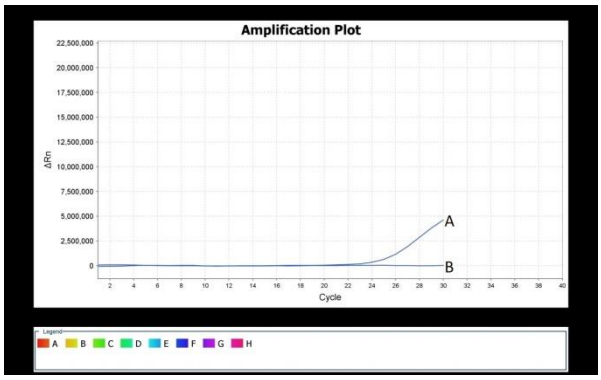
According to the results of the analysis, goat milk was identified in 12 of the 60 goat cheese samples, cow's milk and goat milk mixtures were identified in 23 samples, and only cow's milk was identified in 25 samples. For the sheep cheese samples, sheep's milk was identified in 11 of the 60 sheep cheese samples, sheep's milk and cow's milk mixtures were identified in 30 cheese samples, and only cow's milk was identified in 19 cheese samples. The details of the results of the analysis conducted on the goat and sheep cheese samples are shown in Table 1. The Real-Time PCR image result of the analysed goat and sheep cheese samples is presented in Figure 2.

Table 1. The analysis results of goat and sheep cheeses samples

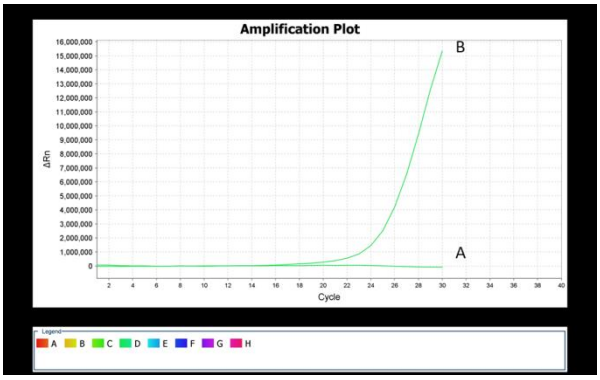
Samples		n	Original (Goat-Sheep) n (%)	Mix n (%)	Cow n (%)
Goat Cheeses	White Cheese	32	10 (31.25)	7 (21.88)	15 (46.88)
	Tulum Cheese	28	2 (7.14)	16 (57.14)	10 (35.71)
	Goat Cheese Total	**60**	**12 (20.00)**	**23 (38.33)**	**25 (41.67)**
Sheep Cheeses	White Cheese	26	4 (15.38)	12 (46.15)	10 (38.46)
	Tulum Cheese	34	7 (20.29)	18 (52.94)	9 (26.47)
	Sheep Cheese Total	**60**	**11 (18.33)**	**30 (50.00)**	**19 (31.67)**
TOTAL		**120**	**23 (19.17)**	**53 (44.57)**	**44 (36.67)**



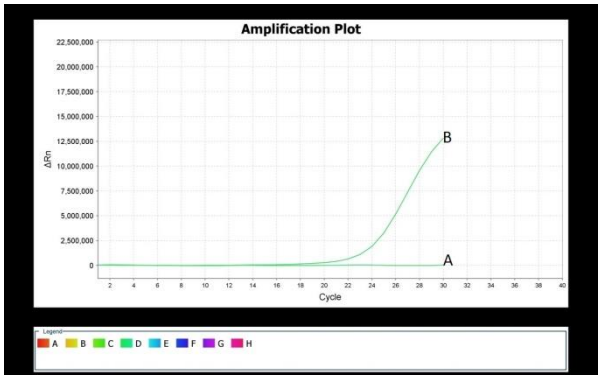
Goat type positive (A) and cow type negative (B) in the goat cheese sample



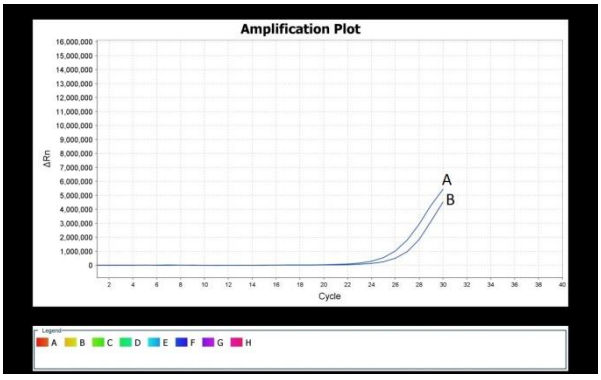
Sheep type positive (A) and cow type negative (B) in sheep cheese sample



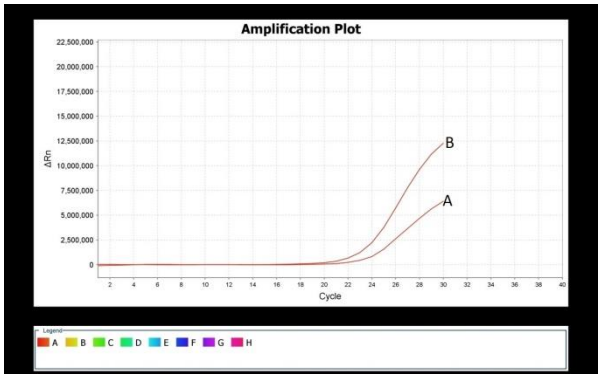
Goat type negative (A) and cow type positive (B) in the goat cheese sample



Sheep type negative (A) and cow type positive (B) in sheep cheese sample



Goat (A) and cow (B) type positive in the goat cheese sample



Sheep (A) and cow (B) type positive in sheep cheese sample

Figure 2. The result of goat and sheep cheese samples in Real Time PCR

DISCUSSION

There are many similar studies about determining the presence of various milk types in dairy products using various methods. In their study conducted in the Czech Republic, Maskova et al. (2006) determined that only 3 of 17 cheese samples sold as goat cheeses were actually goat cheeses, and only 1 of 7 cheese samples sold as sheep cheeses were actually sheep cheeses by using the PCR method. Kara et al. (2016) reported in the province of Afyonkarahisar in Turkey that, based on their Real-Time PCR results, among 100 cream samples sold as buffalo cream, only 28% were produced from buffalo-cattle milk, and 59% were produced by from bovine milk. In their study conducted in Italy, Bottero et al. (2003) used the multiplex PCR method and found cow's milk in 21% of 19 dairy products sold as goat dairy products.

Colak et al. (2005) analysed 100 cheese samples sold as sheep cheeses, and 48% of the samples were found to contain mixtures of cow's milk and sheep's milk, and 52% of the samples contained sheep's milk, according to the researchers' immunochromatographic test conducted in Istanbul.

Darwish et al. (2009) determined by using the PCR method that 47.62% of 21 samples of milk sold as water buffalo milk contained water buffalo milk, 14.29% contained cow's milk, and 38% contained water buffalo-bovine milk. These milk samples were bought in local Egyptian markets. Khanzadi et al. (2013) detected the undeclared presence of cow's milk in 31.5% (33) and goat milk in 65% (68) of 105 dairy product samples sold as sheep's milk products using the PCR method. These 105 sheep milk and dairy products were bought from markets in the Mashhad city of Iran. Zachar et al. (2011) analysed 30 sheep cheese samples in Slovakia and found that 12 samples contained mixtures including cow's milk.

According to Di Pinto et al. (2004), among 30 mozzarella cheese samples sold as buffalo mozzarella, cow milk was detected in 22 (%73.3) samples. The cheese samples were analysed with the PCR method and had been collected from different regional producers in Southern Italy.

Cow's milk is used as a adulteration method in other dairy products as it is more accessible and affordable. Moreover, if different dairy products from different milks are produced in the same production machine, different mixtures of milk types may be detected in analyses on these dairy products. In the literature, different studies have reported the presence of milk from animals other than what was specified by the vendor or the product label in different dairy products that were claimed to be authentic (Ertas and Topal, 2009). Researchers have developed analytical methods especially for detecting the origins of milk and dairy products (Mayer et al., 2012).

In general, immunological, electrophoretic, chromatographic, ELISA and PCR methods are used to determine the origin of meat and dairy products (Zachar et al., 2011). The most common and sensitive method, PCR was used to determine the origin of dairy products in this study. In this study, cow's milk was found in dairy products sold as goat and sheep cheese, and this showed that considerable numbers of these cheeses were imitated and adulterated.

CONCLUSION

Cow's milk DNA was detected in 45% of the 120 cheese samples that were investigated by PCR test in this study. At the same time, as a result of the analysis, mixtures of goat and cow DNA were identified in 38.33% (23) of the goat cheese samples, and mixtures of sheep and cow DNA were identified in 50% (30) of the sheep cheese samples. Accordingly, it was concluded

that cow milk was used in the production of some goat and sheep cheese products. Problems arising in the supply of goat-sheep's milk should be eliminated at first. Especially for the production of authentic and traditional sheep-goat cheese, cow's milk should not be used. In the production processes of dairy products which include different animal sources, the tools, equipment and processes must be separated. If goat, sheep's and cow's milk is used in cheese production, this information should be included on the product label, and consumers should be informed by the vendor about unlabelled products. Inspections and controls on food products should be carried out regularly, and sensitive analyses that provide results in a short time are recommended determining the origins of products.

ACKNOWLEDGMENT

It was supported by the Afyon Kocatepe University Scientific research projects coordination unit (Project code: 18.SAĞ.BIL.28).

This study is summarized from the Master's Thesis (2019/050) with the same name.

This study was presented as a summary report at Turkey 13. Food Congress; (21-23 October 2020, Çanakkale).

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

Conflict of interest: The author declares no potential conflict of interest.

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Investigation of the effects of mesenchymal stem cell administration on liver recovery in experimental hepatotoxicity model

ABSTRACT

Hepatotoxicity refers to liver dysfunction associated with certain medical drugs and chemicals. Studies have shown that mesenchymal stem cells have a positive effect on the improvement of liver diseases. This study aimed to investigate the potential protective effects of fetal kidney-induced mesenchymal stem cells on Doxorubicin-induced hepatotoxicity in rats. Sprague dawley rats were divided into three groups: Control, sham, and treatment group. Intraperitoneally administered mesenchymal stem cells were treated with BrdU before transplantation so that they could be followed up after invivo transplantation. After completion of the experimental steps, the groups were monitored for 5 weeks. Then the rats were terminated and liver tissues were taken for histopathological and immunohistochemical evaluation. In immunohistochemical examinations performed with TNF-α, Caspase-3, and COX-2 primary antibodies, the most severe positivity was in the sham group, followed by the control and treatment groups. While the control and sham groups were found to be statistically similar in immunohistochemical staining with anti-BrdU antibody, the treatment group was found to be significantly different from the other groups ($p<0.05$). As a result, it has been revealed that intraperitoneally administered mesenchymal stem cells prevent degeneration and necrosis in hepatocytes, significantly decreasing TNF-α, COX-2, and Caspase-3 levels thus increasing liver regeneration in rats with Doxorubicin-induced hepatotoxicity.

Keywords: Doxorubicin, Hepatotoxicity, Mesenchymal Stem Cell, Rat

INTRODUCTION

Doxorubicin (DOX) is an anthracycline and an anti-neoplastic agent with a wide range of activity in human cancers (Alegra et al., 2006). Detailed pharmacokinetic studies have been performed in humans and animals. In humans, plasma clearance of DOX is rapid and has substantial tissue binding. DOX is predominantly metabolized in the liver and accumulates mainly in the kidney, but it is also found in the heart, liver, and small intestine (Singh et al., 2015). The application of DOX as a therapeutic drug is limited by its adverse effects on the bone marrow, intestinal epithelium, heart, liver, and kidney, as well as causing cancer cell resistance (Jacevic et al., 2017).

Recently, DOX has been widely used for the treatment of different types of cancer, such as hepatocellular carcinoma, due to its ability to kill transformed liver cells. During the treatment of other cancers, the most common side effect of this drug is DOX-related liver damage. Several experimental studies reported DOX-induced hepatotoxicity.

How to cite this article
Özgermen, BB., Yavuz, O., Ali Evren Haydardedeoğlu AE. (2022). Investigation of the effects of mesenchymal stem cell administration on liver recovery in experimental hepatotoxicity model. *Journal of Advances in VetBio Science and Techniques*, *7*(2), 185-193.
https://doi.org/10.31797/vetbio.1029373

Research Article

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Article info
Submission: 28-11-2021
Accepted: 12-05-2022
Online First: 06-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

Hepatotoxicity refers to the dysfunction of the liver caused by drugs and chemicals or chemotherapeutic drugs. The mechanism of DOX-induced toxicity includes an oxidative stress state characterized by excessive production of reactive oxygen species (ROS) and/or a decrease in antioxidant defenses leading to an imbalance in normal oxygen metabolism (Mansouri et al., 2017).

Once the oxidative stress begins irreversible changes occur, causing hepatocyte apoptosis or tissue necrosis and resulting in increased hepatic enzyme levels in the blood, particularly alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Jacevic et al., 2017). Hepatotoxicity is mainly related to the formation of free radicals, the presence of inflammatory markers, and environmental factors. All these factors contribute to the pathological abnormalities in hepatocytes and result in acute liver disease which, if left undiagnosed, develops into chronic liver disease (CHD). CHD is characterized by the regular destruction and regeneration of hepatic parenchymal cells leading to fibrosis and cirrhosis. CHD is often associated with portal hypertension and liver failure. There is always a high probability for the development of hepatocellular carcinoma (HSC), due to fibrosis and cirrhosis (Iqubala et al., 2016).

Liver transplantation is accepted as the most effective treatment for advanced liver fibrosis. However, the chance of transplantation is limited due to insufficiency of donor organs, surgical complications, immunological rejection, and high medical costs. Recently, it has been suggested that mesenchymal stem cell (MSC) application is an effective alternative for treatment. MSCs have the potential to differentiate into hepatocytes. Its immunomodulatory properties gain therapeutic value with the secretion of trophic factors such as growth factors and cytokines. In addition, MSCs can suppress inflammatory responses, decrease hepatocyte apoptosis, increase regeneration, help regress liver fibrosis, and restore liver function (Kim et al, 2015).

Autologous bone marrow-derived MSC transplantations have been reported for the treatment of liver fibrosis in humans, and improvements in serum albumin levels and liver histology 3-4 months after transplantation have been reported (Amin et al., 2013; Jang et al., 2014). In experimental studies, it has been reported that MSCs obtained from different tissues treat liver failure, increase regeneration, and can be an alternative treatment method to organ transplantation (Kuo et al., 2008; Ramanathan et al., 2017; Gazdic et al., 2018; Luo et al., 2018). No specific and effective therapeutic agent for DOX-related hepatotoxicity is known. This study aimed to investigate the effect of intraperitoneally transplanted fetal kidney-derived MSCs (FKD-MSCs) on the improvement of hepatotoxicity by histopathological and immunohistochemical methods.

MATERIAL and METHOD

Preparation of the FKD – MSCs

To obtain FKD-MSCs, 19-day pregnant rats were subjected to hysterectomy under sterile conditions and the fetuses were obtained. Fetuses were euthanized by providing general anesthesia with ether. The kidneys were removed immediately and the tissues were transferred to the laboratory fresh. The tissues were stored in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Belgium). Fetal kidney tissues were mechanically dissected into small pieces using a sterile scalpel. Tissues were dissected, obtained by explant culture method, then placed in T25 flasks and incubated at 37°C, 5% CO_2 humidified atmosphere. Medium consisting of 77% DMEM (Lonza, Belgium), 20% fetal bovine serum-FBS (Lonza, Belgium), 2% L-Glutamine (Lonza, Belgium), 1% Penicillin, Streptomycin,

Amphotericin (Biological Industries, Israel) added. Non-adherent cells were removed by changing the medium once every 2-3 days. When there was approximately 70% adhesion, the adherent MSCs were passaged and aliquoted 1:2 with 0.25% Trypsin in PBS. The cells were grown to the 3rd passage. Obtained FKD-MSCs were labeled with BrdU before the transplantation.

Animal model

Thirty Sprague Dawley rats (males, 10 weeks old) were randomly divided into 3 groups: control, sham, and treatment. 10 mg/kg DOX dissolved in 0.9% NaCl was administered as a single dose injection through the tail vein in the sham and treatment groups to induce hepatotoxicity. All rats were kept under standard laboratory conditions (21 ± 2°C, 65% humidity, and 12 h light / 12 h dark). The animals were fed ad libitum with standard rat chow and allowed access to water continuously.

Control group (n=10): Did not receive DOX and/or FKD-MSCs. 0.9% NaCl (the same volume as MSC) was administered to the rats intraperitoneally 3 times at one-week intervals.

Sham group (n=10): Received DOX only, no treatment. 0.9% NaCl (the same volume as MSC) was administered to the rats intraperitoneally 3 times at one-week intervals, 7 days after DOX injection.

Treatment group (n=10): Received DOX and FKD-MSCs. 2 x 10^6 MSC injections were administered to the rats intraperitoneally 3 times at one-week intervals, 7 days after DOX injection.

The animals were followed for 5 weeks from the end of the treatment and they were euthanized under general anesthesia with xylazine/ketamine. The experimental protocol was approved by the Local Animal Ethics Committee (Approval number: 2014/55).

Histological study

The liver tissues obtained from the rats were fixed in 10% buffered formol. Paraffin blocks were obtained by embedding in paraffin after dehydration and transparency processes. Sections of 5-micron thickness from the prepared paraffin blocks were stained with Hematoxylin and Eosin (H&E) (Luna, 1968), examined under binocular head light microscopes (Olympus CX23, Tokyo, Japan), and photographed.

In the histopathological examination, H&E-stained sections were evaluated and scored as reported by Öz et al., (2020). In the histopathological evaluation of liver tissues, degeneration and necrosis in hepatocytes, mononuclear cell infiltrations in portal areas, bile duct hyperplasia, and fibrosis in parenchyma were evaluated. The histopathological changes were evaluated as follows:

0: No lesion.

+1: “Mild” if the relevant changes are only localized in one region.

+2: “Moderate” if the relevant changes were multifocal but limited.

+3: If the pathological change was diffusely distributed, it was considered as "severe".

Immunohistochemical study

A polymer-based indirect-immunoperoxidase method was used for immunohistochemical studies. For this purpose, the sections were taken on 5-micron thick poly-lysine slides in the microtome, deparaffinized in xylol, and rehydrated in grade alcohols. Then an antigen retrieval procedure was performed with Proteinase K enzyme (RE7330-K, Novocastra) for 15 minutes at room temperature. After washing with deionized water, endogenous peroxidase activity was removed by dripping 3% hydrogen peroxide solution on the sections. It was washed 3 times with TBS for 5 minutes, then Protein Block (Novocastra RE7102) for 10

minutes, 3 times with TBS for 5 minutes, Primary antibody [Caspase 3 Recombinant Rabbit Monoclonal Antibody (9H19L2), TNF alpha Monoclonal Antibody (MP6-XT22), PE, eBioscience™, COX2 Monoclonal Antibody (COX 229)] at room temperature for 1 hour, washing 3 times with TBS for 5 minutes, in post-primary block solution (NovoLinkTM Max Polymer Detection System RE7280-K, Novocastra) 30 min, 3 washes with TBS, 30 min, 3 washes with TBS in Novalink polymer solution (NovoLinkTM Max Polymer Detection System (RE7280-K, Novocastra), and DAB (3,3'-Diaminobenzidine) (NovoLinkTM Max Polymer Detection System RE7280-) K, Novocastra) at room temperature for 3-5 minutes. After washing with distilled water, counterstaining with Hematoxylin was performed, after 2 changes in alcohol and xylol, and covered with entellan. Negative controls used in each staining were also stained according to the same procedure, but primary TBS was used instead of antibodies. All sections were evaluated under a light microscope (Olympus CX23, Tokyo, Japan).

Immunohistochemical scoring method

The Allred system was used for immunohistochemical scoring of the cases. The method used by Qureshi and Pervez (2010) was modified and applied to the study. Similar to standard scoring systems, staining intensity and staining prevalence were evaluated by this scoring system. Staining intensity (darkness) score was determined as: 0 (no staining), 1 (weak), 2 (medium), 3 (intense/dark). The extent of staining was determined based on the ratio of stained cells to all cells in the examined area as follows: 0 (no staining), 1 (> 0 - 1/100), 2 (> 1/100 - 1/10), 3 (> 1/ 10 - 1/3), 4 (> 1/3 - 2/3), 5 (> 2/3 - 1). Allred score between 0 and 8 was determined for each case by summing the staining intensity score and the staining prevalence value. 10 different microscope fields were examined randomly at 40 magnification and the average of the obtained scores was accepted as the score of the relevant case.

Statistical analysis

Statistical analyzes in the study were obtained with the IBM SPSS Statistics 22 program. Kruskal-Wallis and Tukey tests were used for statistical evaluation of histopathological findings; Kruskal-Wallis and Duncan tests were used for evaluation of immunohistochemical findings. The $p<0.05$ criteria were used for all statistical evaluations.

RESULTS

Histopathological findings

Histopathological results and statistical values are given in Table-1 and Table-2. Accordingly, hepatocytes in the control group had a normal histological appearance. The lumens of the vena centralis in the liver tissues in this group were empty (Figure 1A). In addition, no inflammatory cell infiltrates were found in the portal areas. Degeneration of hepatocytes was found only in 3 cases. The most severe lesions were found in the sham group. Severe hydropic and vacuolar degenerations were observed in hepatocytes of 3 cases (Figure 1B). It was determined that these degenerative hepatocytes were mostly located in the centrilobular regions. Statistically, the sham group was found to be significantly different from the other groups ($p<0.05$).

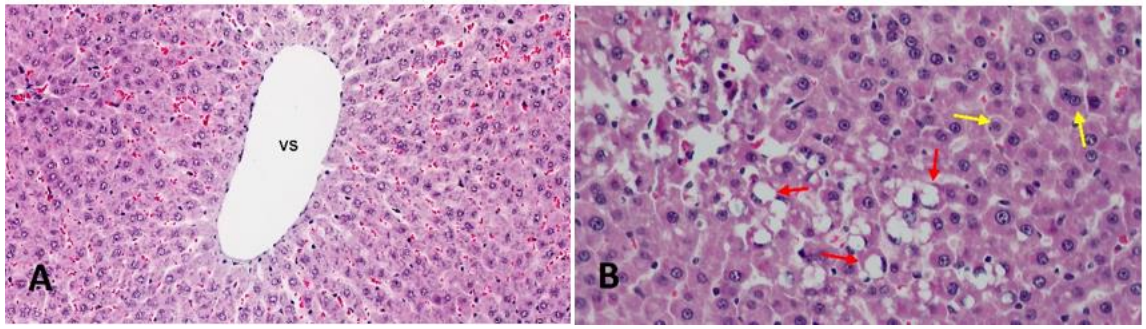

Figure 1. A. Empty lumens of the vena centralis (VS) and normal histological appearance in the liver of rats in the control group. X200. B. Hydropic and vacuolar degenerations in hepatocytes (red arrows) and normal hepatocytes (yellow arrows) of rats in the sham group. X400, H&E.

Table 1. Number of histopathological changes observed in the cases

n=10	Control Group				Sham Group				Treatment Group			
Lesion score	0	+1	+2	+3	0	+1	+2	+3	0	+1	+2	+3
Degeneration and necrosis in hepatocytes	7	3	-	-	1	1	5	3	5	4	1	-
Mononuclear cell in the portal area and bile duct hyperplasia	10	-	-	-	4	4	2	-	3	4	1	2
Parenchymal fibrosis	10	-	-	-	10	-	-	-	10	-	-	-

Table 2. Statistical values of histopathological results

	Control Group (Mean ± Std Error)	Sham Group (Mean ± Std Error)	Treatment Group (Mean ± Std Error)
Degeneration and necrosis in hepatocytes	0.300 ± 0.152^{b}	2.000 ± 0.298^{a}	0.600 ± 0.221^{b}
Mononuclear cell in the portal area and bile duct hyperplasia	0^{b}	0.800 ± 0.249^{ab}	1.200 ± 0.359^{a}
Parenchymal fibrosis	0	0	0

Values in rows that do not contain a-b common superscripts are significantly different according to the Kruskal Wallis – Tukey test ($p<0.05$).

Moderate mononuclear cell infiltrations and bile duct hyperplasia were observed in portal areas of 2 cases. When the treatment group was examined, it was determined that the degenerations in hepatocytes were moderately severe only in one case. On the other hand, severely enlarged bile ducts in the portal areas of 2 cases were observed (Figure 2). Hepatic necrosis and/or parenchymal fibrosis were not observed in any of the groups.

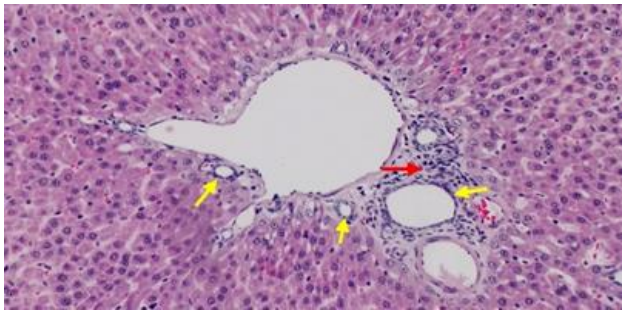

Figure 2. Mononuclear cell infiltrations in portal areas (red arrow) and bile duct hyperplasia (yellow arrows) of rats in the treatment group, X200, H&E.

Immunohistochemical findings

The statistical results of the Allred scoring method, in which the staining intensity and the extent of staining were evaluated together, are given in Table-3. According to this, while all groups were found to be statistically different in immunohistochemical staining with TNF-α, the sham group had the most severe positivity. This was followed by the control and treatment groups, respectively. It was noted that positive staining was mostly randomly distributed throughout the lobe and intracytoplasmic immunoreactions were observed in hepatocytes (Figure 3). No positive reactions were found in other cell groups in the liver tissue.

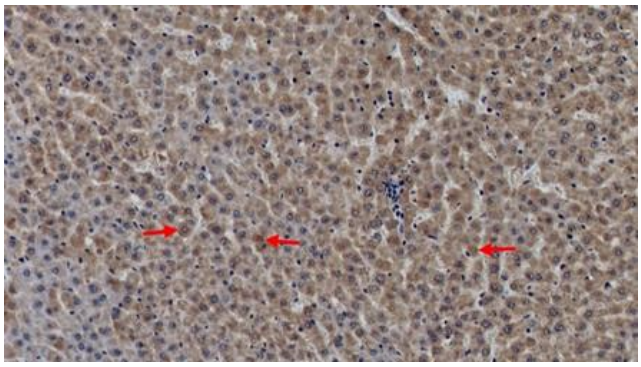

Figure 3. Anti-TNF-α intracytoplasmic immunopositive reactions were randomly distributed in hepatocytes (arrows) in the sham group, X200, DAB

In immunohistochemical analyzes performed with Caspase-3 primary antibody, the most intense positive staining was observed in the sham group. Treatment and control groups were statistically significantly different from the sham group ($p<0.05$). Positive staining was observed mostly in the hepatocytes and bile duct epithelium in portal areas (Figure 4). Positive immunoreactions were found in the cytoplasm of the cells, similar to those in TNF-α.

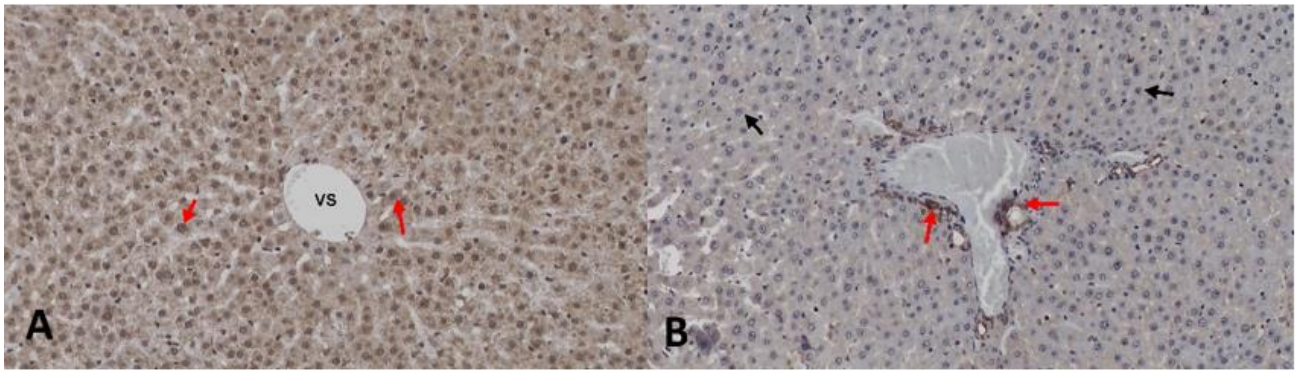

Figure 4. A. Anti-Caspase3 positive immunohistochemical stains (arrows) randomly scattered among hepatocytes around the vena centralis (VS), X200, DAB. B. Anti-Caspase3 positive immunoreactions (red arrows) in bile duct epithelium of portal areas and immunonegative hepatocytes (black arrows), X200, DAB.

Similarly, as a result of the immunohistochemical staining of COX-2 primary antibody, the most severe reactions were obtained in the sham group, followed by the treatment and control groups, respectively. While these two groups were statistically similar ($p>0.05$), the sham group was different ($p<0.05$). Although intranuclear staining was observed more intensely in hepatocytes, intracytoplasmic staining was also observed (Figure 5).

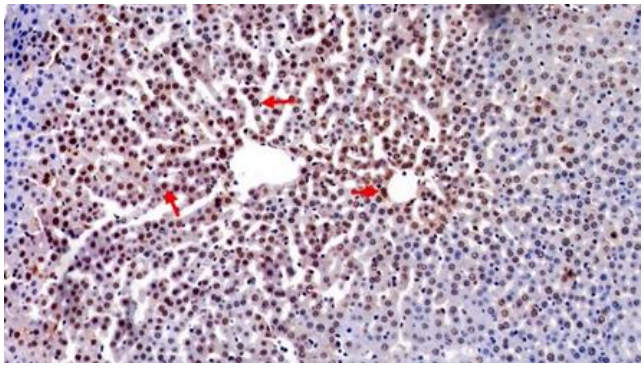

Figure 5. Anti-COX-2 intranuclear positive immunohistochemical staining with centrilobular distribution (arrows) in the sham group, X100, DAB.

The most severe reactions were observed in the treatment group in immunohistochemical staining with anti-BrdU antibody. In these cases, immunopositive staining was noted especially in the nuclei of hepatocytes (Figure 6A). Weak positive and negative immunoreactions were observed in the control and sham groups (Figure 6B). According to these findings, the control and sham groups were found to be statistically similar, while the treatment group was significantly different from the other two groups ($p<0.05$).

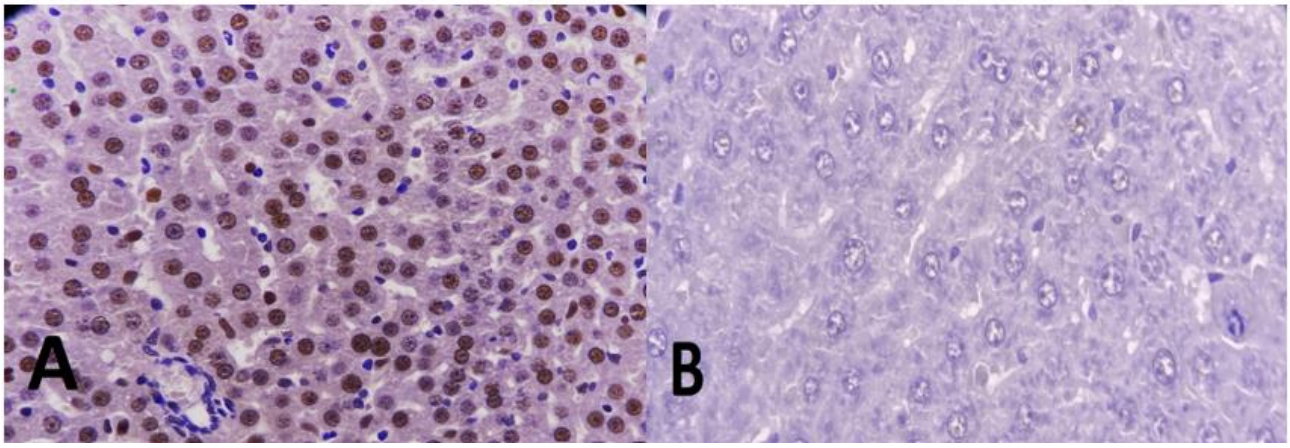

Figure 6. A. Intranuclear Anti-BrdU immunopositive reactions in hepatocytes, treatment group X630, DAB. B. Negative Anti-BrdU immunoreactions in hepatocytes, sham group X630, DAB.

Table 3. Statistical values of immunohistochemical results

	Control Group (Mean ± Std Error)	Sham Group (Mean ± Std Error)	Treatment Group (Mean ± Std Error)
TNF-α	4.860 ± 0.172^{b}	6.040 ± 0.394^{a}	3.890 ± 0.355^{c}
Caspase 3	2.610 ± 0.302^{b}	6.420 ± 0.226^{a}	3.520 ± 0.512^{b}
COX-2	3.300 ± 0.222^{b}	4.850 ± .0290^{a}	3.660 ± 0.302^{b}
BrdU	0.280 ± 0.035^{b}	1.220 ± 0.213^{b}	3.370 ± 0.800^{a}

Values in rows that do not contain a-c common superscripts are significantly different according to the Kruskal Wallis – Duncan test ($p<0.05$).

DISCUSSION

Various results have been reported based on structural examination of liver tissue extracted from animal models treated with DOX. Marked hyperplasia in the bile duct, dilatation of the sinusoidal space and central vein congestion, vacuolization in hepatocytes, dilatation of sinusoids, condensation in nuclei and degeneration of hepatocyte cords, cellular edema, focal necrosis, and irregularity of the hepatic trabecular necrosis, proximal trabeculae ducts, dilatation and vacuolization and swelling of mitochondria, lymphocyte infiltration were observed in the intercellular spaces as a result of the histopathological examination of hepatic tissue after DOX administration (Prasanna et al., 2020).

Previous studies reported that DOX has toxic effects on organs such as the heart, kidneys, and testicles as well as the liver (Pugazhendhi et al., 2018). Shivakumar et al. (2012) reported that they encountered degenerative changes in the liver after exposure to DOX in Wistar albino rats in an experimental study. In another study,

It was reported that DOX at different concentrations caused hydropic and vacuolar degenerations in hepatocytes (Jacevic et al., 2017). In our study, in accordance with the literature, it was revealed that both hydropic and vacuolar degenerations in hepatocytes occurred mostly in the sham group, and it was determined that the number and severity of these findings decreased in the treatment group. Thus, it was thought that the transplantation of MCSs might have a histopathological preventive effect on DOX-related degenerative changes in the liver.

Wang et al. (2016) revealed that the use of anthracycline group antitumoral drugs caused a systemic inflammation in the patient. In our study, however, it was noticed that inflammatory reactions were mostly seen in the sham and treatment groups. In addition, the more frequent observation of bile duct hyperplasia in the treatment group was considered as a response of the liver to

degenerative events, and it was interpreted that the use of stem cells led the liver to regenerate.

A previous study reported that DOX did not cause liver fibrosis and did not cause histological changes (Di Stefano et al., 2006). Similarly, the absence of fibrosis findings in any of the groups in our study shows that the results obtained are compatible with the literature. In general terms, it can be stated that stem cell therapy has a positive effect on the histological changes in the liver.

In a previous study revealed that apoptosis occur in DOX-induced hepatotoxicity (El Sayyad et al., 2009). In another study, it was determined that stem cells have a strong anti-apoptotic and mitogenic effect on the liver (Yu et al., 2007). In our study, it was thought that Caspase-3 activity is more effective in the sham group and doxorubicin administration triggers apoptosis in animals. It was noted that Caspase-3 expressions were decreased in the treatment group compared to the sham group, and this decrease was statistically similar to the control group. It was concluded that stem cell therapy prevents programmed cell death. However, it can be said that the expression of other cytokines such as Caspase-8 and Caspase-9 should be investigated to determine whether apoptosis is triggered internally or externally.

As stated in many previous studies, TNF-α, the proinflammatory cytokine of DOX, is expressed more in cells (Supriya et al., 2016; Cengiz et al., 2021). In our study, it was interpreted that TNF-α was expressed significantly less in the treatment group compared to the sham group, this could also prevent inflammatory reactions. In addition to these findings, the histologically lower severity of inflammatory reactions in the treatment group also proves the accuracy of the aforementioned immunohistochemical findings.

Yu et al. (2007) reported that after the transplantation of stem cells, they encountered “hepatocyte-like” cell formations in ischemic damaged rat livers. It has been revealed that while it takes 5-7 days for stem cells to reach the sinusoids in the liver tissue, it takes at least 10-14 days for them to transform into the above-mentioned cells. In the same study, it was reported that BrdU positive reactions in the liver were observed in hepatocytes 24 hours after the transplantation of stem cells. In our study, BrdU positive immunoreactions were observed in the treatment group, especially in the nuclei of hepatocytes. This shows that FKD-MSCs have reached the liver tissue after being transplanted intraperitoneally.

CONCLUSION

As a result, it can be stated that intraperitoneally administered FKD-MSCs to rats with DOX-induced hepatotoxicity, prevent degeneration and necrosis in hepatocytes. It was determined that TNF-α, COX-2, and Caspase-3 levels were significantly reduced in the treatment group and this shows that FKD-MSCs provided regeneration in the liver. In addition, strong BrdU positive reactions in the treatment group was interpreted as intraperitoneally transplanted stem cells being able to reach the liver tissue and helping reduce tissue damage. However, further studies are required to investigate the effects of stem cells on some other parameters such as liver enzymes, angiogenesis, and oxidative stress.

ACKNOWLEDGMENT

This work was supported by Aksaray University Scientific Research Fund (Grant number 2020-029).

Ethical approval:

This study was approved by Dışkapı Yıldırım Beyazıt Education and Research Hospital Local Animal Ethics Committee (Approval number: 2014/55).

Conflict of interest: The authors declared that there is no conflict of interest.

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Effects of thymoquinone on some cytokine levels in cerulein-induced acute pancreatitis

Research Article

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ABSTRACT

In this study, it was aimed to evaluate the possible effects of thymoquinone administration on some cytokine levels in rats with experimental acute pancreatitis. No application was made the animals in group K. TQ group animals were intraperitoneally given 20 mg/kg thymoquinone daily for 9 days. In the AP group animals, acute pancreatitis was induced by intraperitoneal administration of cerulein as first dose 50 µg/kg and 2 hours later 25 µg/kg second dose on the 7th day of the study. Animals in the AP+TQ group were intraperitoneally administered 20 mg/kg thymoquinone daily for 9 days. On the 7th day of the study, after 2 hours from thymoquinone administration, acute pancreatitis was induced by intraperitoneal administration of cerulean as 50 µg/kg and 2 hours later 25 µg/kg. TNF-α, IL-6, IL-8, IL-10, AST and ALT levels were determined in the blood samples taken from all animals. In the study, TNF-α level was found to be importantly higher in the acute pancreatitis group compared to the control group, while TNF-α level was significantly lower in the acute pancreatitis group treated with thymoquinone than the acute pancreatitis group. IL-6 and IL-8 levels were higher in the acute pancreatitis group compared to the control group. IL-6 and IL-8 levels were found to be significantly lower in rats with acute pancreatitis treated with thymoquinone compared to the group with acute pancreatitis. While AST and ALT levels in the acute pancreatitis group were significantly increased when compared with the control group, both enzyme levels in the acute pancreatitis group treated with thymoquinone administration were found to be significantly lower than the rats with acute pancreatitis. In the study, the findings obtained in rats with acute pancreatitis which were pre-treated with thymoquinone can be evaluated as that thymoquinone alleviates inflammation due to pancreatitis.

Keywords: Cerulein, Cytokine, Rats, Thymoquinone

INTRODUCTION

The incidence of acute pancreatitis (AP) is increasing globally, and mortality can be high in case of organ failure and severe necrosis. Acute pancreatitis is an acute inflammatory disease of the pancreas and mainly characterized by acinar cells atrophy, necrosis, activation of digestive enzymes (amylase and lipase), and inflammatory cell aggregation in the pancreas. In addition, intrapancreatic/acinar activation of trypsin causes a series of varying degrees of severity, which include multiple organ failure and death (Petrov et al., 2010). In the early stage of acute pancreatitis, there are release of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and the activation of zymogens in acinar cells. The degrees of systemic inflammatory response determine serious multiorgan dysfunction and mortality (Banks et al., 2013). Reversing of proinflammatory cytokine activation and cytokine storm play a crucial role in prognosis of acute pancreatitis (Zhang et al., 2018).

Article info
Submission: 31-01-2022
Accepted: 26-06-2022
Online First: 06-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

How to cite this article
Kanaç, S., Keski, E., Uluışık D. (2022). Effects of thymoquinone on some cytokine levels in cerulein-induced acute pancreatitis. *Journal of Advances in VetBio Science and Techniques, 7*(2), 194-201. https://doi.org/10.31797/vetbio.1063971

In recent years, the use of herbal substances for the treatment or prevention of many ailments has been very popular. As one of these plants, Nigella sativa is medicinal plant belonging to family Ranunculaceae. Many cultures have used seeds of Nigella sativa to treat different disease for centuries. In many studies, it was investigated the pharmacological properties and potential medicinal benefits. The well-known properties of Nigella sativa are hepatoprotective, immunomodulatory, gastroprotective, anti-inflammatory, renal protective and antioxidant effects (Al Rowais, 2002). Thymoquinone is the fundamental constituent of the black seed oil which has shown strong anti-inflammatory effects by suppressing prostaglandins, leukotrienes and modulating cytokines (Salem, 2005; Arjumand et al., 2019).

In the light of these data, we evaluated the antiinflammatory effect of Thymoquinone in rats with cerulein-induced acute pancreatitis by measuring some proinflamatory and antiinflamatory cytokines.

MATERIAL and METHOD

In the study, 32 healthy adult male Wistar Albino rats were used. During the study, the suitable living conditions (heat, humidity and light) for the rats were provided. All animals were fed with Purina brand standard rat diet during the experiment. The animals were allowed to drink water while fasting for 16 hours before the start of the study. Animals were divided into four groups.

Group I (K) (n=6): No application was made the animals in group K.

Group II (TQ) (n=6): Animals in TQ group were intraperitoneally given 20 mg/kg thymoquinone daily for 9 days.

Group III (AP) (n=10): In the AP group animals, acute pancreatitis was induced by intraperitoneal administration of cerulein as first dose 50 µg/kg and 2 hours later 25 µg/kg second dose on the 7th day of the study.

Group IV (AP+TQ) (n=10): Animals in the AP+TQ group were intraperitoneally administered 20 mg/kg thymoquinone daily for 9 days. On the 7th day of the study, acute pancreatitis was induced by intraperitoneal administration of cerulein as 50 µg/kg and 2 hours later 25 µg/kg after 2 hours from thymoquinone administration.

At the end of the 9th day of the study, blood samples taken from animals in all groups. TNF-α, IL-6, IL-8, IL-10, AST and ALT levels were determined in the blood samples taken from all animals. In plasma samples, TNF-α, IL-6, IL-8 and IL-10 levels were determined with ELISA (Biotek ELx800, Biotek Instrumentations, Inc, Winooski, VT, USA) using sandwich enzyme-linked immunosorbent method via commercial kits (Elabscience), while it was determined AST and ALT levels in the Siemens Centaur CP autoanalyzer using Siemens kits.

The data obtained from the study were analyzed by one-way ANOVA (SPSS). Differences among the groups were determined by Duncan's multiple range test. Differences were considered significant at $p<0.05$.

RESULTS

In the study, the effects of thymoquinone administration on TNF-α, IL-6, IL-8 and IL-10 levels in rats with cerulein-induced acute pancreatitis are given in Table 1, AST and ALT levels are given in Table 2.

Table 1. The effects of thymoquinone on TNF-α, IL-6, IL-8 and IL-10 levels in rats with cerulein-induced acute pancreatitis (Mean±SE).

	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-10 (pg/ml)
K	46,65±6,26^{c}	38,34±3,68^{b}	141,34±6,98^{c}	61,37±6,62^{b}
TQ	43,84±5,83^{c}	40,95±4,63^{b}	132,01±6,65^{c}	63,56±7,13^{b}
AP	113,19±5,09^{a}	67,07±4,35^{a}	329,57±20,03^{a}	78,15±4,55^{ab}
AP+TQ	79,34±5,94^{b}	51,58±4,04^{b}	268,71±24,19^{b}	81,42±4,65^{a}

a-c The difference between mean values with different superscripts in the same column are significant ($p<0.05$)

Table 2. The effects of thymoquinone on AST and ALT levels in rats with cerulein-induced acute pancreatitis (Mean±SE).

	AST (U/L)	ALT (U/L)
K	84,17±6,83^{c}	23,50±3,57^{c}
TQ	79,33±6,14^{c}	24,67±2,94^{c}
AP	146,10±4,39^{a}	51,20±2,89^{a}
AP+TQ	115,40±6,34^{b}	38,90±3,14^{b}

a-c The difference between mean values with different superscripts in the same column are significant ($p<0.05$)

DISCUSSION

Acute pancreatitis is a rapidly developing inflammatory disease in which peripancreatic tissues and other organ systems is often involved (Bhatia et al., 2005). Although the development and progression of the disease has not been fully explained, severe necrotic tissue cases result in a very high morbidity and mortality rate (Pandol et al., 2007).

The systemic inflammatory response syndrome and multi-organ failure syndrome in acute pancreatitis are associated with the severity of pancreatitis. This response to pancreatitis includes increments in leukocyte activation and production of various cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-10 and IL-18 (Wereszczynska-Siemiatkowska et al., 2003; Štimac et al., 2006).

Although TNF-α is produced by many cells, its main source is macrophages and monocytes. TNF-α has a leading role in the development of the inflammatory response (Lane et al., 2001). It has shown that TNF production in mononuclear cell cultures taken from patients with acute pancreatitis is increased compared to healthy ones (McKay et al., 1996). While it has been reported that elevated circulating TNF-α levels might cause shock in many conditions, including acute pancreatitis, TNF-α is now considered one of the most important mediators of shock. It has been reported that elevations in TNF concentration are directly related to the severity of inflammation and pancreatic damage (Norman et al., 1995; Bhatia et al., 2000).

IL-10, synthesized by Th2 cells, monocytes, and B cells, is a naturally occurring anti-inflammatory cytokine. IL-10 reduces the release of Th1 proinflammatory cytokines (Fiorentino et al., 1991). In experimental studies, IL-10 has been shown to reduce the levels of inflammatory markers and the severity of pancreatitis (Kusske et al., 1996; Rongione et al., 1997). It has been reported that pre-application of synthetic IL-10 agonists reduces mortality and lung damage caused by experimental pancreatitis (Osman et al., 1998). However, IL-10 levels in humans have been shown to be related with the severity of pancreatitis and tissue damage. Pezzilli et al. (1997) have stated that IL-10 levels in healthy individuals were so low that it cannot be determined, while it increased in the first day of acute pancreatitis and gradually decreased in the following days.

IL-6 is produced by immunologically active cells such as monocytes/macrophages, endothelial cells and fibroblasts in response to stimuli. It was stated that IL-6 is produced in pancreatic tissue by periacinar myofibroblasts in response to TNF-α and IL-1β after experimentally induced pancreatitis (Norman et al., 1997; Shimada et al., 2002). This event is considered to play important role in the regulation of the generation and secretion of acute phase reactants by hepatocytes (Geiger et al., 1988; Castell et al., 1989). High levels of IL-6 are a marker of the severity of pancreatitis and also serve to determine the course of the disease (Galloway and Kingsnorth, 1994; Kingsnorth et al., 1995).

IL-8 is a potent chemokine that has neutrophil chemoattractant properties (Baggiolini et al., 1989). Following stimulation by proinflammatory mediators, IL-8 is released by wide cell types such as monocytes/macrophages, endothelial cells, neutrophils, fibroblasts, epithelial cells, keratinocytes, T cells, NK cells, and chondrocytes (Jeannin et al., 1994). IL-8 is known to be an important mediator in many inflammatory diseases. The increments in IL-8 level of the circulation in systemic inflammatory diseases help in predicting morbidity and mortality (Hack et al., 1992).

Throughout history, people have used various herbs and plants to prevent or treat diseases (Majdalawieh and Fayyad, 2015). Such plants are considered as an excellent treatment option with their easy obtaining, nutritious and synergistic effects (Donaldson, 1998). Treatment approaches based on the properties and effects of natural plants have played an important role in the development of conventional medicine and modern medicines.

Nigella Sativa is botanically a dicotyledonous flowering plant belonging to the Ranunculaceae family and is commonly known as black seed or black cumin (Ali and Blunden, 2003; Salem, 2005). Nigella Sativa seeds are traditionally used as a food preservative, nutritional supplement or spice in various cultures. Thymoquinone with a chemical formula of C10H12O2 and a molecular weight of 164.2 g/mol is the most important bioactive phytochemical found in Nigella Sativa oil and extracts (Woo et al., 2012; AbuKhader, 2013; Schneider-Stock et al., 2014). It is claimed that some of the medical benefits of Nigella Sativa and thymoquinone are related to its antihistaminic, anti-inflammatory, antihypertensive, hypoglycemic, anticancer and immune-enhancing effects (Ahmad et al., 2013; Shabana et al., 2013; Rahmani et al., 2014; Schneider-Stock et al., 2014).

It is reported that Nigella Sativa extracts suppress IL-6, TNF-α and NO expressions in a dose-dependent manner (Majdalawieh et al., 2010). It has been supported by various findings that Nigella Sativa has anti-inflammatory and antioxidant effects in patients with rheumatoid arthritis (Hadi et al., 2016). It is stated that these findings are consistent with the findings that NO production in mouse macrophages is inhibited by Nigella Sativa (Mahmood et al., 2003).

Increases in glutathione levels appear to be a mechanism underlying the beneficial effects of thymoquinone against inflammatory disorders. Nuclear Factor Kappa Beta (NF-κB) is a eukaryotic transcription factor critically involved in various biological processes, including inflammation (Majdalawieh and Ro, 2010). It has been shown that thymoquinone administration inhibits NF-κB signaling in the medulla spinalis and brains of Experimental Allergic Encephalitis (EAE) induced rats. This suggests that NF-κB is potentially a target molecule for thymoquinone (Mohamed et al., 2005). It is reported that thymoquinone suppresses Advanced Glycation End Product (AGE)-induced NF-κB activation and IL-6 expression (Sayed and Morcos, 2007). In another study, it was determined that

thymoquinone administration significantly suppressed Lipopolysaccharide (LPS)-induced TNF-α production in the basophil cell line (El Gazzar, 2007). Consistent with above data, Chehl et al. (2009) reported that thymoquinone significantly decreased the expressions of IL-1β, TNF-α, monocyte chemoattractant protein 1 (MCP-1), and cyclooxygenase 2 in pancreatic ductal adenocarcinoma depending on dose and time. On the other hand, it has been reported that LPS-induced NF-κB and p38-mitogen activating protein kinase (MAPK) activation is blocked by thymoquinone, resulting in suppression of the expression of proinflammatory mediators such as IL-1β and TNF-α (Vaillancourt et al., 2011). It has been reported that thymoquinone reduces serum levels of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α (Badr et al., 2011).

In our study, while TNF-α level, which is a proinflammatory cytokine and plays a key role in inflammatory events, was found to be significantly higher in the acute pancreatitis group than in the control group ($p<0.05$, Table 1), this difference was expected situation and was consistent with the above data. In this study, the significantly lower TNF-α level in the acute pancreatitis group with thymoquinone administration compared to the pancreatitis group ($p<0.05$, Table 1) supports the reports that thymoquinone suppresses the increments in TNF-α levels in various inflammatory conditions. The levels of IL-6 and IL-8, which are proinflammatory cytokines, increased in parallel with the changes in TNF-α levels in the acute pancreatitis group ($p<0.05$, Table 1). IL-8 levels were found to be significantly lower in rats with acute pancreatitis due to thymoquinone administration compared to the group with acute pancreatitis ($p<0.05$, Table 1). IL-6 level in acute pancreatitis group treated with thymoquinone was different from the group with acute pancreatitis and it was observed that it approached the control group values. The increase in the level of IL-10, an anti-inflammatory cytokine, was not statistically significant in the acute pancreatitis group compared to the control group. This increase became more evident with the administration of thymoquinone to rats with acute pancreatitis ($p<0.05$, Table 1). The changes in proinflammatory cytokine levels determined by the thymoquinone administration to rats with acute pancreatitis are consistent with the reports that thymoquinone inhibits the NF-κB signal, an inflammation transcription factor, and suppresses the expression of cytokines such as IL-1β, IL-6 and TNF-α. In the study, the significant increase in IL-10 level determined by the application of thymoquinone to rats with acute pancreatitis can be evaluated as thymoquinone increases IL-10 production, which suppresses proinflammatory cytokines against inflammation.

It has been shown that pancreatic enzymes spread into the systemic circulation as a result of inflammatory changes in the pancreas and retroperitoneum. These enzymes cause damage to its own tissue and the other organs such as the liver by producing various inflammatory mediators (Gallagher et al., 2004; Rahimian et al., 2017). In this study, increases in AST and ALT levels showed that cerulein administration also caused liver damage, while thymoquinone administration to the acute pancreatitis group significantly limited the increase in hepatic enzyme levels such as AST and ALT ($p<0.05$, Table 2). Increases in serum AST, ALT and total bilirubin levels are accepted as an indicator of hepatic damage in experimental acute pancreatitis. While it has been reported that TNF-α and IL-6 levels increase in acute pancreatitis, it is suggested that apoptosis-related gene expression is upregulated in parallel to the increases in these cytokines. It has also been suggested that this increase in hepatic cell apoptosis may be the main cause of liver dysfunction in the early stages of severe acute pancreatitis (Sha et al., 2008). In the study, the significant changes in AST and ALT

levels in the acute pancreatitis group with thymoquinone administration can be considered as a result of the positive reflection on liver damage by suppressing of inflammatory mediators due to the antiinflammatory and antioxidant effects of thymoquinone.

CONCLUSION

Depend on the obtained changes in the cytokine levels and liver enzymes levels may be considered that thymoquinone showed beneficial effect in rats with acute pancreatitis in this study.

ACKNOWLEDGMENT

This research was supported by Selcuk University Scientific Research Projects Coordinatorship (Project no: 19202064).

Ethical approval: The Ethical Committee of Selcuk University Experimental Medicine Research and Application Center (Report no. 2019-29) approved the study protocol.

Conflict of interest: The author declares no conflict of interest.

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The extraction of Peste Des Petits ruminants Virus RNA from paraffin-embedded tissues using a modified extraction method

Research Article

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ABSTRACT

Peste des petits ruminants (PPR) which is caused by small ruminant morbillivirus (PPRV) has an important economic impact on small ruminant farming. Molecular assays are commonly used in the diagnosis of the disease. Extraction of RNA from formalin-fixed paraffin-embedded (FFPE) tissues is challenging because of the RNA is often degraded by formalin fixation process. Although commercial kits have been developed for extraction of nucleic acids from FFPE tissues, they are expensive than other extraction kits. In this study, a modified extraction method was evaluated for detection of PPRV from FFPE tissues. A total of 20 FFPE tissue samples including 15 PPRV positive and 5 PPRV negative FFPE tissue samples were used. Two years ago, these selected FFPE tissue samples were analysed by nucleoprotein gene based real time RT-PCR method before they were fixed with formalin and embedded in paraffin. FFPE tissue samples were extracted using modified extraction method and were tested by fusion (F) gene based one step RT-PCR. PPRV specific RNA was detected in 12 FFPE tissue samples whereas 3 positive samples were found negative by one-step RT-PCR. Furthermore, 5 negative FFPE tissue samples were also found negative. Three false negative results were from samples with high real-time RT-PCR cycle threshold. Therefore, false negative results could be related with lower viral loads which might be lower than detection limit of the one-step RT-PCR. The results of the study show that modified extraction method could be used for RNA extraction from FFPE tissues which had been stored for 2 years.

Keywords: Formalin-fixed tissues, peste des petits ruminants, RNA, RT-PCR

INTRODUCTION

Peste des petits ruminants (PPR) is an economically important transboundary viral disease of sheep and goats characterized by high fever (40°- 41.5°C), diarrhoea, necrotic stomatitis, muco-purulent nasal discharge, enteritis and abortion (Couacy-Hymann, 2015; OIE, 2022; Şevik and Sait, 2015). Primary hosts of the disease are sheep and goats. Although cattle, pigs and camels can also be infected, clinical signs are not seen in these animals (Abraham et al., 2005; Fakri et al., 2019). The disease has also been reported in wild ruminant species including ibexes, gazelles, wild goats (*Capra aegagrus*) and sheep (*Ovis orientalis*) (Abubakar et al., 2011; Dou et al., 2020; Li et al., 2017; Mahapatra et al., 2015).

PPR causes high mortality rates in naïve goat and sheep populations (Couacy- Dou et al., 2020; Hymann et al., 2007; Şevik and Sait, 2015). In endemic areas, mortality rates may be 20% or less, but it may reach 100% naive population (Mapaco et al., 2019). The disease is spread through direct contact with infected animals or contact with infected fomites (Parida et al., 2019).

Article info
Submission: 23-02-2022
Accepted: 12-07-2022
Online First: 06-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

How to cite this article
Şevik M. (2022). The extraction of Peste Des Petits ruminants Virus RNA from paraffin-embedded tissues using a modified extraction method. *Journal of Advances in VetBio Science and Techniques*, *7*(2), 202-209. https://doi.org/10.31797/vetbio.1078235

The disease has been reported in southern Asia, Africa, the Arabian Peninsula and the Middle East (Banyard et al., 2014; Kerur et al., 2008; Li et al., 2017; OIE, 2022). Turkey first reported PPR in 1999 (OIE, 1999). Due to its economic impact, PPR has been declared as a notifiable disease by the World Organization for Animal Health (OIE) (OIE, 2022). The Food and Agriculture Organization and OIE aim to eradicate PPR until 2030 (Dou et al., 2020).

Peste des petits ruminants virus (PPRV), renamed as small ruminant morbillivirus, is the causative agent of the disease classified in the genus *Morbillivirus* within the family *Paramyxoviridae*, and closely related with rinderpest, measles and canine distemper viruses (Gibbs et al., 1979; ICTV, 2022). Four genetically distinct lineages of PPRV have been identified based on molecular characterization of the nucleoprotein (N) and fusion protein (F) of PPRV (Bailey et al., 2005; Kerur et al., 2008).

Virus isolation, immunocapture enzyme-linked immunosorbent assay, agar gel immunodiffusion, immunohistochemistry and molecular detection methods are routinely used for confirmation of the disease (OIE, 2022). Molecular detection methods are highly specific and sensitive. However, these methods require high-quality RNA from the extracted samples. Mostly, fresh tissues and swab specimens are used for the detection of PPRV nucleic acid (Şevik, 2014). However, it may not be possible to find fresh tissue samples in some cases. Therefore, in this study, a commercial kit which developed for total nucleic acid isolation from fresh tissues was modified, and it was evaluated for detection of PPRV from formalin-fixed paraffin-embedded (FFPE) tissues.

MATERIAL and METHOD

Samples

This study was conducted at Konya Veterinary Control Institute in 2017. A total of twenty FFPE lung tissue samples that were fixed with formalin and embedded in paraffin two years ago were selected. Selected tissue samples were analysed by N gene based one step real time RT-PCR method before they were fixed with formalin and embedded in paraffin. One step real time RT-PCR method was performed using primers and probe described by Batten et al. (2011) with the One-Step RT-PCR Kit (Qiagen, Hilden, Germany). Reaction mixture was prepared containing 0.4 µM of each primer and 5 µl of the extracted RNA, in a final volume of 25 µl. Amplification protocol was performed according to Batten et al. (2011) using a real time PCR machine (Roche Applied Science, Indiana, USA).

Of the 20 FFPE tissue samples, 15 samples were detected as positive whereas 5 samples were detected as negative by one step real time RT-PCR method. Samples that were positive by real-time RT-PCR had cycle threshold (Ct) values ranged from 24 to 39 (Figure 1). A FFPE lung tissue sample that was border disease virus positive was used as negative control for the analyses.

RNA Extraction

Extraction of RNA was carried out from the FFPE samples using a commercial kit which developed for total nucleic acid isolation from fresh tissues (Roche Applied Science, Indiana, USA). However, extraction kit procedure was modified to obtain enough quality RNA from the FFPE tissue samples.

First, 5 µm paraffin tissue sections were collected from FFPE tissue samples, and were put into the eppendorf tubes. Then, they were deparaffinized by addition of xylene (1200 µl) and were incubated for 15 min at 65°C, followed by centrifugation for 5 min at 14000 × rpm. Deparaffinization steps were repeated three times. Then, samples were centrifuged for 5 min at 14000 × rpm with adding 80%, 90% and 100% ethanol, respectively. After samples washed with 100% ethanol, ethanol was

aspirated for 30 min at 37°C. Proteinase K (1:20) was added to tissue pellets which were into eppendorf tubes, and they were incubated at 56°C overnight. To inactivate proteinase K, samples were incubated for 15 min at 100°C before the RNA extraction procedure. After proteinase K inactivation, RNA extraction was performed according to the manufacturer's instructions. RNA extracts were stored at -20°C until analyses.

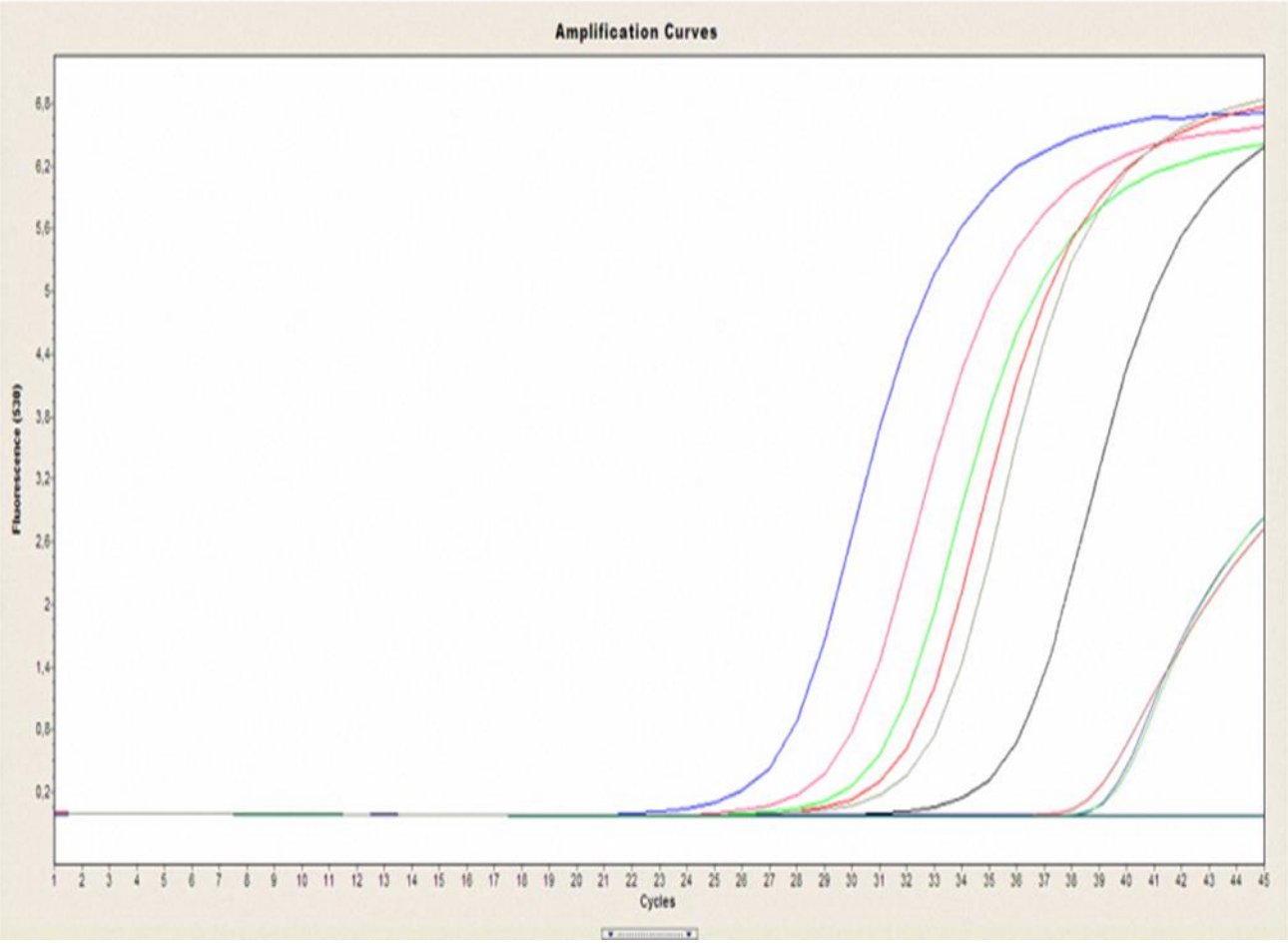

Figure 1. Results of the one step real-time RT-PCR, before selected tissues were fixed with formalin.

RNA quality measurement

Quality of the extracted RNA was assessed using a spectrophotometer (DeNovix Inc., Wilmington, USA). The absorbances of samples were measured at 260 nm and 280 nm. Purity of the samples was determined with ratio of 260/280.

Detection of PPRV by one step RT-PCR

PPRV specific RNA was detected using one-step RT-PCR kit (Qiagen, Hilden, Germany). One step RT-PCR reaction mixture was prepared containing, 0.4 µM of each primer and 2.6 µl of the extracted RNA, in a final volume of 20 µl. One-step RT-PCR was performed with primers described by Forsyth and Barrett (1995) which amplify the 448 bp of the F gene of PPRV. Amplification conditions were performed according to previous report (Şevik and Sait, 2015) using a thermal cycler (Techne, UK). PCR products were assessed in 1.5% agarose gel stained with GelRed (Biotium, Fremont, CA, USA).

RESULTS

PPRV-RNA was detected in 12 FFPE tissue samples by one-step RT-PCR. However, three FFPE tissue samples that had been found positive by one step real time RT-PCR were found negative by one-step RT-PCR.

Additionally, five FFPE tissue samples that had been found negative by one step real time RT-PCR were also found negative by one-step RT-PCR. Detailed one-step real time RT-PCR and RT-PCR results are shown in Table 1.

Table 1. Results of the one step RT-PCR with modified extraction method

Sample No	Animal species	One step Real time RT-PCR Ct values before FFPE procedures	One step RT-PCR	
			Results	Product intensity
1	Lamb	24.70	Positive	Strong
2	Lamb	39.20	Negative	
3	Lamb	32.40	Positive	Weak
4	Kid	24.62	Positive	Strong
5	Sheep	26.50	Positive	Strong
6	Kid	39.55	Negative	
7	Lamb	38.44	Negative	
8	Lamb	25.45	Positive	Strong
9	Lamb	27.54	Positive	Strong
10	Lamb	28.33	Positive	Strong
11	Lamb	31.85	Positive	Weak
12	Sheep	26.98	Positive	Strong
13	Lamb	30.96	Positive	Weak
14	Lamb	27.23	Positive	Strong
15	Lamb	32.96	Positive	Weak
16	Lamb	-	Negative	
17	Kid	-	Negative	
18	Lamb	-	Negative	
19	Lamb	-	Negative	
20	Lamb	-	Negative	
N.C	Lamb	-	Negative	

NC = Negative control

Purity of the extracted RNA analyses based on A260/280 ratio revealed that 8 samples had ratios around 2.0 whereas 4 samples had a ration 1.8-1.9. Additionally, three samples had a ration < 1.8.

Samples with Ct values between 24 and 28 had strong product intensity whereas samples with Ct values between 30 and 32 had weak product intensity (Figure 2). However, samples with Ct values between 38 and 39 had no PCR product on one step RT-PCR.

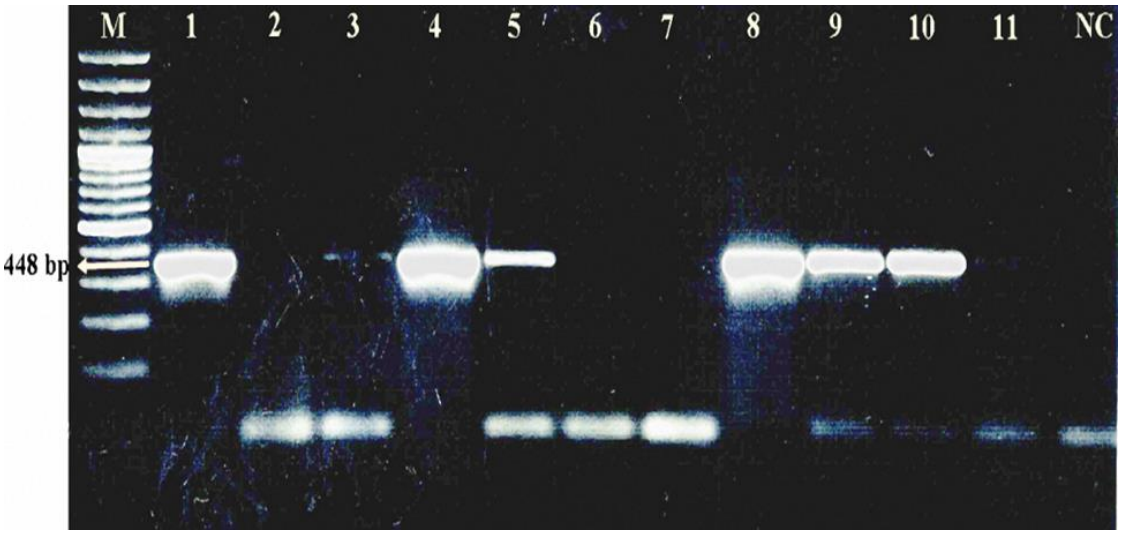

Figure 2. One step RT-PCR products based on F gene of PPRV. M: Marker (100 bp), Lane 1-11: Samples; Lane 3 and Lane 11 were weak positive; Lane 2, 6 and 7 were false negative results. NC: Negative control.

DISCUSSION

PPR is one of the notifiable viral diseases of goats and sheep, and has been diagnosed in different regions of Turkey (OIE, 1999; Ozkul et al., 2002; Şevik and Sait, 2015). Different techniques can be used for confirmation of PPRV infection such as molecular techniques, virus isolation, imunocapture ELISA and agar gel immunodiffusion. Virus isolation is not commonly used for diagnosis of PPR because it is expensive and labour-intensive (Hudu et al., 2016). Molecular diagnostic techniques are commonly used currently to diagnose viral diseases. They are more sensitive than virus culture and provide a more rapid diagnosis of viral infections (van Elden et al., 2002). Furthermore, molecular techniques are recommended for confirmation of PPR suspected cases by OIE (OIE, 2022). Therefore, RT-PCR assays are commonly used in diagnostic laboratories.

RT-PCR methods are dependent upon high-quality nucleic acids extractions from samples (Browne et al., 2020). Fresh tissues and swab specimens are recommended for obtaining high quality RNA (Şevik, 2014). FFPE processing is an economical approach to storage of the specimens for extended periods of time, and widely used in research and diagnostics (Guo et al., 2017). Also, in some cases, it may not be possible to find fresh tissue samples. Therefore, FFPE tissue samples can be used for RNA isolation. However, extraction of amplifiable RNA from FFPE tissues is challenging because of nucleic acid degradation due to the fixation process (von Ahlfen et al., 2007). Therefore, special commercial kits have been developed for detection of nucleic acids from FFPE tissues. However, FFPE DNA/RNA purification kits are expensive than other extraction kits. In this study, a commercial kit which developed for total nucleic acid isolation from fresh tissues was modified, and it was evaluated for detection of PPRV from FFPE tissues. To improve RNA yields and purity and to degrade cross-linked RNAs, overnight proteinase K incubation period was added to the procedure of modified extraction method.

In the current study, PPRV specific RNA was detected in 12 of 15 lung tissue samples which were previously found positive by N gene based one step real time RT-PCR. Three positive samples, had been found positive by one step real time RT-PCR, were found negative by one-step RT-PCR. Furthermore, five FFPE tissue samples that had been found negative by one step real time RT-PCR method were also found negative by one-step RT-PCR. The difference between results of one step RT-PCR and one step real time RT-PCR can be explained by extraction method, analytical sensitivities of the methods and RNA quality of the three samples that were found negative by RT-PCR method. Because these three samples that had been found positive by one step real time RT-PCR had high Ct values when compared other samples (Table 1). It has been reported that viral load is inversely related with Ct values, a high Ct value indicating a low viral load (Bonacorsi et al., 2021). Furthermore, these three samples that were found negative by one step RT-PCR had an A260/280 ratio under 1.8. It has been reported that ratios under 1.8 could indicate the presence phenol, proteins and other contaminants (Glasel, 1995). The purity of the RNA is very important for RT-PCR (Banko et al., 2021). Therefore, these false negative results can be explained by low purity of the samples.

Conventional RT-PCR has lower sensitivity compared with real time RT-PCR. Therefore, real time RT-PCR can detect lower viral loads which were below the detection limit of the one-step RT-PCR (Banko et al., 2021; Ramamurthy et al., 2011). Furthermore, it has been reported that formalin fixation causes RNA degradation, poly-A tail damage and RNA

modification by adding methylol groups (-CH_2OH) which are reduce PCR efficiency (Evers et al., 2011; Masuda et al., 1999). Therefore, these modifications because of formalin fixation may lead to false negative results.

In this study, PPRV F gene sequences were successfully amplified by one step RT-PCR assay using extracted RNA from FFPE lung tissues. Limited number of studies has been performed for detection of morbillivirus RNA from FFPE tissues (Liang et al., 2012; Seimon et al., 2013). To detect canine distemper virus RNA in FFPE tissues, Liang et al. (2012) used in situ hybridization (ISH) whereas Seimon et al. (2013) used RT-PCR and ISH. Furthermore, detection of PPRV specific RNA in FFPE tissues by real time RT-PCR has been reported (Kihu et al., 2015). Detection of PPRV RNA in FFPE samples allow to use FFPE tissues in the confirmation of PPR in laboratories where fresh tissues are not available for RNA extraction.

In this study, one step RT-PCR assay was performed with primers targeting F gene of PPRV. Primers targeting nucleoprotein (N) gene of the PPRV were also chosen for detection of PPRV in FFPE tissues (Kihu et al., 2015). Therefore, results of the studies suggest that both of the primers can be used for detection of PPRV in FFPE tissues.

CONCLUSION

In conclusion, results of the current study show that modified extraction method could be used for PPRV RNA extraction from FFPE tissues which had been stored for 2 years. However, viral load in FFPE tissues should be considered when using this modified extraction method. This provides an opportunity to field veterinary laboratories that may lack cold storage facilities to keep fresh pathological samples for PPR diagnoses.

ACKNOWLEDGMENT

The part of the study was presented as an oral presentation in the 4th International Conference on Medical and Health Sciences in Burdur, Turkey, 19-20 March, 2022.

Ethical approval: This study data was used with the permission of General Directorate of Food and Control dated 13.11.2017 and numbered E.2852005.

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Clinical and immunohistochemical evaluation of penile tumors in bulls

ABSTRACT

Fibropapilloma is the most common neoplasic formation associated with bovine papillomavirus type I. It occurs inside the penis of bulls and causes clinically adverse effects, such as phimosis, paraphimosis, urethral stricture, and urinary retention. This study aimed to conduct a clinical and immunohistochemical evaluation of penile tumors, which are associated with adverse outcomes with regard to breeding value and yield in bulls.A total of 40 bulls of different breeds aged between 10 months to 3 years were included in the study. Tumor tissue samples collected postoperatively were fixed in 10% buffered formaldehyde solution. The avidin–biotin–peroxidase method was used for immunohistochemical staining. Three separate areas were examined under 40X objective lens for each fibropapilloma tissue. The immunoreactivity was classified as none (-), mild (+), moderate (++), and severe (+++). Fibropapillomas ranged from 2 to 10 cm in diameter. Thirty-two papillomas were pedunculated and eight were sessile and attached to the body; 30 were solitary and 10 were multiple. There were 13 relapsed cases and 27 non-relapsed cases. Immunohistochemical examination revealed a statistically significant difference in the binary comparisons of proliferating cell nuclear antigen (PCNA) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) based on positive cell scoring between the relapsed and non-relapsed cases. In conclusion, cancer markers such as PCNA and HPRT can be used to evaluate the prognosis and malignancy of penile tumors that cause significant economic losses.

Keywords: Fibropapilloma, cattle, hypoxanthine-guanine phosphoribosyltransferase, proliferating cell nuclear antigen, penile tumor.

INTRODUCTION

Fibropapilloma is the most common neoplasic formation associated with bovine papillomavirus type I. It occurs inside the penis of male cattle. Similar lesions can be observed on the vulvae and teats of cows in the same herd as an infected bull. Fibropapillomas, which generally occur at the tip of the penis, are more prevalent in young bulls aged <3 years than in older bulls due to the development of immune response by age. Penile tumors are associated with phimosis, paraphimosis, or penile prolapse. Furthermore, fibropapillomas with ulcerated surfaces can cause contamination through microorganisms and blood during cryopreservation of collected semen (Kamiloğlu et al., 2004; Khodakaram et al., 2009; Kaya et al., 2010; Heppelman et al., 2019). Their attachment to the penile tissue can be pendulous or broad-based (Bulut and Ünsaldı., 2001; Heppelman et al., 2019). Although the fact that the cauliflower-like appearance of fibropapillomas during clinical observation may help with diagnosis, the definitive diagnosis is made following histopathological evaluation (Kaya et al., 2010).

How to cite this article
Aydın, U., Karakurt, E., Dağ, S., Beytut, E., Özaydın, İ., Nuhoğlu, H., Yıldız, U., Yıldız, A., Kurtbaş E. (2022). Clinical and immunohistochemical evaluation of penile tumors in bulls. *Journal of Advances in VetBio Science and Techniques*, *7*(2), 210-219.
https://doi.org/10.31797/vetbio.1086702

Research Article

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Article info
Submission: 12-03-2022
Accepted: 24-07-2022
Online First: 06-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

Surgical removal may prove to be a better option in bulls that are intended for breeding on the grounds that spontaneous resolution of fibropapillomas may occur but at a slower pace. When untreated, pain and mechanical interference during copulation can adversely affect intromission and fertility (Bulut and Ünsaldı., 2001; Kamiloğlu et al., 2004; Heppelman et al., 2019). Treatment options such as excision, electrocauterization, and cryosurgery are used in the treatment of penile tumors (Kamiloğlu et al., 2004; Kaya et al., 2010; Heppelman et al., 2019).

The present study aimed to conduct a clinical and immunohistochemical evaluation of penile tumors, which are associated with significant economic losses in terms of breeding value and yield in bulls.

MATERIAL and METHOD

Animal material

The animal material of this study comprised 40 cattle presented to the animal hospital polyclinics of the Faculty of Veterinary Medicine, Kafkas University, with the complaint of a mass in their penis.

Clinical observations and treatment procedure

The bulls that were presented to the Animal Hospital of the Faculty of Veterinary Medicine, Kafkas University, underwent detailed clinical examinations. Bulls that were clinically diagnosed with tumors upon taking anamneses, as well as inspection and palpation examinations, underwent a surgical operation after the necessary preparations. Furthermore, the presence of tumor structures in different regions was investigated by inspection, rectal palpation, and radiographic examination prior to the surgical intervention. The inspection included the whole body along with the penis, radiographical examination included the lungs and chest cavity in particular, and palpation and ultrasound-guided rectal examination included the pelvic urethra, urinary bladder, kidneys, abdominal cavity, spleen, and liver. After completion of all the clinical examinations, the animals were placed in the lateral position using Reuff's method following sedation by injecting 0.02 mg/kg IV xylazine HCI (Rompun® 2% 25 ml Bayer). Then, the penis was taken out of the preputium. The coarse dirt was removed by an assistant using antiseptic solutions. Then, 70% ethyl alcohol and 10% povidone-iodine (PVP-I) were used for the purposes of asepsis and antisepsis of the surgical site, which was thereafter isolated using surgical drapes. In addition to sedation, local infiltrative anesthesia of the penis was performed using lidocaine HCI (Vilcain® 50 mL, Vilsan). Following anesthesia, the mass in the penile tissue was removed using electrocautery. The mass was referred to the Department of Pathology for histopathological examination. Postoperatively, analgesics (0.5 mg/kg meloxicam-Anafleks® 0.5%, Hektaş) and antibiotics (procaine benzylpenicillin 200,000 IU, dihydrostreptomycin sulfate 200 mg, Reptopen® S, CEVA-DIF) were recommended for use for 3 days and 7 days, respectively, together with nitrofurazone ointment (Furacin® 0.2% Sanofi İlaç San. Tic. A.Ş.) for use around the penis and inside the preputium.

Tissue samples

The study material included samples from 40 cases of fibropapilloma, which were collected from the penile sites of bulls presented to the Department of Surgery and Pathology, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey.

Histopathological examinations

Tumor tissue samples collected postoperatively were fixed in 10% buffered formaldehyde solution and embedded in paraffin blocks. Next, serial sections of 5-µm thickness were cut for hematoxylin and eosin (H&E) staining, where serial sections of 4-µm thickness were cut for immunohistochemical staining of poly-l-lysine-coated slides.

H&E staining was performed for the sections to investigate the histopathological changes, which were evaluated under a light microscope (Olympus Bx53). Images were taken using CellΛP software (Olympus Soft Imaging Solutions, GmbH, 3.4).

Masson trichrome staining with aniline blue

The staining procedure was performed according to the manufacturer's instructions (Facepath, Barcode No: 8681065132824).

Immunohistochemical examination

The avidin–biotin–peroxidase method was preferred for immunohistochemical staining. The sections were deparaffinized for 10 min in three separate xylol series. The sections were rehydrated in graded alcohol series (100%, 96%, 90%, 80%, and 70%). The sections were soaked in distilled water for 3 min, followed by incubation for 20 min in 3% hydrogen peroxide solution to block endogenous peroxidase activity. The microwave method was used for the sections, which were soaked in phosphate-buffered saline (PBS) for 3 min to expose the antigenic receptors (Citrate buffer solution, pH 6, 800 watts, 10 min). After 20 min of cooling, non-immune serum (Thermo Scientific Histostain IHC Kit, HRP, broad spectrum, REF:TP-125-HL) was added to the sections for 10 min to prevent nonspecific staining. The primary antibodies, which were diluted at different ratios in PBS (anti-bovine papillomavirus [BPV], MyBioSource, MBS320197, dilution ratio 1/100; proliferating cell nuclear antigen [PCNA], Santa Cruz, SC-56, dilution ratio 1/100; and hypoxanthine-guanine phosphoribosyltransferase [HPRT], Bioss Antibodies, BS-9026R, dilution ratio 1/400), were then incubated in the fridge (4°C) overnight. Biotinylated secondary antibody (Thermo Scientific Histostain IHC Kit, HRP, broad spectrum, REF:TP-125-HL) was added to the sections for 10 min at room temperature, following which the sections were rinsed in PBS buffer three times for 3 min each. Next, all the sections were incubated for 10 min using peroxidase-conjugated Strep Avidin (Thermo Scientific Histostain IHC Kit, HRP, broad spectrum, REF:TP-125-HL). The chromogen substrate 3,3′-diaminobenzidine-tetrahydrochloride solution (Thermo Scientific, REF:TA-125-HD) was incubated for 15 min by dripping into the sections. The sections, which were rinsed in distilled water for 5 min, were stained using Mayer's hematoxylin solution and covered with immune mount.

The preparations were observed under a light microscope (Olympus Bx53), and images were taken using CellΛP software (Olympus Soft Imaging Solutions GmbH, 3.4). Detailed analysis of the images were performed using ImageJ (1.51j8).

Analysis of the immunohistochemical staining results of BPV, PCNA, and HPRT antibodies were performed upon investigation of immune positive reactions in the sites that most prominently reflected the staining character using a rating system based on the number of positive cells. To quantify the immune positive reactions in the tissues, the analyses were started based on the high-intensity reaction sites. Three separate areas were examined under 40X objective lens for each fibropapilloma tissue. Immunoreactivity was classified as none (-), mild (+), moderate (++), and severe (+++) (Beytut 2017).

Statistical analysis

Statistical Package for the Social Sciences (SPSS® 26.0, Chicago, IL, USA) software was used to analyze the results. The Mann–Whitney U Test was used in binary comparisons of PCNA and HPRT based on the positive cell scoring. The results were expressed as mean ± standard error (SE). A P value of <0.05 was considered statistically significant.

RESULTS

Clinical findings

The ages of the bulls ranged from 10 months to 2 years. Eighteen bulls were aged 2 years, 11 were aged 1 year, 7 were aged 1.5 years, 1 was aged 3 years, and 3 were aged 10 months. In addition, 19 bulls were of Brown Swiss breed and 21 were of the Simmental breed.

Pedunculated or sessile wart-like growth with a cauliflower-like appearance was observed during the macroscopic examination of the fibropapillomas. Some of the masses were highly hemorrhagic and necrotic. Most of the papillomas (32) were located at the tip of the penis (Figure 1A), and the others (8) were located in the penile body (Figure 1B).

Figure 1a. Image of tumoral mass in the glans penis, **b.** Image of tumoral mass in the penis body. Thirty were solitary and 10 were multiple.

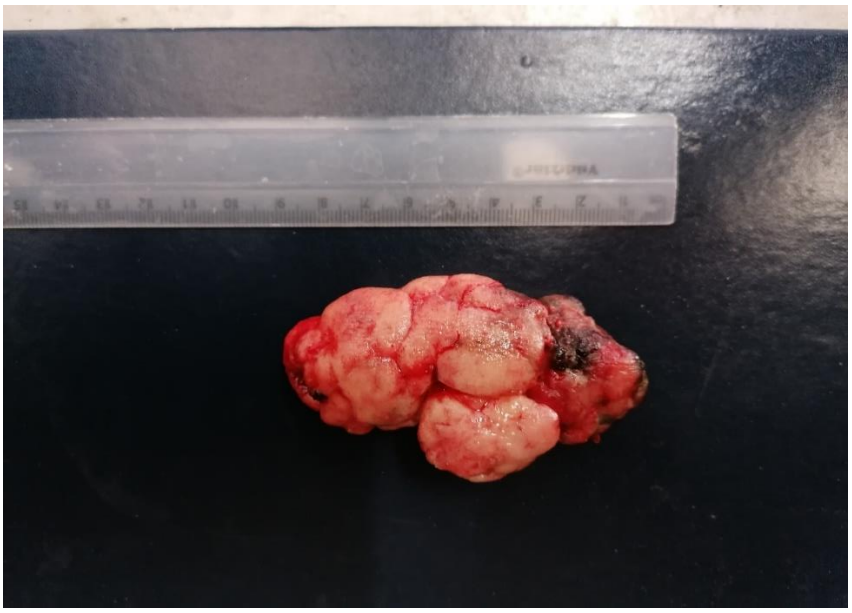

Figure 2. Postoperative macroscopic view of the tumoral mass. The diameter of penile tumors ranged from 2 to 10 cm.

Upon inspection, palpation, ultrasonography, and radiographic examination for the presence of tumor formations, there were no tumor-like find.

Microscopic findings

Hyperkeratosis, acanthosis, and finger-like protrusions from the epidermis to the dermis and dense bundles of connective tissues between these structures that form vortex structures were detected upon histopathological examination of the masses. Degeneration of keratinocytes, increase in the abundance of keratohyalin granules, and koilocytosis were observed. Other remarkable histopathological manifestations included inflammatory cell infiltration in the dermis layer, sporadic mitotic figures, hemorrhage and ulcers, and large necrotic areas (Figure 3-a-b).

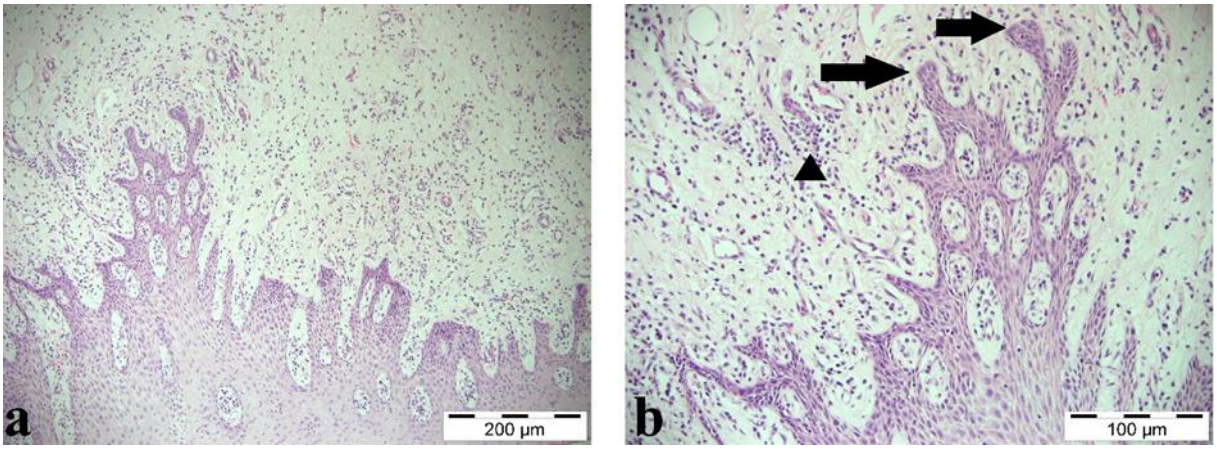

Figure 3a. Fibropapilloma, H&E, Bar= 200 µm, **b.** Higher magnification, rete pegs (arrows) and perivascular inflammatory infiltration (arrowhead), H&E, Bar= 100 µm

Masson trichrome staining with aniline blue

Masson trichrome staining with Aniline Blue indicated very intensive collagen accumulation in cases with fibropapilloma. There was increased fibroblast activity in the rete peg areas and in the dermis immediately below the epidermis layer, and fibroblast activity was decreased toward the deeper sections of the masses (Figure 4 a-b).

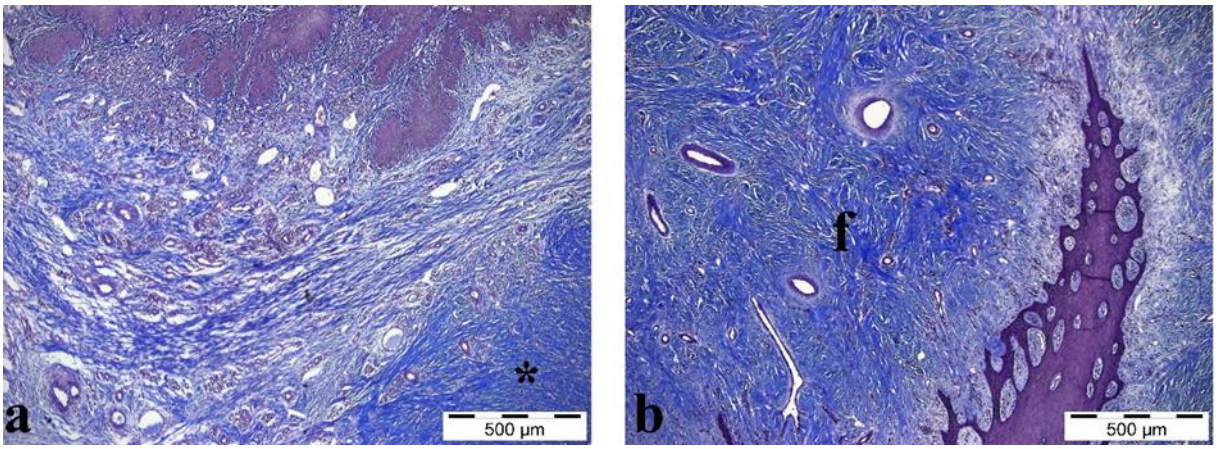

Figure 4. Masson Trichrome Aniline Blue Staining, **a-b.** Presence of connective tissue (star and f), Bar= 500 µm

Immunohistochemical findings

The number of positive cells in terms of PCNA and HPRT expressions of relapsed and non-relapsed cases after treatment are given in Table 1. It was determined that there was a statistically significant difference in PCNA and HPRT expressions in the relapsed group compared to the non-relapsed group. Immunohistochemical examination revealed

BPV immune positive reactions in the nuclei of cells in the stratum granulosum layer of the epidermis (Figure 5-a-b). The PCNA immune positive reactions were more intense in epithelial hyperplasia and acantholytic areas. The PCNA cell expressions were especially yellow brownish in the nuclei of tumor cells in the periphery of rete peg structures. The cellular proliferation of HPRT immune positive reactions were increased, characterized by epithelial hyperplasia, and more intense in the acantholytic sites. The HPRT cell expressions in a dark brown granular form were localized in the cytoplasm of fibrocytes and fibroblasts.

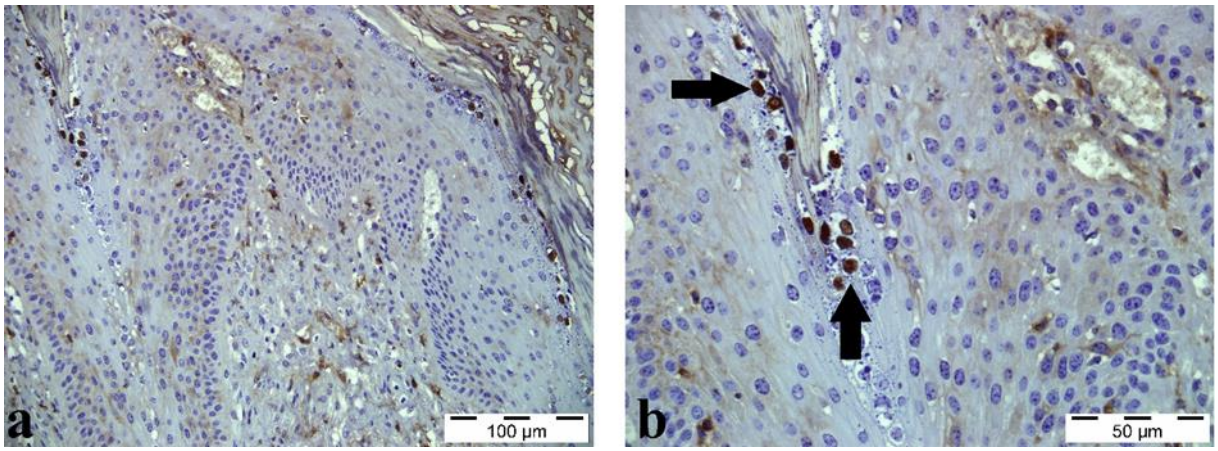

Figure 5 a. BPV immunopositive reactions in the nuclei of cells in the stratum granulosum layer, Bar= 100 µm, **b.** Higher magnification, intranuclear BPV expressions (arrows), IHC, Bar= 50 µm

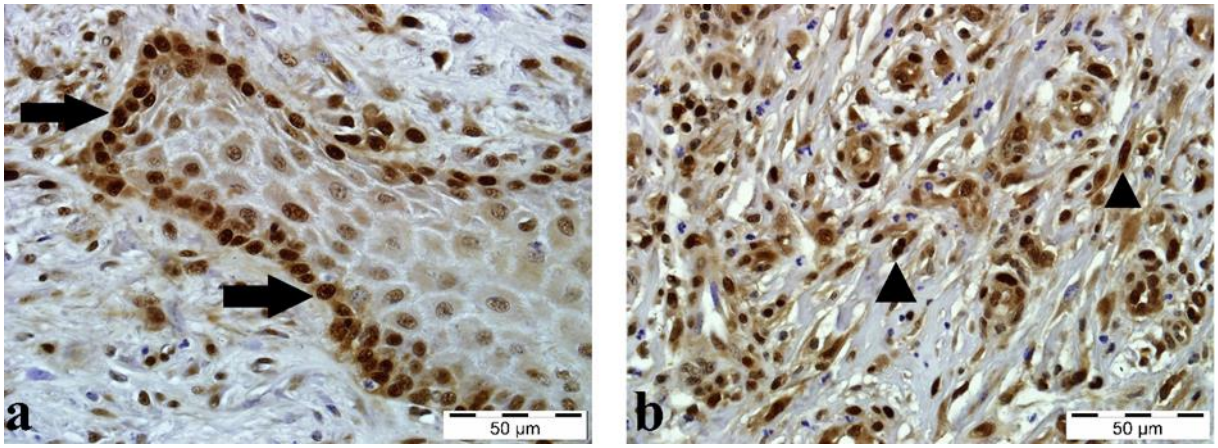

Figure 6. PCNA, IHC, **a.** Immune positive reactions in the nuclei of tumoral cells (arrows) in the periphery of the rete peg structures, Bar= 50 µm, **b.** PCNA immunoreactivity in the nuclei and cytoplasm of fibrocyte and fibroblasts (arrowheads), Bar= 50 µm

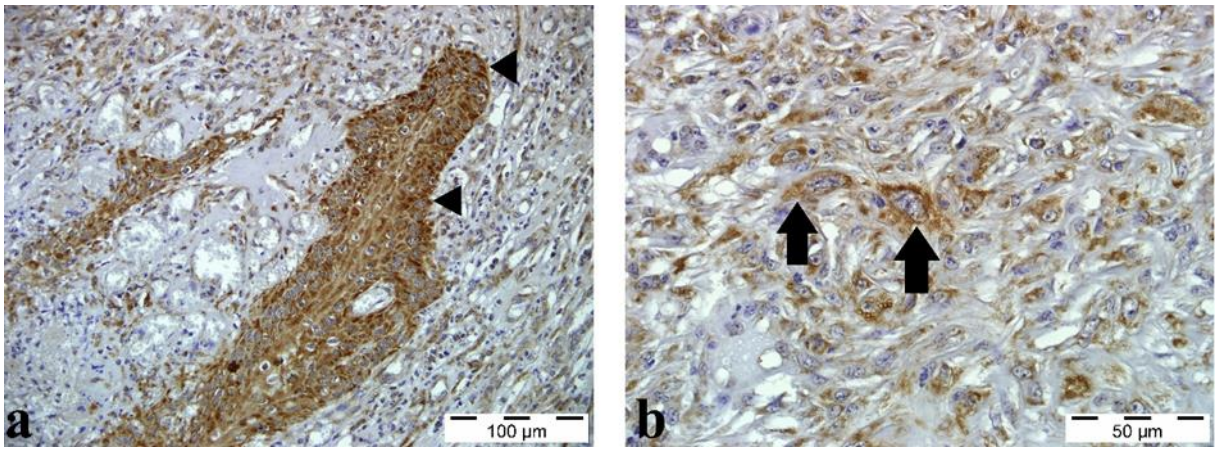

Figure 7. HPRT, IHC, **a.** Intense intracytoplasmic dark brown positive reactions in rete peg structures, Bar= 100 µm, **b.** Granular HPRT expressions in the cytoplasm of connective tissue cells, Bar= 50 µm

Postoperative period

All patient owners were contacted by phone for information. There were no relapses in 27 of the 40 cases, whereas there were 13 cases of relapse; 2 relapsed cases were re-operated, with no recurrence thereafter. In all the cases of relapse, the bulls were fattened for a period of 1–3 months and reserved for beef consumption. Eleven out of the 27 bulls without relapse after treatment were still used as breeders; there was no transmission to the offspring and their mothers, and 16 bulls were reserved for beef consumption after an adequate fattening period of approximately 1 year. The three relapsed cases had urinary retention, whereas there were no urination-related problems in 37 cases.

Positive cell scoring

Immunohistochemical examination showed a statistically significant difference as a result of the binary comparisons of PCNA and HPRT by positive cell scoring between the relapsed cases and non-relapsed cases.

Table 1. Statistical analysis of PCNA and HPRT cell expression between relapsed and non-relapsed cases after treatment

Groups	PCNA	HPRT
Recurrence	2.85±0.38	2.77±0.44
No recurrence	1.41±0.50	1.19±0.40
P Value	<0.001	<0.001

DISCUSSION

Penis tumors in cattle are associated with significant economic losses if not treated in a timely manner. Therefore, it is important for the prognosis of the disease to choose both an effective treatment option and an advanced diagnosis method for penile tumors. A significant loss of productivity was prevented because of the treatment option and diagnostic method used in the present study and the results will contribute to clinical practice.

Fibropapillomas are benign tumors caused by papillomavirus; they originate from fibrocytes and fibroblasts and have a cauliflower-like appearance, as observed in the bulls, especially those aged 1–3 years, located around the penis, and preputium and in the vulva and teats of cows in the same herd as the infected bull. Fibropapillomas are usually localized at the junction of the glans and corpus penis and the craniodorsal part of the penis (Bulut and Ünsaldı, 2001; Biricik et al., 2002; Khodarakam and Kargar, 2009; Kaya et al., 2010; Heppelman et al., 2019). These tumors, which are fed by vessels, may appear pedunculated or sessile (Bulut and Ünsaldı, 2001). In the present study, the fibropapillomas in the operated bulls occurred on the glans and corpus penis and had a cauliflower-like appearance. With regards to the 11 breeding bulls, which were also used for insemination, there was no post-treatment transmission in the mated cows and the offspring born from these animals. In 22 cases, there was a pedunculated attachment to the penile tissues, whereas in 18 cases, there was a sessile attachment. The majority of the 40 cases (36 cases) were aged 1–3 years. The fact that elder animals had a comparatively more advanced immune system may account for the fact that the vast majority of fibropapillomas occurred in younger bulls.

Although fibropapillomas can vary between a hazelnut and an apple in size, they may usually occur in a solitary or multiple structure (Bulut and Ünsaldı, 2001; Khodarakam and Kargar, 2009). In the present cases, fibropapillomas were closer in size to an apple in those with solitary structure, whereas those

with multiple structures reached the size of nuts or walnuts and in some cases were the size of an apple. The fact that the owners of the bulls failed to notice papillomas until hemorrhage and reluctance to copulate was observed might be the underlying reason for the large-sized fibropapillomas in the present cases. In addition, the owners of the bulls with papillomas with solitary structure, especially those on the glans, may not notice the condition until the penis hangs out due to the effect of gravity. This may result in excessive growth of papillomas.

Fibropapillomas on the glans caused urethral stricture, urinary retention, and even complete closure of the orificium urethra externa, and rarely led to ischemic necrosis upon combined pressure on the penis of preputium hair and tumor (Bulut and Ünsaldı, 2001; Kamiloğlu et al., 2004). Urinary retention was observed in only 3 of the 40 cases in the present study, whereas there was no stricture, closure, and resultant urinary retention in the other 37 cases.

Usually, electrocautery is performed for the treatment of penile tumors in bulls because this method is associated with a decreased incidence of hemorrhage and relapse. The tumor can also be removed using traditional surgical procedures. Nevertheless, the risk of hemorrhage and transmission is slightly higher in the traditional surgical methods (Bulut and Ünsaldı, 2001; Kamiloğlu et al., 2004; Kaya et al., 2010; Heppelman et al., 2019). In all the present cases, the tumor was removed using electrocautery. The reason for the small number of recurrent cases during the postoperative period can be considered the complete removal of the tumor using electrocautery.

Although penile tumors can be clinically diagnosed, the definitive diagnosis requires histopathological and immunohistochemical examination (Kaya et al., 2010). The macroscopic and microscopic characteristics of the fibropapilloma cases in the present study were consistent with the data reported in the literature (Kelman, 1997; Jelinek and Tachezy, 2005; Hatipoğlu et al., 2009). Similar to that reported in previous studies (Biricik et al., 2002; Hatipoğlu et al., 2009), intranuclear BPV immune positive reactions were observed in cells in the stratum granulosum layer.

Proliferating cell nuclear antigen (PCNA) plays an important role in nucleic acid metabolism as a component of the replication and repair mechanism (Kelman, 1997). It has many functions, such as DNA repair, translation synthesis of DNA, DNA methylation, chromate remodeling, and cell cycle regulation (Maga and Hübscher, 2003). Furthermore, it is used to investigate tumor cell proliferation and tumor malignancy (Hatioğlu et al., 2009; Özsoy et al., 2011). Özsoy et al. (2011), investigated PCNA immunoreactivity in 2011 to determine cell proliferation in cases of bovine cutaneous papillomatosis. They found that the PCNA expression was more intense in epithelial hyperplasia and acantholytic areas. Similarly, in 2005, Jelínek and Tachezy (2005) reported that there was intensive PCNA immunoactivity in the basal layer of the epidermis and dermis in cases of bovine cutaneous papillomatosis. In 2007, Maeda et al. (2007) investigated PCNA expression to determine cell proliferation in cases of breast papillomatosis caused by papilloma virus type-6 and found that the reaction was more intense in the spinous layer of the epidermis and in the basal layer. Consistent with the results reported in the literature, there was intensive intranuclear PCNA expressions in the periphery of rete peg structures in the present study. This increase in PCNA expression indicates that fibropapillomas have a very high proliferative index. When the postoperative long-term outcomes in the present cases were evaluated, it was found that all relapsed cases were directly correlated with the increases in PCNA expression. There was a statistically significant difference upon binary

comparisons of PCNA cases with and without relapse by positive cell scoring.

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is a common marker used to determine the frequency of mutations in the development of cancer and potential carcinogens (Townsend et al., 2017). HPRT is a recovery pathway enzyme that is involved in the production of both guanine and inosine bases. The enzyme acts by transferring phosphoribose from phosphoribosyl diphosphate to hypoxanthine or guanine bases, forming inosine monophosphate and guanosine monophosphate, respectively (Townsend et al., 2017). Due to the constant need for guanosine-5'-triphosphate as both a nucleotide for DNA synthesis and an energy molecule, HPRT is reliably produced as a cleaning gene across the cell and is found at low levels in all somatic tissues (Townsend et al., 2019). HPRT has shown potential as a surface antigen in several malignancies, including lung cancer, colorectal cancer, and Burkitt's lymphoma, and is used as an important parameter in determining malignant structures along with increased metastasis and tumor proliferation. Nevertheless, there is no detailed study that fully explains the effect of HPRT on tumor proliferation and migration (Townsend et al., 2021; Wang et al., 2021). Townsend et al. (2018) investigated enzymes in a colorectal cancer cell line and reported that HPRT had excessive expression in cancer tissues compared with that in normal tissues because of its role in proliferation and cell cycle regulation. Townsend et al. (2019) investigated HPRT expression in benign and malignant cancer cases using immunohistochemical and PCR methods and reported that HPRT immunoactivity was more intense in malignant cancer cases than in benign cancer cases. Wang et al. (2021) found that HPRT supported proliferation and metastasis in head and neck squamous cell carcinoma by directly interacting with signal transducer and activator of transcription 3 and that suppression of HPRT expression stopped cancer development. In addition, it was suggested that HPRT expression indicated poor prognosis. Similarly, Sedano et al. (2020) investigated the role of HPRT1 in breast cancer and found that HPRT1 expression was higher in tumor tissues than in normal tissues. As a result of the above research, it was concluded that there was an inverse correlation between HPRT expression and survival. Similar to the studies by Townsend et al. (2019), Sedano et al. (2020), and Wang et al. (2021) the present study investigated the immunoactivity of HPRT in cases of fibropapilloma, a benign tumor, and found that the reaction was more intense in sites with increased proliferation, characterized by epithelial hyperplasia, and in acantholytic sites. A review of the postoperative long-term outcomes of the cases suggested that there was a direct correlation between the increased HPRT and PCNA expressions and the relapsed cases. Furthermore, there was a statistically significant difference upon binary comparisons of PCNA cases with and without relapse by positive cell scoring.

CONCLUSION

In conclusion, it was shown that cancer markers, which are of great importance terms of the prognosis of penile tumors, which are among the most important causes that have a negatively impact on the breeding value and meat yield of bulls, are useful parameters in evaluating the malignancy of this disease. Moreover, the clinical results of the study will significantly contribute to clinical practice in terms of the prognosis of penile tumors and thus help with preventing the loss of productivity to a large extent.

ACKNOWLEDGMENT

The authors thank Assoc. Prof. Mushap KURU for his help to statistical analyses.

Ethical approval: The study was conducted following the approval of the Kafkas University Animal Experiments Local Ethics Committee (approval no: KAÜ-HADYEK-2020-172).

Conflict of interest: There is no conflict of interest between the authors.

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Koyunlarda kan BHBA seviyesinin fertilite üzerine etkisi

The effect of blood BHBA level on fertility in sheep

ÖZET

Bu çalışmada koyunlarda sezon dışı dönemde senkronizasyon öncesi kan BHBA seviyesinin gebelik oranlarına üzerine etkisinin incelenmesi amaçlandı. Bu amaçla, Burdur ilinde bulunan en az 1 doğum yapmış 2-4 yaşlı 100 baş Merinos/Merinos melezi koyun kullanıldı. Senkronizasyon amacıyla hayvanlara progesteron emdirilmiş süngerler 60 mg Medroxy progesteron acetat (MPA) intravaginal olarak 14 gün süre ile uygulandı. Uygulama günü hayvanlardan kan alınarak, BHBA (betahidroksibütirik asit) ölçümü yapıldı. Süngerler çıkartılırken, 500-700 IU aralığında kas içi PMSG uygulaması yapıldı. Süngerlerin çıkarılmasından 24 saat sonra arama koçu yardımı ile östruslar gözlendi ve östrusta olduğu belirlenen hayvanlar elde sıfat yöntemi ile çiftleştirildi. Çiftleştirmeleri takiben 35-45. gün aralığında transrektal prob kullanılarak ultrason yardımı ile gebelikler kaydedildi. Senkronizasyona alınan hayvanların tamamının östrusta olduğu belirlendi ve aşımları gerçekleştirildi. Yapılan gebelik muayenesi sonucunda hayvanların 57 baş hayvan gebe (%57) iken 43 baş (%43) hayvan gebe kalmamıştır. Çalışmada, kan BHBA değerleri 0,12 mmol/L -0,66 mmol/L (n:100) aralığında ölçülmüş olup ortalama 0,35±0,083 mmol/L olarak tespit edilmiştir. Gebe kalan hayvanların kan BHBA seviyesi 0,29±0,005 mmol/L olarak tespit edilirken, gebe kalmayan koyunların kan BHBA seviyesi 0,41±0,073 mmol/L olarak tespit edilmiştir. ($p<0,001$). Ayrıca çalışmada kullanılan koyunlarda kan BHBA düzeyi ile gebelik oranları arasında güçlü bir negatif korelasyon olduğu belirlenmiştir ($r = −0,719$, $p<0,001$). Sonuç olarak literatürde anılan dönemde koyunların fertilite parametreleri ile kan BHBA düzeyi ile arasındaki ilişki hakkında bir çalışma olmaması sunulan çalışmanın literatüre önemli bir katkı sağladığı kanısına varılmıştır. Düşük BHBA seviyelerine sahip olan hayvanlardan daha yüksek bir fertilite başarısı elde edilebileceği, yüksek BHBA seviyesine sahip hayvanların enerji dengelerinin düzenlenerek fertilite oranlarının yükseltilebileceği, yapılan çalışmanın küçükbaş hayvanlarda sürü yönetimi açısından 2 yılda 3 kuzulatma hedefinin sağlanmasında dikkat edilmesi gereken bir kriter olabileceği sonucuna varılmıştır.

Anahtar Kelimeler: BHBA, Fertilite, Koyun, Senkronizasyon.

ABSTRACT

The aim of this study was the effect of pre-synchronization blood BHBA levels on pregnancy rates of sheep in the non-breeding season. For this purpose, 100 Merino/Merino crossbred ewe, aged 2-4 years, who have given at least one birth before, were used in the study. For synchronization purposes, progesterone containing sponges 60 mg Medroxyprogesterone acetate (MPA) analogue, was administered intravaginally for 14 days. BHBA (beta hydroxybutyric acid) measurement was made by drawing blood in each animal on the day of administration. 500-700 IU PMSG was injected at the time of sponge withdrawal. Oestrus was observed 24 hours after sponge withdrawal with the help of teaser ram. Animals which was in estrus were hand mated. Pregnancy status was recorded with the help of ultrasound using the transrectal probe in the interval of 35-45 days after mating. All of the synchronized animals were determined to be in estrus and their mating was performed. As a result of the pregnancy examination, 57 (57%) of the animals became pregnant and 43 (43%) did not become pregnant. Blood BHBA values were measured in the range of 0.12 mmol/L -0.66 mmol/L (n:100) in the present study and the average of BHBA as 0.35±0.083 mmol/L was determined. While the blood BHBA level of pregnant animals was found as 0.29±0.005 mmol/L, blood BHBA level of non-pregnant sheep was found to be 0.41±0.073 mmol/L, which was statistically significant ($p<0.001$). In addition, it was determined that there was a strong negative correlation between blood BHBA level and pregnancy rates in sheep ($r = −0.719$, $p<0.001$).

How to cite this article
Güngör, Ş., Özkara, H., İnanç, ME., Herdoğan, M., Mart, F., Çay, HA., Yıldız, R., Gümüş, H., Akın, AC., Ata A. (2022). The effect of blood BHBA level on fertility in sheep. *Journal of Advances in VetBio Science and Techniques, 7*(2), 220-227.
https://doi.org/10.31797/vetbio.1117222

Research Article

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Article info
Submission: 16-05-2022
Accepted: 24-07-2022
Online First: 06-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

As a result, it was concluded that there was no study in the literature about the relationship between the fertility parameters of sheep and the blood BHBA level in the mentioned period, and it was concluded that the presented study made a significant contribution to the literature. It has been concluded that a higher fertility success can be achieved from animals with low BHBA levels, that fertility rates can be increased by regulating the energy balance of animals with high BHBA levels, and that the study can be a criterion to be considered in achieving the target of 3 lambing in 2 years in terms of herd management in sheep breeding.

Keywords: BHBA, Fertility, Sheep, Synchronization

GİRİŞ

Koyun yetiştiriciliği eski çağlardan itibaren insanların et, süt, yapağı, kürk, deri, post, gübre gibi çeşitli ihtiyaçlarını karşılamaktadır. Günümüzde yetiştiriciliği yapılan koyun ırkları doğal şartların olumsuz etkilerinin yanı sıra, yetiştiricinin damızlık seçimi ve yetiştirme faaliyetleri sonucu oluşmuştur (Akdağ vd., 2018). Ayrıca koyunculuk, ülke ekonomilerinde yer alan önemli bir üretim faaliyetidir (Akçapınar, 2000). Bu kapsamda küçükbaş hayvan yetiştiriciliğinde en ekonomik yollar ile iyi bir verimlilik indeksi oluşturmak ve üreme performanslarının üst seviyelere çıkartılması hedeflenmektedir (Özyurtlu vd., 2010).

Koyunlarda üreme faaliyetlerinin denetlenmesi, yüksek verimli hayvanların genetik özelliklerinin muhafaza edilmesi, döl veriminin artırılması ve hayvan materyalinin en verimli bir şekilde kullanılması için yapılan uygulamalardır. Üremenin kontrol edilmesi ile mevsimsel poliöstrik hayvanlarda üreme sezonu dışında da gebelik elde edilebilir, ovulasyon şansı ve oranı yükseltilebilir (Özyurtlu vd., 2010). Üremenin denetlenmesi ile doğacak kuzular daha erken yaşta cinsel olgunluğa erişebilir, üreme mevsiminin süresi kontrol edilebilir ve daha iyi döl verimi elde edilebilir. Koyunlarda üreme fonksiyonları üzerine yapılan uygulamalar genellikle mevsim dışında östrusun uyarılması veya üreme mevsiminde östrus ve ovulasyon senkronizasyonu şeklinde olmaktadır (Gordon, 1997). Östrusların senkronizasyonu ile luteal veya folliküler evreler kontrol altına alınır. Hayvanların sabit zamanlı tohumlanması veya elde sıfat yapılarak, belirli bir zaman diliminde doğumları gerçekleştirilerek piyasaya tek örnek sürü arzı sağlanır. Aynı zamanda, kuzulama oranının yükseltilmesi amaçlanır. Böylece 2 yılda 3 defa kuzulama hedeflenir (Alaçam, 1993). Tekniğine uygun bir şekilde yapılan senkronizasyon faaliyetleri sonucunda koyunlarda döl verimininin yanı sıra genetik ıslah da hız kazanmaktadır (Jainudeen vd., 2000).

Östrus senkronizasyonu için progesteron emdirilmiş intravaginal süngerler koyunlarda sıklıkla kullanılmaktadır. Ticari olarak satılan, FGA (Fluorogestone acetate) veya MAP (Medroxyprogesterone acetate) içeren intravaginal süngerler bulunmaktadır. İntravaginal sünger uygulamasın da genellikle 9-14 günlük periyotlarda uygulanır. Üreme mevsimi dışındaki uygulamalarda ovulasyonsuz östruslar şekillenebileceğinden dolayı follikül gelişimini desteklemek, ikizliği arttırmak ve ovulasyonu sağlamak amacıyla da kas içi PMSG (gebe kısrak serum gonadropin) enjeksiyonları önerilmektedir. Koyunlarda PMSG dozu östrusların uyarılması için 600-800IU, ikizliğin uyarılması amacıyla 600-1000IU arasında uygulanır (Uçar vd., 2002; Vinoles vd., 2001; Wildeus, 2000). Uygulamadan yaklaşık olarak 24-48 saat sonra östruslar görülmektedir (Wildeus, 2000).

Koyunlarda ovaryum üzerinde bulunan follikülerin gelişimi besin alınımına oldukça duyarlıdır. Günlük ihtiyacını karşılayacak dengeli ve yeterli bir beslenme olmaması durumunda negatif enerji dengesi (NED)

oluşur. Negatif enerji dengesi sonucu hayvanlarda fizyolojik olarak beyinde hipotalamo-hipofizyal aks üzerine etki etmektedir. Bu etkisini hipoinsülinemi, hipoglisemi ve plazma Insulin-Like Growth Factor-1 (IGF-1) düzeyini baskılayarak oluşturmaktadır. NED olan hayvanlarda kandaki insülin ve IGF-1 düşük seviyelerdedir. İnsülin ve IGF-1 ovaryum üzerine doğrudan uyarıcı etkiye sahip olup, ovaryum korteksinde yer alan folliküllerin gelişiminde direkt etkilidir (Yang ve Fortune, 2015). Dolaşımdaki IGF-1 konsantrasyonun düşmesi büyüme hormonunun (GH) üzerindeki negatif feedback etkisini azaltır ve dolaşımdaki GH konsantrasyonunun artmasına yol açar. Artan GH konsantrasyonu karaciğer de glikoneogenezisi arttırır ve vücuttaki depo yağların lipolizini hızlandırır, lipoliz sonucu esterleşmemiş yağ asidinin (NEFA) salınımı artar. GH ve NEFA konsantrasyonlarının yüksek düzeyde seyretmesi hayvanlarda insülin direnci oluşturur. Dolaşımdaki düşük insülin, IGF-1 ve glikoz seviyeleri aktif primer follikül üretimini kısıtlayarak LH'nın pulsatil salınımını baskılar. Bunun sonucunda da ovulasyon mekanizmasında aksamalar görülür (Lucy, 2007).

Keton cisimleri (BHBA, asetoasetik asit, aseton), yağ asitlerinin oksidasyonu sonucu oluşan ara ürünlerdir. Karaciğere ulaşan NEFA düzeyi, oksidasyon kapasitesinin üzerinde olması sonucu keton cisimlerinin üretimi artar (Ospina vd., 2010). Asetoasetik asit ve aseton, keton grubundadırlar. BHBA hidroksil grubunda yer almaktadır. Keton cisimlerinin %80'lik bir kısmını BHBA oluşturur. BHBA düzeyi ketoziste artar ve ketozis teşhisinde önemli bir parametredir (Duffield, 2000; Ospina vd., 2010). BHBA düzeyinin çalışılan örneklerde daha stabil olması asetoasetat ve aseton yerine daha fazla kullanılmasına neden olur. NED tespitinde en uygun zaman postpartum dönemin ilk 2 haftasıdır (Dann vd., 2005). NED teşhisi için kanda bulunan metabolik indikatörler üzerine yıllarca çalışılmıştır. Negatif enerji dengesini yansıtan bu indikatörlerden, BHBA karaciğerdeki yağ asitlerinin oksidasyon düzeyini, NEFA ise vücut depolarından mobilize olan yağ asit miktarını göstermektedir (LeBlanc, 2006). Ayrıca kas dokusunda yer alan hücreler keton cisimlerini glikoz yerine enerji kaynağı olarak kullanılabilir (Drackley vd., 2001; Janovick ve Drackley, 2011).

Laktasyon döneminde sağlık problemi olmayan ineklerde BHBA seviyesi 1 mmol/L'nin altında olmalıdır. Serum BHBA seviyesi 1,4 mmol/L'nin üzerindeyse; ruminantlarda, klinik ketozis riski oldukça yüksektir. Bir işletmedeki ineklerin %10'undan fazlasında serum BHBA değeri 1,4 mmol/L'nin üzerinde ise işletmede yer alan ineklerin sürü sağlığı açısından çok kritiktir (Carrier vd., 2004). Güç doğum, ikizlik, retensiyo sekundinaryum, mevsim, vücut kondisyon skoru gibi çeşitli faktörlerle birlikte BHBA, NEFA ve haptoglobin (hb) gibi metabolitlerin de periparturent süreçte metabolik stres oluşumunun metritis ile ilişkisi vurgulanmıştır (Dubuc vd., 2010). BHBA'in koyunlardaki düzeyi ile ilgili yapılan çalışmalar ineklerde olduğu gibi verim parametrelerinden ziyade yetersiz bakım ve besleme, çoklu gebelikler ve yaşlı hayvanları etkileyen ketonemi ve ketonüriyle seyreden gebelik toksemisi olarak adlandırılan metabolizma hastalığıdır (Sargison vd., 1994).

Bu çalışmada, yapılan literatür taramaları sonucu koyunlarda kan BHBA düzeyi ile gebelik oranları arasında ilişkinin olup olmadığına dair bir çalışma olmaması sonucu, sezon dışında intravaginal yöntemle senkronize edilen koyunların gebelik oranları ile kan BHBA düzeyleri arasındaki ilişkinin ortaya konması amaçlandı.

MATERYAL ve METHOD

Hayvanların seçimi ve bakım beslemesi

Çalışma, Burdur'da halk elinde yetiştiriciliği yapılan 2-4 yaşlı Merinos/merinos melezi en az bir doğum yapmış hayvanlardan toplamda 100 baş koyun kullanıldı. Koyunların günlük besin madde ihtiyaçları NRC standartlarına göre belirlenmiş (NRC, 2007) ve günde iki kez yemleme yapılmıştır, çalışma süresince hayvanların tükettikleri yemlerin besin madde analizi yapılarak Tablo 1'de verilmiştir.

Tablo 1. Çalışma süresince kullanılan yemlerin besin madde içerikleri

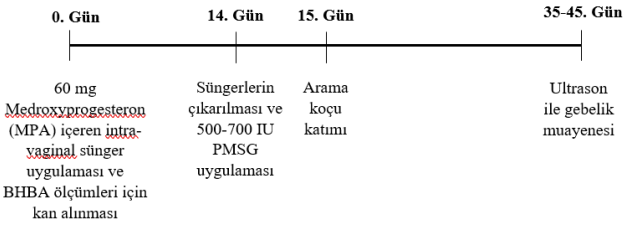
Yemler	Besin madde içerikleri[a]								
	KM	OM	HP	HS	HY	NDF	ADF	NFE	NFC
Konsantre yem karması[b]	89.25	93.27	12.53	8.52	3.12	21.75	9.17	58.38	55.87
Buğday samanı	91.55	92.60	4.45	42.55	1.55	73.15	53.75	35.60	13.45
Çayır kuru otu	89.90	91.50	10.25	28.15	2.30	63.25	39.25	40.70	15.70

a; KM: Kuru madde; OM: Organik madde; HP: Ham protein; HS: Ham selüloz; NDF: Nötr detergent fiber (Nötral deterjan lif), ADF: Acid detergent fiber (Asit deterjan lif), NFE: Nitrogen-free Extract = [KM%-(HP%+HS%+HK%+HY%)]; NFC: Non-fiber carbohydrate = [OM%– (NDF%+HP%+HY%)]. b; Her bir kilogram vitamin-mineral karışımı içerir; 1,000,000 IU vit A, 200,000 IU Vit D, 1,800 mg vit E, 8,400 mg Zn.

Rasyonun, %45'ini konsantre yem karması ve %55'ini kaba yem oluşturmuştur. Kaba yem olarak çayır kuru otu ve buğday samanı kullanılmıştır. Kullanılan yemler etüvde kurutulmuş ve analiz edilmek üzere öğütülmüştür. Yemlerde sırasıyla; (a) kuru madde analizi, 105 °C'de yemler 12 saat kurutularak (AOAC, metot 934.01); (b) ham kül analizi, 500 °C'de yemler kül fırınında 5 saat yakılarak (AOAC, metot 942.05); (c) ham yağ analizi Soxhlet yöntemi ile (AOAC, metot 920.39), ham protein analizi, Kjeldahl yöntemi ile (AOAC, metot 984.13); (d) ham selüloz analizi Crampton and Maynard (1938) belirttiği yönteme göre; (e) nötral deterjan lif analizi Goering ve Van Soest'in (1970) belirttiği yöntem esas alınarak ısı-dayanıklı amilaz ve sodyum sülfit ile; (f) asit deterjan lif analizi Goering ve Van Soest'in (1970) belirttiği yöntem esas alınarak özel ADF solüsyonu ile yapılmıştır. Temiz ve taze içme suyu ad libitum olarak verilmiştir. Çalışmadan önce yemliklerin ve sulukların dezenfeksiyonu yapılmış ayrıca hijyen kurallarına dikkat edilmiştir.

Senkronizasyon protokolü

Koyunlara sezon dışı dönemde 60 mg Medroxyprogesteron asetat (MPA) içeren süngerler (Esponjavet®/Sünger; Hipra, İstanbul, TÜRKİYE) uygulandı, 14. günün sonunda 500-700 IU PMSG (Gonaser®/PMSG; Hipra, İstanbul, TÜRKİYE) kas içi (im) enjekte edildikten sonra son uygulamadan 24 saat sonra 120. saate kadar arama koçu yardımı ile östrüs tespiti yapılarak östrus bulgusu gösterenler elde aşım yöntemi ile çiftleştirildi.



Şekil 1. Koyunlara uygulanan senkronizasyon, kan alma ve gebelik muayenesi protokolü

Gebelik muayenesi ve doğumların takibi

Aşımları takiben 35-45 gün aralığında ultrason yardımı ile gebelik muayeneleri yapıldıktan sonra gebelik sonuçları kaydedildi.

Kan BHBA düzeyinin tespiti

Senkronizasyon protokolünün başlangıç günü (0. gün) alınan kan örnekleri 3000 devirde 10 dakika santrifüj işleminden sonra serumları alınarak değerlendirme yapılıncaya kadar -86 °C'de saklandı. BHBA seviyesini ölçmek için keton ölçüm cihazı (Precision Xtra, Abbott Diabetes Care, Abingdon, UK) / ticari kit

kullanılarak spektrofotometrik (EPOCH, USA) yöntemle BHBA (Randox Ranbut, USA) ölçümleri yapıldı. Elde edilen sonuçlar mmol/L olarak kaydedildi (Ölmez vd., 2020).

İstatistiksel analiz

Elde edilen sonuçlara göre gebelik oranlarının analizinde student T testinden yararlanıldı. Kan BHBA ve döl verimi parametrelerinin korelasyonları pearson korelasyon yöntemi ile incelendi. Tüm istatistiksel analizler minimum %5 hata payı ile incelenerek ve SPSS 20.00 paket programı kullanıldı.

BULGULAR

Çalışma sonucunda gebelik oranları, gebe ve gebe olmayan hayvanları kan BHBA ortalamaları ayrıca gebelik ile kan BHBA düzeyi arasındaki ilişki Tablo 2'de verilmiştir. Buna göre senkronizasyon alınan hayvanların 57 başı gebe kalmış (%57), 43 baş (%43) gebe kalmamıştır. Koyunların sezon dışı süt verimin olmadığı dönemde kan BHBA değerleri 0,12 mmol/L -0,66 mmol/L (n:100) aralığında ölçülmüş olup ortalama 0,35±0,083 mmol/L olarak tespit edilmiştir. Gebe kalan hayvanların kan BHBA seviyesi 0,29±0,005 mmol/L olarak tespit edilirken, gebe kalmayan koyunların kan BHBA seviyesi 0,41±0,073 mmol/L olarak tespit edilerek istatiksel olarak anlamlı bulunmuştur ($p<0,001$). Ayrıca çalışmada kullanılan koyunlarda kan BHBA düzeyi ile gebelik oranları arasında güçlü bir negatif korelasyon olduğu belirlenmiştir ($r = -0,719$, $p<0,001$).

Tablo 2. Koyunlarda Senkronizasyon sonrası gebelik (%), BHBA (mmol/L) ile gebelik oranı ile BHBA seviyesi arasındaki ilişki

Grup	Gebe (n:57 (%57)	Boş (n:43 (%43)	P
BHBA (mmol/L)	0,29±0,005	0,41±0,073	**
Korelasyon (r)	−719**	381	**

P<0,001 **

TARTIŞMA

BHBA negatif enerji dengesinin en önemli laboratuvar yansımasıdır (Şentürk, 2013). Keton maddelerinden biri olan beta hidroksi butirik asit (BHBA), enerji dengesinin bozulmasıyla açığa çıkan, yağ asit oksidasyonu ara ürünüdür. Diğer keton maddeleri; asetoasetik asit ve asetondur. BHBA seviyesi, karaciğerdeki yağ asitlerinin oksidasyon düzeylerini yansıtmaktadır. Yani, karaciğerde esterleşmemiş yağ asit seviyesi, oksidasyon kapasitesinden fazla olduğunda keton maddeleri adını verdiğimiz asetoasetik asit, aseton ve beta hidroksi butirik asit üretimi artar (Duffield, 2006). Koyun ve keçilerde BHBA değeri, özellikle gebelik toksemisi adını verdiğimiz metabolizma hastalığında önem kazanmaktadır (Ağaoğlu ve Akgül, 2006; Gürgöze vd., 2009; Ramin vd., 2005). Sunulan bir çalışmada koyunlarda kan BHBA normal değeri 0.1-0.7 mmol/L arasında (ort: 0.3 mmol/L) bildirilmiştir. Elde edilen bu değerler çalışmamızda da benzer şekilde tespit edilmiştir. Gebelik toksemisinde bu değer genellikle 3 mmol/L'nin üzerine çıkmaktadır (Ramin vd., 2005). BHBA, geçiş döneminde enerji durumu ile ilgili en önemli indikatör olarak belirtilmektedir (Duffield, 2006). Bu nedenden dolayı, Navarre vd. (2002)'in koyunlar üzerinde yaptıkları bir çalışmada, BHBA'nın kandaki konsantrasyonunun 0,8 ila 1,6 mmol/L olmasının, koyunlarda NED'in göstergesi olduğunu öne sürmüşlerdir.

Moghaddam ve Hassanpour (2008)'un koyunlar üzerinde yaptığı bir diğer çalışmada ise BHBA konsantrasyonu, keçilerinkinin aksine doğum öncesi dönemde daha yüksek olduğunu ifade etmektedir. Sadjadian vd. (2012)'nin Saanen keçilerinde yaptıkları bir araştırmada, BHBA konsantrasyonlarının özellikle doğuma 15 gün öncesinden itibaren doğumdan sonraki 21. günlere kadar yükseldiği, sonraki günlerde de azaldığı tespit edilmiştir. BHBA'nın koyunlardaki düzeyi ile ilgili araştırmalar ineklerde olduğu gibi verim parametrelerinden ziyade ketonemi ve ketonüri ile seyreden ve daha çok multiparus gebelikleri ve yaşlı hayvanları etkileyen bir metabolizma hastalığı olan gebelik toksemisi üzerine yoğunlaşmıştır (Sargison vd., 1994). Çalışmada, bu çalışmalardan farklı olarak süt veriminin olmadığı, ovaryum aktivitenin eksojen olarak uyarılması ile senkronize edilen sezon dışı dönemde planlanması ve bu dönem kan BHBA seviyelerinin gebelik üzerine etkinliğini araştırılması amaçlanmıştır. Yapılan literatür taramalarında koyunlarda sezon dışı dönemde yapılan senkronizasyon yöntemleri ile kan BHBA düzeyinin incelenerek döl verimi parametreleri üzerine etkinliğini inceleyen bir çalışma tespit edilmemiş olup çalışmamızın özgünlüğünü oluşturmaktadır. Çalışmada kullanılan koyunların gebe olanlarının BHBA seviyelerinin düşük, gebe kalmayanlarda ise yüksek bulunması, ineklerde fertilite üzerine yapılan çalışmalar ile benzerlik göstermektedir (Dann vd., 2005; Walsh vd., 2007). İneklerde propilen glikol kullanımının gebelik oranları üzerine yapılan çalışmada BHBA değerleri, propilen glikol grubunda (0,72±0,10 mmol/L), ve kontrol grubunda (0,81±0,10 mmol/L) olarak bulunmuş, propilen glikol grubundan elde edilen gebelik oranının kontrol grubuna göre daha yüksek elde edildiği ifade edilmiştir. Elde edilen bu sonuç çalışmamızla benzerlik göstermektedir. Yine başka bir çalışmada artmış BHBA konsantrasyonları, postpartum ilk tohumlama sonrası gebelik olasılığı ile negatif olarak ilişkilendirilmiştir (Walsh vd., 2007). Gebelik toksemisinin insidensinin araştırıldığı çalışma sonuçlarına göre BHBA seviyesi gebe hayvanlarda gebe olmayanlara göre, ikizlikte ise tek gebelikten fazla olduğu bildirilmektedir (Walsh vd., 2007).

SONUÇ

Sonuç olarak üreme mevsimi dışında laktasyon döneminde olmayan hayvanların gebelik oranları üzerine BHBA seviyesinin araştırıldığı çalışmamız literatürdeki boşluğu gidermek adına önemli bulunmuştur. Gebelik istenen koyunların senkronizasyon öncesi dönemlerde kan BHBA seviyeleri kontrol edilerek kan BHBA seviyesi yüksek olanların enerji dengelerinin düzenlendikten sonra senkronizasyon prosedürüne alınmasının gebelik oranları üzerine olumlu etkisi olacağı düşünülmektedir. Elde edilen veriler ışığında yapılacak yeni çalışmalarda, çiftleşme öncesi, gebeliğin son dönemi, doğumdan sonraki ilk 3 hafta kan BHBA düzeylerinin incelenmesi, elde edilen gebeliklerdeki doğum sayıları gibi diğer döl verimi parametrelerinin de incelenmesinin sonuçların analizinde önemli katkı sağlayacağı kanısına varılmıştır.

AÇIKLAMALAR

Sunulan bu çalışma Tübitak 2209-A (Proje no: 1919B012003009) tarafından desteklenmiştir.

Etik beyan: MAKÜ-HADYEK başkanlığından, 04.12.2020 tarih ve 702 nolu etik kurul izni ile çalışma yürütülmüştür.

Çıkar çatışması: Yazarlar çıkar çatışması olmadığını beyan ederler.

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İskenderun Körfezi'nden avlanan derin su pembe karidesi (Parapenaeus longirostris)'nin atıklarından elde edilen astaksantinin ekstraksiyonu ve karakterizasyonu

Extraction and characterization of astaxantin from the waste of deep water pink shrimp (Parapenaeus longirostris) obtained from İskenderun Bay

ÖZET

Bu çalışmanın amacı, düşük değerli bir hammadde olan karides atıklarından yüksek değerli bir pigment olan astaksantinin izolasyonu için basit ve etkili bir yöntem önermektir. Bu çalışmada, ekstraksiyon çözücüsü olarak aseton kullanılmıştır. Thin Layer Chromatography (TLC) için mobil faz olarak 3/7 (v/v) oranında aseton/ hekzan kullanılmıştır. Çalışmada kullanılan örnekler Parapenaeus longirostris türüne ait olup İskenderun Körfezi (Türkiye)'nde faaliyette bulunan yerel balıkçılardan elde edilmiştir. Karides atıkları, buzla dolu steril bir kapta laboratuvara taşınmıştır. Karideslerin kullanılan atıkları sefalotoraks, abdomen ve abdominal uzantı (telson ve üropodlar) kısmıdır. Sefalotoraks'a yapışan etler temizlenmiş, atıklar su ile yıkanmış ve örnekler 50 °C' de etüvde kurutulmuştur. Polietilen torbalarla paketlenmiş ve kullanılıncaya kadar -18 °C'de saklanmıştır. Bu araştırmada astaksantin, organik çözücüler (petrol eteri ve aseton) kullanılarak karides kabuk atıklarından ekstrakte edilmiştir. Astaksantin pigmentinin karakterizasyonu, üç bant olarak serbest Astaksantin (Rf=0,43), Astaksantin monoester (Rf=0,56) ve Astaksantin diester (Rf=0,81) olarak tespit edilen Lorenz Todd standart kromatogramında belirtildiği Retardasyon faktörünü (Rf) karşılaştıran TLC ile gerçekleştirilmiştir.

Anahtar Kelimeler: Astaksantin, Parapenaeus longirostris, Aseton, TLC.

ABSTRACT

The aim of this study is to purpose a simple and effective method for the isolation of high-value pigment astaxanthin from shrimp waste, a low-value raw material. In this study, acetone was used as an extraction solvent. Mobile phase for Thin Layer Chromatography (TLC) was acetone:hexane in the ratio 3:7 (v/v). The samples used in the study belong to the Parapenaeus longirostris species and were obtained from local fishermen operating in the Iskenderun Bay (Türkiye). Shrimp waste was transported to the laboratory in a sterile container filled with ice. The wastes used in shrimp are the cephalothorax, abdominal shell and tail portion. Adhering meat from the cephalothorax was removed and the waste was washed under water and samples were dried at 50 °C with drying oven. Packed in polyethylene bags and stored at -18 °C until use. In this study, astaxanthin was extracted from shrimp shell waste using organic solvents (petroleum ether and acetone). Characterization of astaxanthin pigment was performed with TLC buy comparing the Retardation Factor (Rf) as indicated in the Lorenz Todd standard chromatogram, in which three bands of astaxanthin (Rf=0.36), astaxanthin monoester (Rf=0.60) and astaxanthin diester (Rf=0.75) were detected.

Keywords: Astaxanthin, *Parapenaeus longirostris*, Aceton, TLC

How to cite this article
Dikel, Ç., Yanar, Y., Demirkol O. (2022). Extraction and characterization of astaxantin from the waste of deep water pink shrimp (Parapenaeus longirostris) obtained from İskenderun Bay. *Journal of Advances in VetBio Science and Techniques, 7*(2), 228-232.
https://doi.org/10.31797/vetbio.994594

Research Article

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Article info
Submission: 13-09-2021
Accepted: 30-07-2022
Online First: 20-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

GİRİŞ

Karides ve karides ürünleri tüm dünyada yaygın olarak tüketilmekte ve yüksek besin değeri nedeniyle talep her geçen yıl artmaktadır (Nirmal, vd., 2020). Küresel karides üretimi 2020'de 5,03 milyon tondur ve 2020'den 2025'e kadar %6,1'lik yıllık birleşik büyüme oranı ile (CAGR) 7,28 milyon tona çıkması beklenmektedir (IMARC, 2020). Ayrıca karides pazarı ciro değerinin 2025 yılı sonunda 67,6 milyar ABD dolarına ulaşacağı tahmin edilmektedir (Anonim, 2019.).

Genellikle karidesler, piyasa talebine göre kabuklu veya kabuksuz olarak donmuş halde depolanır ve ihraç edilir. Bu nedenle, karides işleme sırasında, karidesin yaklaşık %50-60'ı baş, iç organ ve kabuk şeklinde atık olarak kabul edilir (Senphan vd., 2012). Bu yan ürünün büyük miktarları boşa harcanmakta ve bu da değerli biyoaktif bileşenlerin kaybına ve çevre kirliliğinin artmasına neden olmaktadır. Biyoaktif moleküllerin atıklardan geri kazanılması, karides işleyicilerin ve ülkenin ekonomisi için faydalı olacaktır. Bu aynı zamanda karides atıklarının boşaltılmasından kaynaklanan çevre kirliliğinin azaltılmasına da yardımcı olacaktır (Nirman, vd., 2020).

Karides atıkları, protein/peptidler (Cahú vd., 2012), kitin/kitosan (Paul vd., 2015), pigmentler (Sila, Ghlissi, vd., 2015), enzimler (Senphan vd., 2014), lipidler (Senphan ve Benjakul, 2012), mineraller (Gomez-Estaca vd., 2019) ve vitaminler (Nair vd., 2017) gibi değerli biyoaktif bileşenleri içermektedir.

Günümüzde her yıl büyük miktarlarda karides kabuğu (neredeyse karides ağırlığının %60'ı kadarı) atık olarak kaybedilmektedir. Bunlar göz önüne alındığında tüm dünyada modern eğilimler geri dönüşümü işaret etmektedir. Ancak karides atıklarının arıtılması kolay bir iş değildir ve gıda endüstrisinin problemlerinden biridir. Bu problemlerin çözümü ile elde edilen karotenoidler çok değerli bileşiklerdir. En önemli, ucuz, doğal karotenoid kaynakları olan astaksantin ve esterleri endüstriyel karotenoidlere iyi bir alternatiftir (Islam vd., 2004; Sindhu ve Sherief, 2011).

Astaksantin sekiz izopren, C5H8 molekülünden oluşan tetraterpenoidler; başlangıç öncüsü olarak izopentenil difosfat (veya IPP) ve dimetilalil difosfat (veya DMAPP) olarak bilinen fitokimyasallar sınıfına ait bir karotenoiddir.

Astaksantin denizel organizmalarda ve mikroorganizmalarda çok yaygın bulunan, çok güçlü bir antioksidan ve anti-lipit peroksit olan kırmızı renkli Ksantofiller grubundaki karotenoidlerdir. Ksantofiller arasında astaksantin, iyonon halkalarının her iki ucundaki oksijen içeren fonksiyonel gruplarıyla tanımlanır. Astaksantin, stereo izomerlerde bulunur ve yapısında bu pigmentin renginden sorumlu olan konjuge çift bağa sahip olduğu geometrik izomerlerin sayısıdır (Chesson. vd., 1997). Birçok karotenoid gibi, astaksantin de renkli, lipidde çözünen bir pigmenttir. Renk, bileşiğin ortasındaki genişletilmiş konjuge (alternatif çift ve tek) çift bağ zincirinden kaynaklanmaktadır. Bu konjuge çift bağlar zinciri aynı zamanda astaksantinin (ve diğer karotenoidlerin) antioksidan özelliklerinden de sorumludur (Margalith, 1999; Choi vd., 2005).

Astaksantin mikroalg, maya, somon, alabalık, kril, karides, kerevit, kabuklular ve bazı kuşların tüylerinde bulunur. Somon etinin ve pişmiş kabuklu deniz hayvanlarının kırmızı rengi bu pigmentten kaynaklanmaktadır (Ambati, vd., 2014).

Astaksantin ve esterleri gıda endüstrisine, yem endüstrisine, kozmetik ve renklendirici maddeye girer ve doğal metabolik reaksiyonlar, fizyolojik stres, hava kirliliği, tütün, duman, kimyasallara maruz kalma veya UV ve ışığa

maruz kalma sırasında vücutta üretilen insan sağlığı sorunlarının çoğunun tedavisinde rol oynar.

Karides kabuğu atıklarının etkin kullanımı, yan etkisi olmayan doğal ilaçların geliştirilmesi için biyomedikal bir araştırma materyali olma potansiyeline sahiptir.

Farklı kabukluların kabuk atıklarından elde edilecek karotenoidlerin ekstraksiyonu için çözücü olarak birkaç organik çözücü rapor edilmiştir. Karotenoidlerin ekstraksiyonu için aseton, etanol, hekzan gibi organik çözücüler kullanılabilir. Bu çalışma, karides atıklarının tam olarak değerlendirilmesini ve katma değer elde edilmesini amaçlamaktadır. Bilim adamları ve mühendisler için katma değerli ürünler için karides atıklarını kullanmanın yeni yolunu açması hedeflenmektedir. Ayrıca bu bilgiler biyoteknolog ve endüstriyel personelin sürdürülebilir çevresel kalkınma için karides işleme atıklarından yararlanmaları için faydalı olacaktır. Yeni ekstraksiyon yöntemleri yalnızca yeni bir astaksantin kaynağına erişme olasılığını değil aynı zamanda her yıl atılan binlerce ton karides atığının çevre dostu bir kullanımını bulma olasılığını da artırmaktad

MATERYAL ve METHOD

Çalışmada kullanılan örnekler Parapenaeus longirostris türüne ait olup, İskenderun Körfezi/ Kuzeydoğu Akdeniz/ Türkiye' de faaliyet gösteren yerel balıkçılardan alınmıştır. Karides atıkları, buzla dolu steril bir kapta laboratuvara taşınmıştır. Karideste kullanılan atıklar sefalotoraks, abdomen ve abdominal uzantı (telson ve üropodlar) kısmıdır. Sefalotoraksa yapışan etler temizlenerek kabuklar akan temiz su altında yıkanmıştır. Örnekler kurutma fırını ile 50°C'de kurutulmuştur. Polietilen torbalarda paketlenmiş ve kullanılıncaya kadar -18°C'de saklanmıştır.

Astaksantin Pigmentinin Ekstraksiyonu

Karides kabuğu atığı (1gr), 10 ml aseton kullanılarak öğütülmüştür. Ekstrakt filtre kağıdı (Whatmann) kullanılarak süzülmüştür. Örnek tekrar tekrar ekstrakte edilmiş ve renksiz bir filtrat elde edilene kadar taze çözücüyle çözülmüştür. Biriken ekstrakt ayrı konik bir şişede toplanmış ve üzerine 12,5 ml petrol eteri (BP 40-60°C) ve 9,4 ml NaCl ilave edilmiştir. İyice karıştırıldıktan sonra epifaz toplanmıştır. Alt faza eşit miktarda su ilave edilmiş, iyice karıştırılmış ve daha sonra tekrar epifaz toplanmıştır. Biriktirilen epifaz, petrol eterinin buharlaştırılması için 60°C'de su banyosuna tutulmuştur (Ushakumari, vd., 2012).

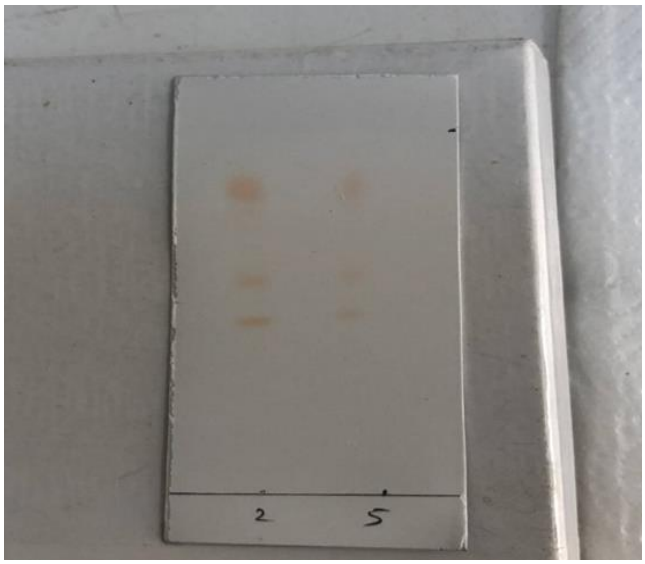
Şekil 1. Astaksantin pigmenti ekstraktı

İnce Tabaka Kromatografisi (TLC) ile Karides Kabuğu Atık Ekstraktında Astaksantin Tanımlanması

Karides kabuğu ekstraktındaki farklı bileşenlerin analizi, Kobayashi ve Sakomoto, 1999 yöntemine dayalı olarak TLC kullanılarak yapılmıştır. Küçük bir miktar ekstrakt silika kaplı alüminyum levhalar üzerine lekelenmiş ve 3:7 oranında hazırlanmış aseton:hekzan kullanılarak yürütülmüştür. Ayrılan bantlar, astaksantin monoester ve astaksantin diester için uluslararası kabul görmüş Rf değerleri kullanılarak belirlenmiştir. RF değeri (Geciktirme Faktörü) TLC'deki herhangi bir çözünen noktanın konumu, Retardasyon faktör olarak karakterize edilir.

Örnekler kurutulmuş, ardından görünen noktaların her biri tarafından kat edilen mesafe ve çözücü tarafından kat edilen mesafe

kullanılarak Rf değeri hesaplanmıştır. Rf= (Ekstraktın kat ettiği mesafe) / (Çözücünün kat ettiği mesafe)



Şekil 1. Parapenaeus longirostris eksraktındaki farklı karotenoidlerin ayrılması ve Rf değerleri

BULGULAR

Tespit edilen astaksantin detayları Tablo 1'de tartışılmıştır. Astaksantin diesterin Rf değeri 0,81 ve monoester için 0,56, astaksantin için 0,43 olarak kaydedilmiştir.

Tablo 1. Karotenoidin Rf değerleri

Karotenoid	*Rf değeri
Astaksantin diester	0,81
Astaksantin monoester	0,56
Astaksantin	0,43

*Rf: Retardasyon Faktörü

TARTIŞMA

Son yıllarda yapılan çalışmalar, doğal astaksantinin güçlü bir nutrasötik ve terapötik potansiyeli olduğunu göstermiştir. Güçlü antioksidan ve diğer biyoaktif özellikleri (anti-inflamatuar, sitotoksik, antiproliferatif ve antikanser aktivitesi) ve ayrıca uygun güvenlik profili, astaksantini farklı sağlık sorunlarını önleme hatta tedavi etme kabiliyetine sahip umut verici bir bileşik haline getirir. Birçok çalışma, kabuklu yan ürünlerinin farklı kısımlarından astaksantin mevcudiyetinin kanıtlamaktadır. Ana zorluk, üretim sürecinin bilinmeyen maliyetinin yanı sıra yan ürünlerden elde edilen miktarlar hakkında bilgi eksikliğidir. Ayrıca endüstriyel düzeyde çıkarma metodolojilerinin başarılı bir şekilde geliştirilmesi için ele alınması gereken zorluklar vardır. Ekstraksiyon işlemleri sırasında, işlemin fizibilitesinin yanı sıra astaksantinin ekstraktının kalitesi de ele alınmalıdır. Yağda çözünen bir madde olduğu için herhangi bir yağ veya aseton, alkol vb. organik çözücüler kullanılarak ekstrakte edilebilir. Karotenoidin karides atıklarından izolasyonu için organik çözücü kullanımı yüksek verim sağlamaktadır.

Astaksantin monoester ve astaksantin diester için elde edilen Rf değerleri, astaksantin monoester için 0,60 ve astaksantin diester için 0,75 – 0,85 olarak belirtilen Rf değerlerine sahip Kobayashi ve Sakamoto (1999) tarafından bildirilen sonuçlarla uyumlu olarak bulunmuştur. Benzer şekilde Dalei ve Sahoo (2022) tarafından aseton ekstraktının Rf değerleri astaksantin, astaksantin monoester ve astaksantin diester için sırasıyla 0,36; 0,60 ve 0,75 olarak bulunmuştur.

SONUÇ

Su ürünleri endüstrilerinden gelen kabuklu kabuk atıkları, astaksantin gibi doğal karotenoid gibi önemli biyoaktif bileşiklerin izolasyonu için kullanılabilir. Yapılan bu çalışmada aseton, organik çözücüler arasında astaksantin için iyi bir ekstraksiyon ortamı olmuştur. TLC ayrımı, kabuklu atıklardan elde edilen karotenoid ekstraktının astaksantin, astaksantin mono ve diester içerdiğini doğrulamıştır.

AÇIKLAMALAR

Bu çalışma 23-27 Ağustos 2021 tarihinde Saraybosna/Bosna Hersek'te düzenlenen 5. ICABB (5th International Congress on Advances in

Bioscience and Biotechnology) Kongresi'nde sözlü bildiri olarak sunulmuştur.

Etik beyan: Bu çalışmada canlı materyal üzerinde çalışılmamıştır.

Çıkar çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

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Gastrointestinal linear foreign bodies in cats: A retrospective study of 12 cases

ABSTRACT

Gastrointestinal foreign bodies are common in animals and may present with various clinical manifestations depending on the location, severity, and duration of the obstruction. Linear foreign body (LFB) obstructions are more common in cats compared to dogs, and the foreign body causing the obstruction is usually thread or threaded needle. In this study, it was aimed to determine the radiography and the localization of the obstruction in the diagnosis of LFB in cats, to investigate the operative treatment options and their effects on the prognosis. The study material consisted of 12 cats of different breeds, ages, and genders, which were referred with the suspicion of foreign body with acute/chronic vomiting and anorexia. After the identification of obstruction due to foreign body, the treatment was planned considering its localization, severity and duration of pathology. All foreign bodies were removed through surgical procedures. The most common finding was the plication in the intestines. Four cats died in the postoperative period. As a result, an early and rapid diagnosis of LFB positively affects the prognosis. Intestinal perforations caused by LFB and infection due to bacterial translocation are inevitable in delayed cases. Another issue to be considered is that extremely important to carry out a detailed examination of the mouth in cats, which show symptoms of gastrointestinal system origin illness. In addition, it is thought that to pull out the LFBs that protrude from the anus increase the perforation risk of intestine.

Keywords: Cat, foreign body, gastrointestinal, linear, vomit

INTRODUCTION

Gastrointestinal (GI) foreign bodies are common in pets and may present with various clinical manifestations depending on the severity, location, and duration of the obstruction (Aronson et al., 2000; Papazoglou et al., 2003). In general, complete obstruction is associated with more dramatic clinical signs and a rapid worsening, whereas partial obstruction may be associated with more chronic signs of inadequate digestion and malabsorption (Papazoglou et al., 2003). Intestinal foreign bodies are easy to treat and have a good prognosis if diagnosed early. However, special situations may occur for surgeons due to linear foreign bodies (LFB) (thread, fabric, tape, cord, etc.). These foreign bodies can easily pass through the GI tract. However, these objects are usually stuck while passing through around the base of the tongue and pylorus (Evans et al., 1994). As peristalsis continues, foreign bodies become tense and embedded in the mesenterium of the GI lumen. Thus, the intestines get compressed due to LFB and causes plication. As a result, perforation may occur in the intestines (Aronson et al., 2000; Hayes, 2009).

How to cite this article
Parlak, K., Akyol, ET., Uzunlu, EO., Zamirbekova, Boran Çayırlı, ÜF., Arıcan M. (2022). Gastrointestinal linear foreign bodies in cats: A retrospective study of 12 cases. *Journal of Advances in VetBio Science and Techniques, 7*(2), 233-241.
https://doi.org/10.31797/vetbio.1131263

Research Article

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Article info
Submission: 15-06-2022
Accepted: 18-08-2022
Online First: 20-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

It has been reported that LFB obstructions are more common in cats than in dogs, and the object causing the obstruction is mostly thread or thread-needle (Bebchuk, 2002; Evans et al., 1994; Felts et al., 1984; Hayes, 2009). The most common clinical manifestations are vomiting, anorexia, and depression for both species (Bebchuk, 2002; Evans et al., 1994).

In this study, it was aimed to investigate the role of radiography in the diagnosis of LFB in cats, determination of obstruction localization, surgical treatment options and their effects on prognosis.

MATERIAL and METHOD

Animal

The study material consisted of 12 cats of different breeds, ages and genders, which were referred to Selcuk University Faculty of Veterinary Medicine Surgery Clinic with acute/chronic vomiting and anorexia, with suspicion of foreign body. Clinical signs and the anamnesis of the cats were recorded on the general examination forms at admission, and owners were asked if they witnessed swallow of the foreign body and the time that had elapsed occur clinical signs and admission.

Clinical Examination

The physical examination started with direct inspection of the oral cavity in all animals and continued with palpation of the esophagus and abdomen. The obtained data were recorded. Vascular catheterization on the vena cephalica antebrachi and hematological examinations (blood gas analysis with GEM Premier 3000, USA and hemogram with MS4e, France) were performed. In order to diagnose the foreign body, radiological examination of the GI tract (right or left lateral and ventro-dorsal positions) were performed. Contrast-enhanced radiography was performed with Barium Sulfate 60% solution (2 mL/kg, PO) in cases without intestinal perforation which do not show signs of peritonitis in physical, radiological and hematological examination.

Surgical Treatment

After the identification of the foreign body that caused the obstruction, treatment was planned considering its localization, severity and duration of pathology. For the surgical procedure, medetomidine HCl (Domitor®-Zoetis, 0.025 mg/kg, IM) and butorphanol (Butomidor-Interhas 0.1 mg/kg, IM) were administered as preanesthetic. Subsequently, anesthesia induction was achieved by administering propofol (Propofol-Lipuro 1%® 1.5-3 mg/kg, IV). Then the cats were intubated and anesthesia was maintained with isoflurane (Isoflurane 2%-Adeka Pharmaceuticals, for maintenance anesthesia) in 100% oxygen with a flow of 2 L/min.

In all cases, abdominal exploration was performed starting from the stomach to the rectum and intestinal segments were carefully examined. After the location of the foreign body was determined enterotomy was used to remove it. In a case with invagination, resection/anastomosis was performed in addition to enterotomy. In addition to enterotomy, gastrotomy was performed in two cats in which the foreign body caused obstruction in the pylorus. In the presence of a foreign body connected with the oral cavity, before removal by enterotomy, the foreign body was released from the oral cavity by an assistant. After all foreign bodies were removed, the incision line on the intestine was routinely closed with a single layer of continuous suture (parallel suture in the antimesenteric region or transverse suture in the stenotic segment). Polydioxanone (PDO 3/0 and 2/0) nontraumatic sutures were used as suture material. The leak test was performed by injecting physiological saline with the syringe from the cranial side of the suture line. After the closure of the incision lines, the omentum was sutured to the cranial and caudal of the incision

lines with simple separate sutures. Abdominal cavity was lavaged at least twice with warm saline and closed routinely.

Metoclopramide HCl (0.2 mg/kg, IM) to counteract vomiting and, fluid therapy (lactated Ringer's solution, 100 mL, ql2h, IV) were given for correcting dehydration and improving tissue perfusion in all cats. For postoperative antibiotic therapy, metronidazole (Polygyl 0.5%, Polifarma 7.5 mg/kg, q24h, IV) for 3 days and cefazolin sodium (Iespor®, Ulagay Pharmaceutical, 30 mg/kg, q24h, IM) for 7 days were administered. As analgesic, meloxicam (Metacam®, Bohringer Ingelheim) was administered (0.1 mg/kg, q24h, PO) on the first day and continued (0.05mg/kg, q24h, PO) for the next 4 days. Soft diet intake was allowed after temporary diet restriction for 8-12 hours postoperatively.

RESULTS

Clinical findings

The animals included in the study were determined as young animals with a mean age of 15 months (6 months-5 years), and gender distribution was 5 males and 7 females. The breed distribution was observed as 7 mixed breeds, 1 Blue Point Siamese, 2 British Shorthairs, 1 Scottish Fold and 1 Siamese (Table 1).

None of the patient owners reported witnessing foreign body ingestion. Only one cat (case 10) had a history of suspected foreign body ingestion, and another two cats (case 6 and 12) had a history of partially protruding foreign body (thread) from the anus at certain intervals. According to the anamnesis, it was noted that food intake decreased after vomiting in early period, and completely stopped with recurrent vomiting.

Table 1. Signalements and diagnosed foreign bodies of the cases

Case	Signalements				Diagnosis	Treatment
	Breed	Age	Sex	History		
1 (EX)	Mix	1 year	Male	Vomiting for 2 days, no defecation	LFB (thread)	Enterotomy and Gastrotomy
2	Siamese	6 months	Female	Loss of appetite and vomiting for 7 days	LFB (thread)	Enterotomy
3	Mix	9 months	Female	Loss of appetite and vomiting lasting more than 1 week	LFB (thread)	Enterotomy
4	Mix	1 year	Female	Loss of appetite and vomiting lasting more than 1 week	LFB (threaded needle), perforation	Enterotomy
5	British shorthair	1 year	Male	Loss of appetite and vomiting lasting more than 1 week	LFB (thread)	Enterotomy
6 (EX)	Mix	5 years	Male	Vomiting and loss of appetite for 4 weeks, protruding LFB through the anus	LFB (thread), seen on the base of the tongue	Enterotomy
7	Scottish fold	2 years	Female	Up to 30 times vomiting per day for 5 days, no defecation	LFB (thread), seen on the base of the tongue	Enteretomy
8	Blue point	7 months	Male	Vomiting and loss of appetite for 3 days, no defecation	LFB (thread), invagination	Enteretomy and resection/ anastomosis
9	Mix	1 year	Female	Loss of appetite and vomiting for 3 weeks	LFB (thread), perforation	Enterotomy
10	Mix	2 years	Female	Loss of appetite and vomiting for 5 days	LFB (thread), perforation	Enterotomy
11 (EX)	British Shorthair	11 months	Male	Loss of appetite and vomiting for 5 days	LFB (thread), perforation	Enterotomy
12 (EX)	Mix	9 months	Female	Loss of appetite and vomiting for 1 week, protruding LFB through the anus for 2 weeks	LFB (thread), perforation	Enterotomy

LFB: Linear Foreign Bodies

The primary findings in the clinical examinations of all cats were varying degrees of vomiting, loss of appetite, tangles and matting of the coat. Dehydration and lying in the sternal position were observed in all cats. The absence of defecation, which suggested a complete obstruction, was noted in three cats (cases 1, 7, 8). Besides these clinical symptoms, abdominal pain was observed in 50% (cases 4, 6, 8, 9, 10, 12) and retching was observed in 17% (cases 6, 7). Severe abdominal pain was observed especially in cases with the presence of intestinal perforation (cases 4, 9, 10, 11 and 12) (Figure 1). In addition, the case with the needle as foreign body were located in the intestine also showed the abdominal pain (case 4). In the clinical examination, foreign body (thread) was encountered under the tongue in two cats (cases 6 and 7) (Figure 2). During the intraoral inspection it was observed that the foreign body extending from the mouth to the intestines. In response to the oral examination, decreased neck movements, and ptyalism were noted in both cats. Lethargy, anorexia, retching, and severe vomiting were mainly observed in cases of the foreign body located in the mouth. However, vomiting and anorexia were often observed in cases of the foreign body which located in the lower GI tract. The owners reported that medical treatment was performed, and no response obtained in these cats. For this reason, only 3 of the cases (cases 1, 8 and 10) were diagnosed in the early period.

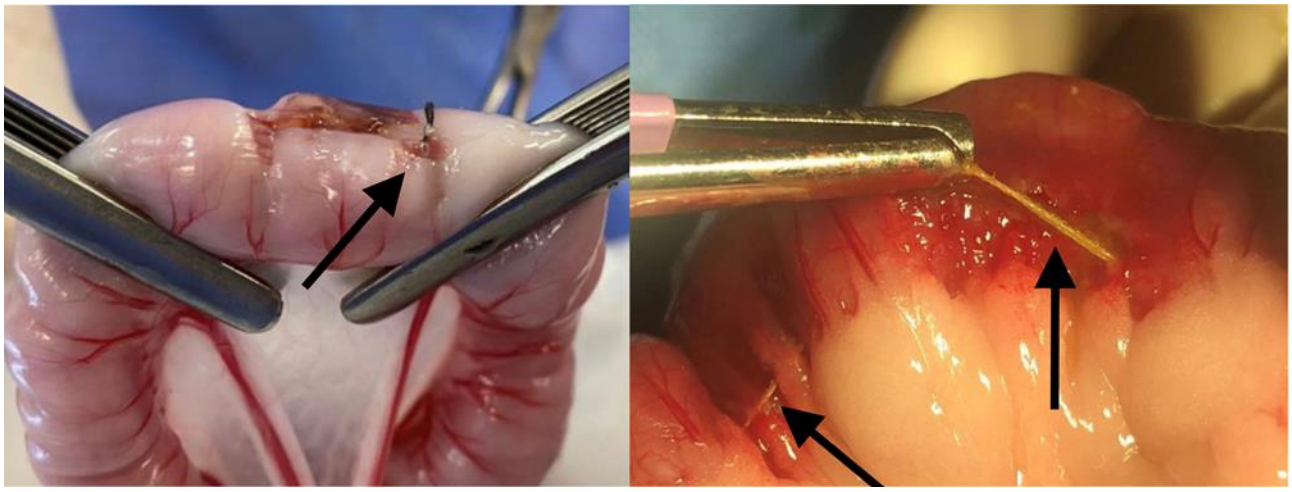

Figure 1. Foreign body (thread) causing perforation (black arrow).

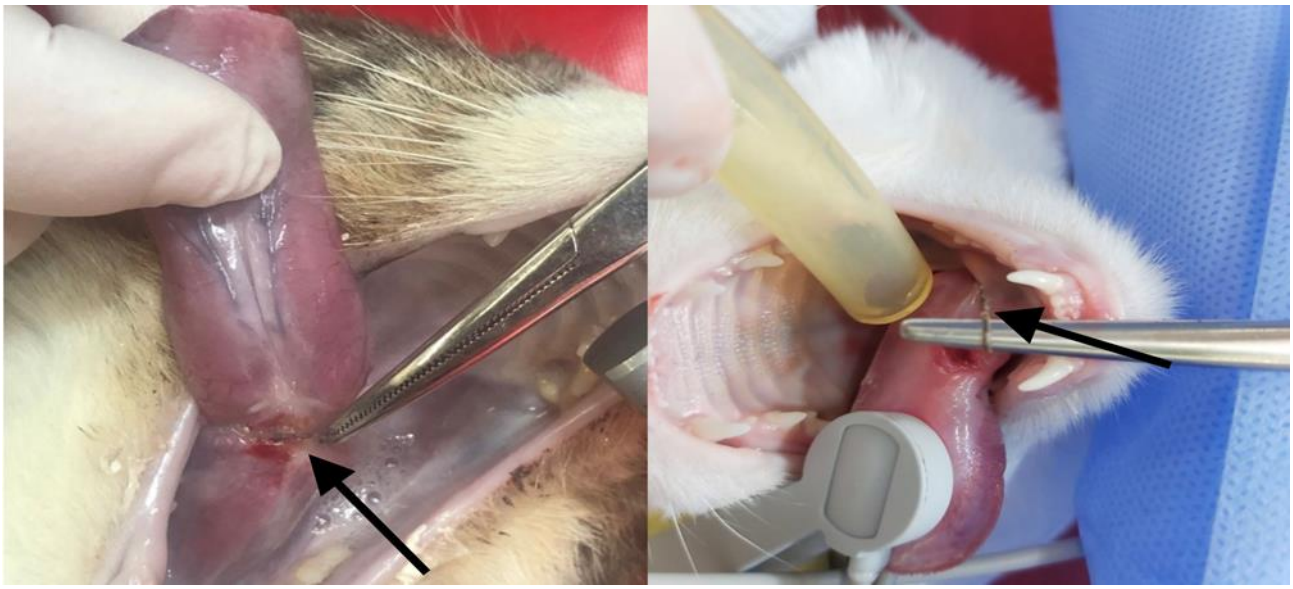

Figure 2. Looped LFB (thread) around the base of the tongue.

Hematological Findings

Some hematological parameters were evaluated (Table 2). Hypokalemia (42%, n=5), hypernatremia (42%, n=5), hyperchloremia (50%, n=6), hypochloremia (20%, n=2) and hyperlactatemia (50%, n=6) were identified. However, it was observed that 60% (n=3) of cats with hypernatremia progressed with hyperchloremia.

Table 2: Hematological and biochemical values

Case	pH (mmol/L) (7.35-7.40)	pO2 (mmHg) (35-100)	K+ (mmol/L) (3.5-5.8)	Na+ (mmol/L) (135-152)	Cl- (mmol/L) (106-115)	cLac (mmol/L) (0.6-2.2)	WBC (x10^3/µL) (5.5-19.5)	HCT (%) (30-57)
1	7.39	40.2	3.6	159	122	1.4	18.4	56.2
2	7.49	40.7	2.9	162	107	2.5	20.1	61.3
3	7.36	43.0	3.4	145	113	7.2	37.4	19.2
4	7.31	38.2	2.8	140	103	5.6	10.5	59.2
5	7.54	28.0	2.1	162	89	2.5	35.9	67.5
6	7.37	55.4	4.4	149	120	3.7	8.9	54
7	7.48	42.5	4.2	151	124	1.5	12.4	61.6
8	7.40	30.1	4.1	159	122	1	29	40.8
9	7.38	38.2	3.9	151	121	1.5	7.2	57.4
10	7.37	31.0	3.6	170	126	1.9	18.5	52
11	7.51	56.2	3.1	148	106	2.4	17.3	57.7
12	7.33	33.7	4.8	137	109	1.5	10.4	36

Radiological Findings

Direct radiographic examination was performed for all cases. The barium sulfate was used in cases requiring contrast-enhanced radiography (cases 6, 8). However, severe vomiting, the vomiting reflex that developed after ingestion of barium sulfate prevented contrast-enhanced radiography. As a result of radiographic examinations, foreign body (needle) was clearly observed in 1 case (case 4). In the other cases after detection of obstruction and plication findings (Figure 3) experimental laparotomy decision was taken and the foreign body was determined during the surgical procedure.

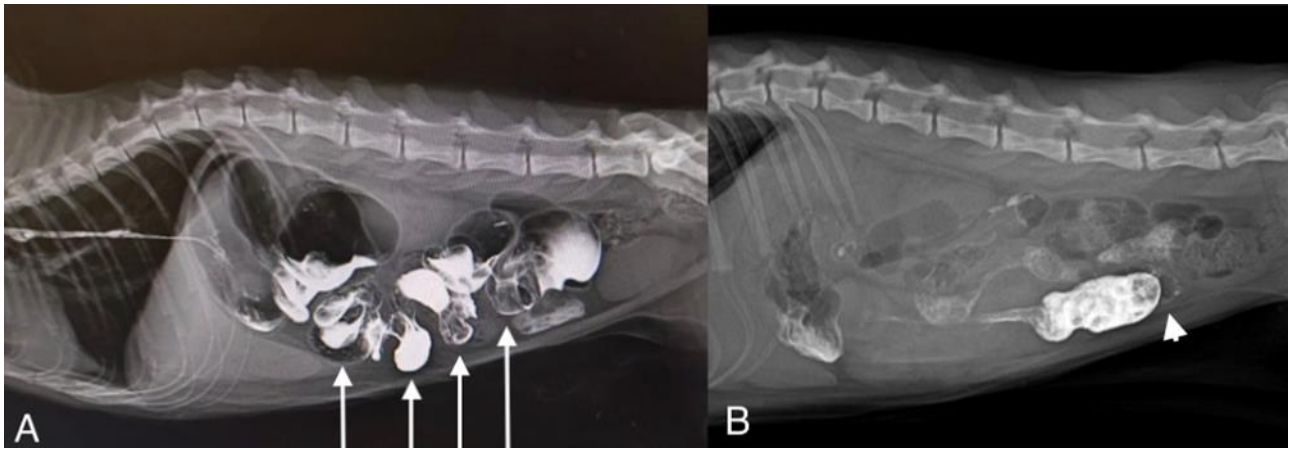

Figure 3. Plication (arrows) and obstruction (arrowhead) findings on contrast-enhanced radiographs.

Surgical Findings

In two cats (cases 6 and 7) the foreign body was detected to locate in upper GI tract, in other cases it was seen in lower GI tract region. In the present study encountered foreign bodies were thread but only one threaded needle found in one case (case 4).

All foreign bodies were removed using one or more of the surgical procedures such as gastrostomy, enterotomy and resection/ anastomosis following laparotomy. Intestinal plication was evident in ten cats (Figure 4). In the present study, intestinal perforation in five cats (cases 4, 9, 10, 11 and 12) and intestinal invagination at three points in one cat (case 8) were observed (Figure 5). Intestinal perforations were caused by ingested foreign bodies that are sharp and elongated, such as needle and thread. In cases with perforation due to thread, there was excessive plication in three cats. However, plication was not observed in two cats with intestinal invagination (case 8) and perforation due to thread as foreign body (case 9). Four cats died in the postoperative period (cases 1, 6, 11 and 12).

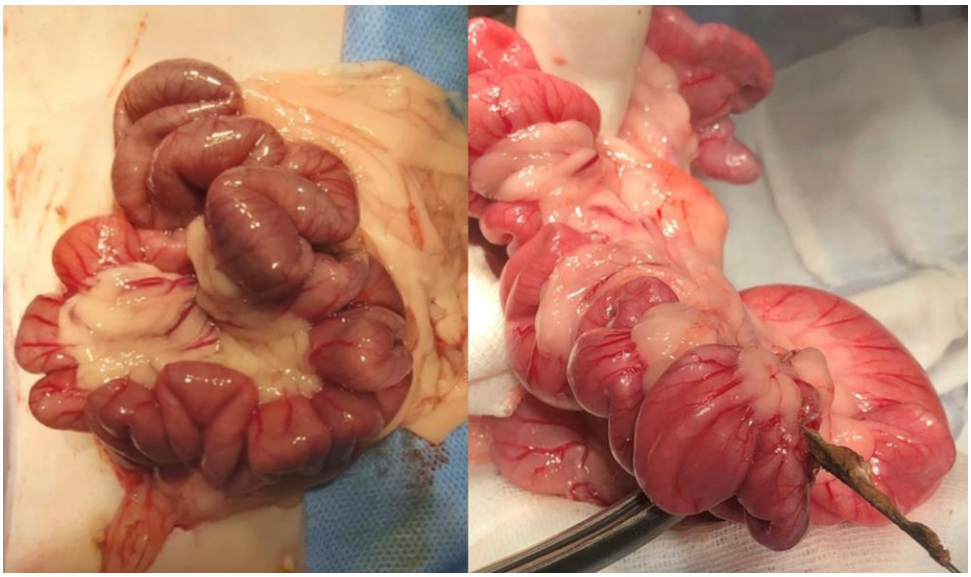

Figure 4. Typical plication of intestines.

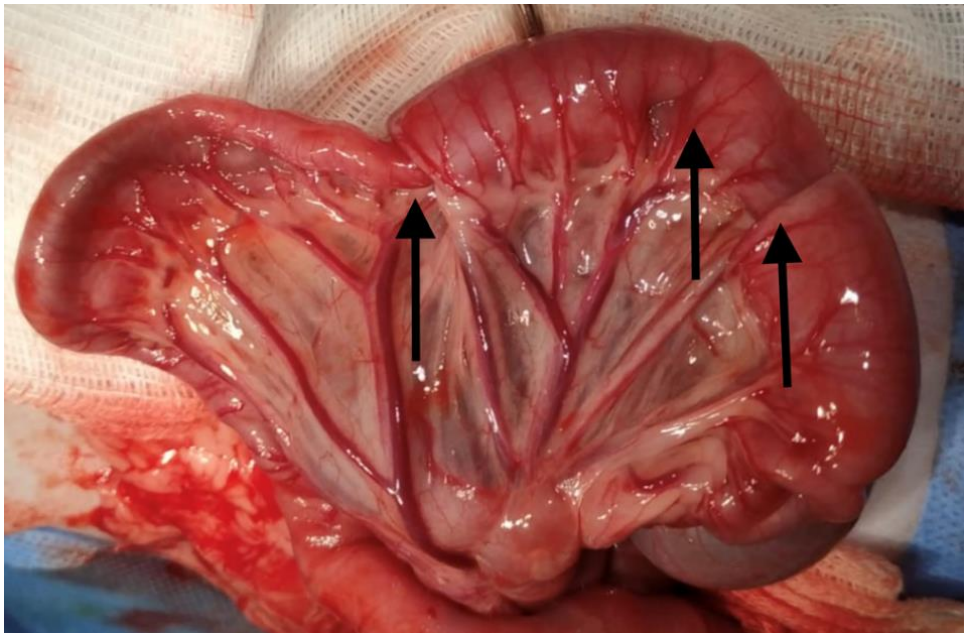

Figure 5. Invagination on the different three sites (black arrow).

DISCUSSION

Direct radiographs are used in the diagnosis of foreign bodies. However, it was not always sufficient for a definitive diagnosis (Elser et al., 2020). Although direct radiographs are very helpful in the diagnosis of needles and similar radiopaque materials, they are quite insufficient in the diagnosis of radiolucent foreign bodies such as threads. The fluid or gas accumulation can be observed in the intestines or stomach, these findings are not pathognomic for the foreign bodies, and contrast-enhanced radiography or USG examination are needed in accordance with the literature (Codrenau et al., 2019; Madany et al., 2020).

Hypochloremia, metabolic alkalosis, hypokalemia and hyponatremia have been reported in dogs with various GI foreign body cases, and LFB cases have generally been associated with serum sodium changes. While these changes were generally accompanied by hyponatremia, they were accompanied by hypernatremia in other foreign body cases. However, the observed biochemical changes were not associated with foreign body localization (Boag et al., 2005). In our study, hypernatremia was observed in five cats and no hyponatremia was encountered. This situation showed inconsistency with the literature data. Literature data have been reported in dogs. In this study, which evaluated cats, it was observed that species differences were an important factor in biochemical data. However, foreign body localization and duration of pathology differed, and no variation was observed among the cats. In accordance with the literature data, foreign body localization did not cause specific biochemical changes.

The survival rate in LFB cases in cats has been reported to be 84-92% (Basher & Fowler, 1987; Felts et al., 1984). These rates were observed in the cats with the duration of clinical findings between 1-10 days. However, the mortality rate was reported to be quite high in cases with symptoms over 14 days (Hayes, 2009). Clinical findings lasting up to 30 days have been observed in some cases. In our study, the clinical findings were similar to the periods in the literature and were observed predominantly for 7 days (2-30 days). The survival rate in our study was 66% (n=8) and it is consistent with the literature data. Although mortality rates were generally associated with more than one enterotomy site in these studies, it was thought that the effect of chronic partial obstructions caused by LFB may also contribute to the mortality rate. In addition, perforation in the lower GI tract and subsequent peritonitis have also been associated with deaths (Aronson et al., 2000; Basher & Fowler, 1987). The association of chronic partial obstructions with mortality is consistent with the presence of a 14- and 30-days foreign body history in two of the cats that died in our study. This may cause an increase in mortality in chronic cases (Aronson et al., 2000). It was thought that the mortality rate could be observed in acute cases as well as in chronic cases, depending on the severity of the obstruction.

It has been reported that partial obstruction usually occurs in LFB cases (Aronson et al., 2000). Similar to this, partial obstruction was encountered in most cases, while complete obstruction was encountered in only one case in our study. This case died after surgical treatment. This showed that complete obstructions can also be encountered in cases of LFB, suggesting that this may be more fatal than in cases of non-LFBs.

The owners' observation in foreign body located in the oropharyngeal region is very important. In our study, the findings of ptyalism, tenderness in the cervical region and loss of appetite before the clinical examination suggested the presence of a foreign body in the mouth. In this situation, a simple intraoral control is done by animal owners could accelerate the diagnosis and contribute to a better prognosis. In our study, the foreign body

(thread on the base of the tongue) detected in the oropharyngeal region at a rate of 17% (n=2) and it was similar to the literature data (Neamtu et al., 2021; Pratt et al., 2014). Anorexia, retching and vomiting were frequently observed clinical findings. Severity of these clinical findings were associated with the interest of the animal owner. In this study, a case was admitted to the clinic after 30 days later of ingested foreign body when the clinical symptoms became more severe. The increase of the mortality rate has also been associated with the patient's admission time to the clinic.

In a previous study, it was reported that the location, severity and duration of the obstruction were not associated with the survival rate (Hayes, 2009). Severity, localization and duration of obstruction due to the LFB were varied in this study. Nevertheless 75% of the cats were survived after the surgical treatments. The cats with chronic and complete obstruction were died. It has been observed that the localization of the foreign body has no effect on the mortality rate, which is consistent with the literature. However, contrary to the literature, it was thought that the severity and especially the duration of the obstruction may have an effect on the mortality rate.

CONCLUSION

As a result, early and rapid diagnosis of LFB positively affects the prognosis in the post-operative period. Secondary infection caused by LFB should be avoided in delayed cases. LFBs that protrude from the anus should not be made to pull out. This increases the risk of plication and perforation. Cat owners should be warned about this. Another issue to be considered is that it is extremely important to carry out a detailed oral examination if the conditions are suitable, in cats that have symptoms generally originating from the GI system, such as vomiting, loss of appetite, and irregular defecation.

ACKNOWLEDGMENT

This manuscript was presented in the Second International Congress on Biological and Health Sciences (ICBH) (Online, 24-27 February 2022), presentation type (oral), publication type (abstract), with the title of “Gastrointestinal Linear Foreign Bodies” by Kurtuluş Parlak.

Ethical approval: This study was approved by the Selcuk University, Faculty of Veterinary Medicine, Laboratory Animal Production and Research Center Ethics Committee (SUVDAMEK) with the decision date (06.01.2022) and number (2021/146). Patient owners of all cases signed an informed consent form.

Conflict of interest: The authors declared that there is no conflict of interest.

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Comparison of enrofloxacin and tulathromycin treatments in sheep with pneumonia caused by *Pasteurella multocida*

ABSTRACT

The objective of the present study was to compare the treatments of enrofloxacin and tulathromycin in sheep with pneumonia caused by *Pasteurella multocida*. A total of 45 female Tuj sheep between the of 2-6 years old were used in the study. Group 1 enrofloxacin administered 15 sheep, group 2 tulathromycin administered 15 sheep, and 15 healthy sheep of the same age group and characteristics enrolled in the study. Bronchoalveolar lavage fluid samples were obtained from sheep with clinical signs (cough, purulent, serous, mucopurulent nasal discharge) of respiratory system disease. After the microbiological examination of the taken samples, those were positive for Pasteuralla multocida included in the study. Blood samples (10 mL) from the *Vena jugularis* were collected in serum tubes with K_2EDTA and gel from the sick animals before and after the treatment as well as once from the control group. In our study, rectal temperature, respiratory rate and heart rate before treatment were found to be statistically significantly higher in patient groups compared to the control group ($P<0.001$). Total leukocyte count was found to be higher in the patient groups before treatment compared to the control group ($P=0.010$). Among the biochemical parameters, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea and creatine kinase levels were found to be statistically significantly higher in the patient groups compared to the control group before treatment ($P<0.05$). Clinical improvement was observed from the 3th day in group 2 and from the 5th day in group 1. In conclusion, administration of a single dose of tulathromycin resulted in earlier clinical improvement than administration of enrofloxacin for one week. At the same time, it was concluded that tulathromycin is more beneficial and practical in terms of a single application.

Keywords: Enrofloxacin, *Pasteurella multocida*, sheep, tulathromycin, tuj.

INTRODUCTION

The term pneumonia is the name given to lung inflammation caused by different etiological factors (Dağ et al., 2018; Eser et al., 2020). Age, breed, immune status and environmental factors have an effect on the emergence of some diseases in sheep (Gülmez et al., 2018). Respiratory system diseases are clinically acute, subacute and chronic. Viral, bacterial, mycotic and parasitic factors play a role in its etiology. Depending on where the inflammation is localized, it progresses as bronchopneumonia, lober and interstitial pneumonia (Çiftçi et al., 2015). In addition to the infectious causes in the etiology, climate changes, cold, stress, bad barn conditions, transport and malnutrition are the main factors that predispose to the disease (Issi et al., 2015). Pneumonia seen in sheep is common in our country as well as all over the world. Respiratory system infections have a share of 5.6% among the diseases seen in sheep. The type of pneumonia seen in sheep is a fibrinous and necrotic bronchopneumonia (Eser et al., 2020).

How to cite this article

Akyüz, E., Erkılıç, EE., Coşkun, MR., Merhan, O., Batı, YU., Sezer, M., Bahçıvan E. (2022). Comparison of enrofloxacin and tulathromycin treatments in sheep with pneumonia caused by *Pasteurella multocida*. *Journal of Advances in VetBio Science and Techniques*, *7*(2), 242-249. https://doi.org/10.31797/vetbio.1124136

Research Article

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Article info
Submission: 31-05-2022
Accepted: 10-08-2022
Online First: 20-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

Pasteurella, caused by *Pasteurella multocida* (*P. multocida*) and *Mannheimia haemolytica* (*M. haemolytica*), usually manifests as a respiratory infection in small farm-raised ruminants and causes significant economic losses worldwide. *P. multocida* is rarely mentioned in pneumonia outbreaks in small ruminants. Infectious *P. multocida* sero groups associated with pneumonic pasteurellosis outbreaks in sheep and goats are groups A and D, which have been shown to be both secondary and primary pneumonia agents (Odugbo et al., 2006).

Developed for veterinary medicine and approved for use in animals in the late 1980s, enrofloxacin in the first fluoroquinolone group is used in septic shock, gastrointestinal, urogenital, respiratory, dermal, mycoplasma and staphylococcal infections in ruminant, equidae, poultry, dogs, cats, and exotic animals (Martinez et al., 2006; Traş et al., 2018). Enrofloxacin can be used in diarrhea caused by *E. coli*, pneumonia caused by *Actinobacillus pleuropneumoniae*, mycoplasma and pasteuralla (Nakamura, 1995). Tulathromycin is approved for use in the treatment and prevention of respiratory tract disease in cattle associated with *M. haemolytica*, *P. multocida*, Histophilus somni and *Mycoplasma bovis* in the United States (Villarino et al., 2014). Apart from cattle and pigs, it can be used for antimicrobial purposes in horses, goats and sheep (Sirochman et al., 2012; Villarino et al., 2013). When administered in a single dose of 2.5 mg/kg (SC) in sheep, it may be safe in terms of heart, liver, kidney and hematological parameters in sheep (Çorum et al., 2015).

The objective of the present study was to compare the treatments of enrofloxacin and tulathromycin in sheep with pneumonia caused by *P. multocida.*

MATERIAL and METHOD

Animals

The study was carried out at Kafkas University, Faculty of Veterinary Medicine, Prof. Dr. Ali Rıza Aksoy Training, Research and Application Farm in Kars. A total of 45 female Tuj sheep between the of 2-6 years old were used in the study. Bronchoalveolar lavage fluid (BALf) samples were obtained from sheep with clinical signs (such as cough, purulent, serous, mucopurulent nasal discharge) of respiratory system disease. After the microbiological examination of the BALf taken samples, those were positive for *P. multocida* included in the study. Group 1 consists of 15 sheep with pasteurellosis that were administered enrofloxacin (Baytril 10%®, Bayer, Germany, 5 mg/kg intramuscularly for 1 week). Group 2 consists of 15 sheep with pasteurellosis that were administered tulathromycin (Draxxin®, Zoetis, USA, 2.5 mg/kg single dose intramuscularly). The control group consisted of 15 healthy sheep of the same age group and characteristics. Blood samples were taken from the sheep in the patient groups twice, before the treatment and 1 week after the treatment. In the control group, blood samples were taken once.

Taken and Processing of Blood Samples

Blood samples were taken from the *Vena jugularis* using a holder and compatible sterile needle tip (Vacuette®, Greiner Bio-One GmbH, Austria) before and after treatment from sick sheeps and once from healthy sheep. For pre- and post-treatment hematological analysis from sheep, 2 mL blood samples were collected into the EDTA tube (BD Vakutainer®, BD, UK). Whole blood analysis was measured using by an automated whole blood analyzer (VG-MS4e®, Melet Schloesing, France) within half an hour. Five mL blood samples taken into vacuum gel serum tubes (BD Vakutainer®, BD, UK) were kept at room temperature for approximately 1 hour, and then centrifuged at 3000 rpm for 10 minutes (Hettich Rotina

380R®, Hettich, Germany), then serum samples were extracted and measurements were performed daily. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, glucose, urea, total protein, albumin, magnesium, and creatin kinase (CK) were measured by using a fully automated biochemistry machine (Mindray BS120®, Mindray Medical Technology Istanbul, Turkey).

Taken of Bronchoalveolar Lavage Fluid Samples

Before taking the bronchoalveolar lavage, both nostrils of the sheep were cleaned with alcohol cotton to prevent nasal contamination. After the head and neck extension of the sheep was achieved, a disposable sterile nasogastric tube (4 mm x 1210 mm, Bicakcilar, Istanbul) was advanced transnasalally through the trachea until it encountered a slight resistance. Whether the carina region was reached or not was followed up with a recurrent cough reflex (Ok et al., 2019, İder and Maden, 2019). When the carina was reached, the nasogastric tube was withdrawn 1-2 cm, 15 mL of sterile saline (37 ºC, 0.9% Isotonic Sodium Chloride-FTS) was infused into the trachea and immediately aspirated. Approximately 3-5 mL of the given fluid was withdrawn. BALf samples were sent to Kafkas University Veterinary Faculty Microbiology Department laboratory for bacterial analysis.

Microbiological Procedure

The samples of bronchoalveolar lavage fluid samples were centrifuged at 3000 rpm for 3 minutes and the supernatant was removed, the sediment was homogenized with 100 µL FTS and cultivated in blood agar medium supplemented with 7% sheep blood. At the end of 24-48 hours of incubation at 37ºC under aerobic conditions, colonies with a diameter of 1-2 mm, colony morphology, gray, smooth or mucoid and without hemolysis were passed into blood agar with 7% blood for further biochemical tests and incubated under the same conditions. In biochemical tests, colonies that are catalase, oxidase positive, immobile, indole positive, urease negative, *Mac Conkey non-growing were defined as P*. multocida (Bergey, 1994).

Treatment Management

Sick sheep were kept under surveillance in separate boxes during the treatment. A standard supportive treatment protocol including A, B, C, D and E vitamins were performed to the all diseased sheeps. For vitamin supplementation, the sheeps were given parenterally vitamin B complex (Berovit B12®, Ceva, Australia) in a practical dose of 8-10 mL/sheep for 7 days, vitamin C (Maxivit-C®, Bavette, Turkey) at a dose of 4-6 mg/kg for 3 days, and vitamin ADE at a single subcutaneous dose of 1 mL/50kg (Ademin®, Ceva, Australia). In addition to the standard treatment, enrofloxacin was administered at a dose of 5 mg/kg once a day for 1 week intramuscularly in group 1. In Group 2, a single dose of 2.5 mg/kg of tulathromycin was administered intramuscularly. No drug was applied to the healthy control group sheep.

Statistical Analysis

The normal distribution of the data of the groups before and after the treatment and the control group was evaluated using visual methods (histogram graph and Q-Q graph) and Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used for the multiple comparison of the pre-treatment and post-treatment groups (group 1 and group 2) and the control group, and Tukey HSD test was used for post hoc comparison. The data obtained before and after the treatment were compared with the Paired Sample t-Test. The data obtained in the study were reported as mean ± standard error (SEM). All analyzes were performed with the SPSS® software program (SPSS® Statistics 26.0, Chicago, IL, USA). Differences obtained

in group comparisons were considered significant at P≤0.05.

RESULTS

In our study, physical examination findings such as rectal temperature, respiratory rate and heart rate before treatment were statistically significant compared to the control (P<0.001, Table 1). Total leukocyte count was found to be higher in the patient groups before treatment compared to the control group (P=0.010, Table 1). The physical examination findings and other hematological parameters are given in Table 1. ALT, AST, ALP, urea and CK concentrations were found to be statistically significantly higher in the patient groups compared to the control group before treatment (P<0.05, Table 2). Among the other biochemical parameters presented in the study, GGT, total protein, total bilirubin, albumin, magnesium and glucose levels are given in Table 2 (P>0.05).

Table 1. Physical examination findings and hematology of sick and healthy sheep

Parameters	Groups	N	Before treatment	After treatment	P1 value
			Mean±SEM		
Rectal temperature (°C)	Group 1	15	39.98± 0.14^{B}	38.70±0.08	<0.001
	Group 2	15	39.05±0.21^{A}	38.89±0.13	<0.001
	Control	15	38.48±0.15^{A}		-
	P2 value		<0.001	0.092	-
Heart beats/min	Group 1	15	108.07±4.28^{B}	74.27±5.28	<0.001
	Group 2	15	105.47±8.41^{B}	71.73±2.63	<0.001
	Control	15	72.53±2.47^{A}		-
	P2 value		<0.001	0.885	-
Breaths/min	Group 1	15	38.40±1.22^{B}	26.80±1.66	<0.001
	Group 2	15	31.93±3.85^{AB}	31.47±2.86	0.923
	Control	15	24.53±1.33^{A}		-
	P2 value		<0.001	0.064	-
Total leukocytes count (×10^{3}/µL)	Group 1	15	14.46±2.58^{AB}	9.66±0.81	0.038
	Group 2	15	18.79±2.68^{B}	9.40±0.88	0.004
	Control	15	8.99±0.58^{A}		-
	P2 value		0.010	0.826	-
Lymphocytes count (x10^{3}/µL)	Group 1	15	7.11±2.09	5.21±0.56	0.394
	Group 2	15	10.17±2.50	5.28±0.65	0.077
	Control	15	5.85±0.48		-
	P2 value		0.269	0.686	-
Monocytes count (x10^{3}/µL)	Group 1	15	0.52±0.04	0.59±0.04	0.272
	Group 2	15	0.65±0.06	0.61±0.07	0.693
	Control	15	0.55±0.05		-
	P2 value		0.267	0.766	-
Granulocytes count (x10^{3}/µL)	Group 1	15	6.81±1.01^{B}	3.85±0.57	0.018
	Group 2	15	7.97±1.11^{B}	3.57±0.33	0.002
	Control	15	2.58±0.15^{A}		-
	P2 value		<0.001	0.072	-
Red blood cell count (x10^{6}/µL)	Group 1	15	13.21±0.85	13.69±0.46	0.628
	Group 2	15	13.93±1.39	13.05±0.55	0.564
	Control	15	12.17±0.42		-
	P2 value		0.448	0.098	-
Mean red cell volume (fL)	Group 1	15	37.14±1.51^{A}	38.22±0.93	0.550
	Group 2	15	36.35±0.90^{A}	39.68±1.18	0.211
	Control	15	42.64±2.03^{B}		-
	P2 value		0.002	0.107	-
Hematocrit (%)	Group 1	15	47.42±2.30	51.86±1.41	0.114
	Group 2	15	46.49±4.10	51.13±1.44	0.296

	Control	15	46.01±2.05		-
	P2 value		0.943	0.064	-
Hemoglobin (g/dL)	Group 1	15	12.54±0.63	11.57±0.35	0.189
	Group 2	15	12.28±0.52	11.80±0.33	0.451
	Control	15	11.16±0.20		-
	P2 value		0.117	0.332	-
Platelet count (x10^3/µL)	Group 1	15	305.40±51.05	473.40±40.50^{b}	0.016
	Group 2	15	352.33	349.87±25.78^{ab}	0.962
	Control	15	354±13^{a}		-
	P2 value		0.656	0.015	-

SEM: Standard error of mean. N: Number of calves in the groups. P<0.05: Statistically significant. P1: Expresses the statistical significance level within the group. P2: Expresses the statistical significance level between groups. A,B: Different letters in the same column indicate statistical difference between groups before treatment. a,b: Different letters in the same column indicate statistical difference between groups after treatment.

Table 2. Serum biochemical parameters of sick and healthy sheep

Parameters	Groups	N	Before treatment	After treatment	P1 value
			Mean±SEM		
Alanine aminotransferase (IU/L)	Group 1	15	71.22±6.05^{B}	78.53±9.13^{b}	0.509
	Group 2	15	66.96±5.52^{B}	59.48±5.24^{ab}	0.334
	Control	15	40.54±5.33^{Aa}		-
	P2 value		<0.001	0.001	-
Aspartate aminotransferase (IU/L)	Group 1	15	188.87±10.89^{B}	181.44±15.05	0.819
	Group 2	15	194.40±12.26^{B}	161.12±8.46	0.034
	Control	15	121.09±7.74^{A}		-
	P2 value		0.002	0.077	-
Gamma glutamyl transferase (IU/L)	Group 1	15	44.96±1.91	37.28±3.24	0.061
	Group 2	15	43.58±2.38	44.08±2.13	0.877
	Control	15	42.79±2.24		-
	P2 value		0.779	0.155	-
Alkaline phosphatase (IU/L)	Group 1	15	122.52±4.78^{B}	103.43±5.82^{ab}	0.081
	Group 2	15	133.77±11.99^{B}	128.16±7.04^{b}	0.877
	Control	15	75.06±6.34^{Aa}		-
	P2 value		<0.001	0.022	-
Urea (mg/dL)	Group 1	15	80.60±1.85^{B}	64.16±3.12	0.020
	Group 2	15	71.73±2.70^{B}	57.30±3.23	0.023
	Control	15	52.34±2.98^{A}		-
	P2 value		<0.001	0.281	-
Total bilirubin (mg/dL)	Group 1	15	0.020±0.01	0.010±0.008	0.559
	Group 2	15	0.030±0.01	0.010±0.009	0.699
	Control	15	0.04±0.01		-
	P2 value		0.089	0.205	-
Total protein (g/dL)	Group 1	15	6.87±0.36	7.36±0.47	0.063
	Group 2	15	7.33±0.43	7.86±0.31	0.128
	Control	15	7.16±0.22		-
	P2 value		0.231	0.111	-
Albumin (g/dL)	Group 1	15	3.46±0.10	3.53±0.15	0.719
	Group 2	15	3.31±0.31	3.36±0.23	0.907
	Control	15	3.36±0.12		-
	P2 value		0.867	0.731	-
Magnesium (mg/dL)	Group 1	15	3.32±0.27	2.79±0.12	0.088
	Group 2	15	3.10±0.11	2.84±0.15	0.189
	Control	15	3.29±0.18		-
	P2 value		0.717	0.064	-
Creatine kinase (IU/L)	Group 1	15	385.48±46.06^{B}	438.70±54.44^{b}	0.548
	Group 2	15	292.19±42.24^{AB}	352.59±29.51^{ab}	0.251
	Control	15	199.81±14.46^{Aa}		-

	P2 value		0.004	0.003	-
Glucose (mg/dL)	Group 1	15	128.27±19.19	111.53±15.45	0.503
	Group 2	15	94.27±14.53	96.27±8.07	0.905
	Control	15	80.53±12.02		-
	P2 value		0.094	0.213	-

SEM: Standard error of mean. N: Number of calves in the groups. P<0.05: Statistically significant. P1: Expresses the statistical significance level within the group. P2: Expresses the statistical significance level between groups. A,B: Different letters in the same column indicate statistical difference between groups before treatment. a,b: Different letters in the same column indicate statistical difference between groups after treatment.

DISCUSSION

Anorexia, fever, dyspnea, serous or mucopurulent nasolacrimal discharge, tachypnea, and tachycardia are among the clinical symptoms seen in respiratory system infection (Bulut, 2019). In our study, there were fever, anorexia, tachycardia and tachypnea findings in the sheep in the patient groups, which was consistent with the literature. Along with the treatments, physical examination findings in both group 1 and group 2 were similar to the control.

Leukocytosis is observed as a result of defense mechanisms in respiratory system diseases and bacterial infections (Akyüz & Gökce, 2021; Akyüz et al., 2022). In the study we presented, respiratory system infection caused by *P. multocida* was formed. Probably as a result of the developing bacterial infection, defense mechanisms were activated and it was determined that leukocytosis and granulocytosis were formed in the patient groups. The fact that total leukocyte and granulocyte counts were similar to the control after treatment in groups 1 and 2 showed that tulathromycin and enrofloxacin treatments were successful in controlling the infection. No statistically significant result was observed between the groups in other hematological parameters.

Alkaline phosphatase concentrations increase in conditions such as cholestasis, stress, bone tissue destruction, and disruption of circulation in the hepatobiliary system. ALT and AST concentrations are used to determine muscle breakdown and liver damage (Soltesova et al., 2015; Bozukluhan et al., 2021). Previous studies showed that in pneumonia, liver perfusion is impaired, causing structural and functional damage to the liver (Bozukluhan et al., 2021). In addition, inflammation caused by mediators released from cells such as monocytes and macrophages causes hepatocellular dysfunction (Civelek et al., 2007). In our study, ALT, AST and ALP concentrations were found to be higher in sheep in groups 1 and 2 before treatment compared to the control. It may be due to the deterioration of liver perfusion resulting from pneumonia and stress. After treatment, ALT, AST and ALP concentrations were observed to decrease more significantly, especially in group 2 compared to group 1. The reason for the decrease in AST and ALP concentrations in the patient groups after treatment may be the reduction of stress, control of inflammation and restoration of liver tissue perfusion with treatment.

Urea concentration increases in cases of high protein catabolism, kidney diseases, infections and anorexia (Gokce & Woldehiwet, 1999; Bozukluhan et al., 2021). In our study, urea concentration before treatment was higher in groups 1 and 2 compared to the control. This increase in urea concentration was probably due to impaired kidney function in association with inflammation. This increase may also occur due to high protein catabolism due to anorexia in pneumonia. After treatment, urea reached close to control levels. CK concentration increases in muscle tissue destruction (Aydın et al., 2018). In the presented study, muscle tissue damage caused by infection may have occurred and CK concentration may have increased as a result.

In pneumonia, especially the cranial lobes of the lungs are affected (Milli et al., 2001; Caswel & Williams, 2007; McGavin et al., 2007; Çiftçi et al., 2015). In accordance with the literature, it was determined that especially the cranial lobes were affected more in the lung auscultation performed in our study. Enrofloxacin is a fluoroquinolone antibiotic with a broad spectrum of antibacterial activity. It has been reported to be effective against *P. multocida* (Suckow et al 1996). In the present study, the clinical improvement of the sick sheep in group 1 after enrofloxacin treatment was found to be consistent with the statement of being active against *P. multocida* in the literature.

In comparison of tulathromycin, florfenicol and amoxicillin treatments in goats with pneumonia, the most effective antibacterial was reported as tulathromycin (Ghanem et al., 2015). In another study, it was determined that tulathromycin showed high activity against common bacterial pathogens that cause respiratory tract disease. It has been reported that the reason for this is related to the rapid absorption and high bioavailability of the drug (Zhou et al., 2017). In our study, the faster clinical recovery of sheep in group 2 may be due to the rapid absorption of tulathromycin in the lungs and its high bioavailability. In another study, it was determined that after a single dose of 2.5 mg/kg tulathromycin administration to sheep with respiratory system disease, improvement was observed on the 5th day (Champour and Taghipour, 2015). In our study, clinical improvement in sheep in group 2 was similarly observed from the 3th day, which was found to be compatible with the study.

CONCLUSION

Especially in the infections occurring on the basis of herd, the fastest recovery with the least drug application is desired. In our study, clinical improvement was observed from the 3th day in group 2 and from the 5th day in group 1. We conclude that the administration of a single dose of tulathromycin is more beneficial and practical in *P. multocida*-induced sheep pneumonia in terms of both clinically earlier recovery and a single administration.

ACKNOWLEDGMENT

Ethical approval: Kafkas University KAU-HADYEK-2022-028. Date:22.02.2022 No: KAU-HADYEK-2022-028

Conflict of interest: The authors stated that there is no conflict of interest.

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Histopathological evaluation of the effects of live Infectious bursal disease vaccine originated from WF2512 strain on bursa Fabricius in the broilers

ABSTRACT

Infectious bursal disease (IBD) is a viral disease that causes significant economic losses in young chickens, characterized by lymphoid depletion and inflammation in the bursa Fabricius (BF). The incidence of the disease shows an increasing trend all over the world. Active and passive immunization is very important as well as strict hygiene measures in combating outbreaks. However, the fact that live-attenuated vaccines (mild, intermediate, hot) used for this purpose cause immunosuppression because of bursal damage is seen as an important limitation. In this study, it was aimed to histopathologically investigate the effects of commercial IBD vaccines originating from WF2512 (intermediate plus/hot, orally with drinking water) on BF under routine broiler rearing conditions. For this, BFs of 55 Ross 308 hybrid breed chickens (50 test, 5 controls) from five different broiler farms were used. In addition to standard vaccines, the IBD vaccine was given on day 15, and five samples from each farm were obtained 10 days later (25th day). After the first sampling, the second BF sampling was performed at the age of 38 days. Histopathological bursal lesion score was applied to evaluate the effectiveness of the vaccine. Accordingly, it was determined that the bursal lesion score, which increased slightly to moderately in the first samples, decreased in the second samples (27-61%). This was accepted as an indication that the bursal damage, which increased with IBD vaccine administration, diminishes over time and that histological regeneration was increased.

Keywords: Bursa Fabricius, histopathology, immunosuppression, infectious bursal disease

INTRODUCTION

Infectious bursal disease (IBD) is a highly contagious viral disease that causes severe inflammation in the bursa Fabricius, progresses with immunosuppression, and causes significant economic losses in the poultry industry, especially in young chickens (Berg, 2000). Gumboro disease, also known as avian nephrosis due to kidney damage, was named after the Gumboro region where the first outbreaks occurred (Cosgrove, 1962). However, due to morphological and histopathological changes in bursa Fabricius (BF) it was later dubbed IBD (Hitchner, 1970). In the 3- to 6-week period when the development of bursa Fabricius is the fastest, the clinical form develops in chickens infected with the virulent Infectious bursal disease virus (IBDV) and the disease progresses severely. The subclinical form, in which almost no clinical signs are visible, develops at the age of less than 3 weeks. Immunosuppression develops in both the acute/clinical and subclinical forms, preventing the development of an adequate immune response to subsequent vaccinations and increasing susceptibility to secondary infections (Mazariegos et al., 1990; Müller et al., 2012).

How to cite this article

Ateş, MB., Çelik, Z., Salık, R., Dağar, O., Bulut, A., Tural, A., Özdemir Ö. (2022). Histopathological evaluation of the effects of live Infectious bursal disease vaccine originated from WF2512 strain on bursa Fabricius in the broilers. *Journal of Advances in VetBio Science and Techniques, 7*(2), 250-258. https://doi.org/10.31797/vetbio.1069954

Research Article

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Article info
Submission: 08-02-2022
Accepted: 06-07-2022
Online First: 25-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

In addition to hygiene measures, active and passive immunization play a critical role in the fight against IBD, which is one of the most important viral diseases causing economic losses in commercial chicken breeding around the world, and whose incidence is increasing and remains complex (Müller et al., 2012).

The disease's causative agent (IBDV) is an RNA virus with two strands (A and B) that belongs to the Avibirnavirus genus of the Birnaviridae family (Hon et al., 2008). IBDV is divided into two serotypes (Virulent serotype 1 and apathogenic serotype 2). Although virus neutralization tests and electrophoretically distinguish these two serotypes, they are indistinguishable in fluorescent antibody, agar gel precipitation, and Enzyme-Linked Immunosorbent Assay tests (Sapats & Ignjatovic, 2000). Five different viral polypeptides (VP) are found in the virus genome. VP2-5 is used to encode segment A, and VP1 is used to encode segment B. VP2, which contains at least two epitopes to neutralize antibodies that protect the susceptible host from IBDV, is the most preferred protein for protective immunization in poultry (Eterradossi et al., 1997). Furthermore, VP2 is involved in cell tropism, tissue culture adaptation, and IBDV pathogenicity (Brandt et al., 2001).

Primary viremia occurs via the portal circulation after faecal-oral and inhaled agents replicate primarily in intestinal-associated macrophages and lymphoid cells (Dey et al., 2019). Secondary viremia occurs after viruses reaching the bursa Fabricius replicate in B lymphocytes in the follicles. Thus, viruses that have the ability to spread to other tissues and organs cause specific clinical signs and symptoms, as well as death (Dey et al., 2019). BF is an epithelial and lymphoid organ in the poultry immune system where lymphocyte stem cells mature into mature, immunocompetent B lymphocytes. The virus primarily prefers BF, where the majority of B cells in young chickens are in the active division stage (Dey et al., 2019). Following BF infection, heterophile granulocyte and inflammatory cell infiltrates are observed, as well as lymphoid depletion characterized by degeneration and necrosis, particularly in B cells expressing immunoglobulin M (Eterradossi & Saif, 2020). Depletion of B cells and atrophy of BF in surviving birds results in immunosuppression with inadequate antibody response to other viral diseases or vaccinations (Dey et al., 2019).

In the fight against IBD, strict hygiene measures and live or inactive vaccination methods are widely used. However, because IBDV can be transmitted for up to 122 days in feed and 52 days in water, combating the disease becomes extremely difficult (Müller et al., 2012). Inactivated vaccines are widely used in breeder flocks to control IBD in many countries. Maternal antibody transfer helps protect the offspring until the adaptive immune response is fully functional in hatching chicks (Davison et al., 2008). The half-life of maternal antibodies in broiler lines is generally thought to be about 3 days (Müller et al., 2012).Therefore, it is of great importance to immunize chicks with live-attenuated vaccines to prevent IBD. Live-attenuated vaccines are referred to as mild, intermediate or intermediate plus (hot) vaccines based on their ability to cause varying degrees of histopathological lesions and are preferably administered via drinking water to induce strong cellular and humoral immunity (Dey et al., 2019). It has been reported that while mild vaccines do not cause significant bursal damage in chicks, intermediate or intermediate plus vaccines may cause severe bursal lesions (Dey et al., 2019). However, it is known that mild vaccines have a lower level of protection against maternal antibodies or the very virulent form of IBDV compared to other vaccines (Dey et al., 2019; Müller et al., 2012). This dilemma is common in the poultry industry. As a matter of fact, the damage that may occur in bursa Fabricius after

IBD vaccination may result in serious economic losses by increasing the susceptibility to secondary diseases after immunosuppression, as well as causing insufficiencies in immunization that will occur after other vaccinations.

Poultry farming is an industry that is developing rapidly all over the world and is economically significant. Especially the stress of reaching slaughter weight in a short time after hatching can cause broiler chickens to become susceptible to many diseases. IBD, which was first discovered nearly 60 years ago, is still considered one of the most serious threats to the poultry industry. The fact that live-attenuated vaccines, which are an important tool in the fight against this disease, can cause bursal damage and atrophy, is seen as a major drawback. In addition, although there are experimental or controlled field studies, studies conducted directly in the field conditions where other vaccine programs and routine breeding protocols are applied are very limited. In these circumstances, the use/selection of a vaccine that causes no or minimal damage to the bursa Fabricius is one of the challenges in the poultry industry. In this study, it was aimed to histopathologically investigate the effects of commercial vaccines (intermediate plus) originating from WF2512 on bursa Fabricius under routine broiler rearing conditions and to guide industry stakeholders in vaccine selection with the obtained data.

MATERIAL and METHOD

Animals, Feeding and Hosting

In the study used BF samples from 55 Ross 308 hybrid broilers (50 test, 5 controls) from five different farms. 16 hours of light and 8 hours of darkness were applied to the broiler farms. Water was provided via a nipple system on all farms, and feed was provided ad libitum via automatic feeders. Broiler rations were formulated to suit the National Research Council's basic requirements, which included no antibiotics, anticoccidials, or other additives (Council, 1994) (Table 1).

Table 1. Diet composition according to the growth period

Analytical components (g/kg)	Starter diet (1 to 10 d)	Grower diet (11 to 24 d)	Finisher Diet (25d to 42d)
Crude Protein	230	210	190
Crude Cellulose	37	38	38
Crude Oil	63	64	64
Crude Ash	67	68	68
Calcium	13	11	9
Phosporus	6,5	5,5	4,5
Methionine	5	4	4
Lysine	14	12	11
Energy (kcal)	3025	3150	3200

Study Design

All broilers used in the study were given inactivated Newcastle (NC) vaccine subcutaneously (SC), live Newcastle (La sota, spray), and live infectious bronchitis (IB, H120, spray) vaccines at the age of one day. NC (La sota, spray) and IB (H120, spray) vaccines were given again at 14 days. Then, at the age of 15 days, commercial IBD vaccine (intermediate plus) obtained from the WF2512 strain was mixed with drinking water and given orally to all broilers except the control group. Ten days after IBD vaccination, BF samples were taken from 5 broilers on each farm and control group (25th day, 25 tests, 5 controls). Finally, on the 38th day, a second BF sampling (25 tests) from the same farms was conducted. A summary of the trial design is given in Figure 1.

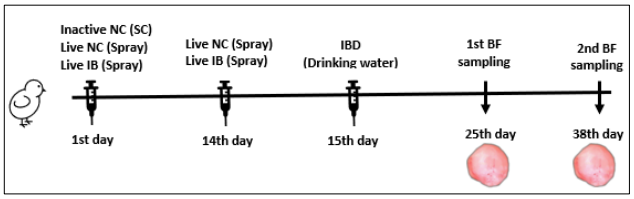

Figure 1. Study Design

Pathological Method

BF samples were fixed in 10% formol solution for 24 hours. Trimming of the hardened tissues was performed. Samples taken into tissue follower cassettes were washed in running tap water for 24 hours. An automatic tissue

processing device (Leica TP1020) was used for routine tissue processing of all tissues. After the tissues were embedded in paraffin, 5 µm thick sections were taken with a microtome (Leica RM 2125RT). The resulting slides were stained with haematoxylin-eosin (HE) (Luna, 1968). Microscopic examination was performed with light microscope and photos were taken from those deemed necessary. The method previously reported by Shaw and Davison (2000) was modified to determine the bursal lesion score. First, lymphoid depletion and accompanying inflammation findings in 10 randomly selected follicles in each BF were evaluated and scoring was done (Follicular lesion score) (Shaw & Davison, 2000). In addition, a lesion spread score (1: mild; 2: moderate; 3: severe) was determined, reflecting the overall microscopic examination of BF, and showing the frequency/spread of lesioned follicles. Finally, the numerical values obtained by multiplying these two scores (minimum:1; maximum:21) were accepted as the final score of each case. The histopathological findings based on this scoring are given in Table 2.

Table 2. Histopathological findings used in the bursal lesion score.

Follicular lesion score	Score	Lesion spread score	Score
No lesions	**1**	Mild	**1**
Interstitial mononuclear cell infiltration, edema, lymphoid depletion (<10%)	**2**	Moderate	**2**
Interstitial mononuclear cell infiltration, edema, lymphoid depletion (11-25%)	**3**	Severe	**3**
Interstitial mononuclear cell infiltration, edema, lymphoid depletion (26-40%), intraepithelial cysts	**4**		
Interstitial mononuclear cell infiltration, edema, lymphoid depletion (41-55%), intraepithelial-intrafollicular cysts, hemorrhage, mild necrosis, and fibrosis	**5**		
Interstitial mononuclear cell infiltration, edema, lymphoid depletion (56-75%), intraepithelial-intrafollicular cysts, hemorrhage, moderate necrosis, and fibrosis	**6**		
Interstitial mononuclear cell infiltration, edema, lymphoid depletion (> 75%), intraepithelial- intrafollicular cysts, hemorrhage, severe necrosis, and fibrosis.	**7**		
Total score= (Follicular lesion score) x (Lesion spread score)			

Statistical Analysis

The Kolmogorov-Smirnov test was used to analyze the histopathological scores for normal distribution. The homogeneity of variances was controlled using Levene's test. Histopathological scores were evaluated by the post-hoc Duncan test after one-way ANOVA (SPSS® Inc. version 26.0 for Windows, Chicago, IL, USA). Statistical significance was defined as a value of $p<0.05$.

RESULTS

One replicate (No:5) of Farm 1 and Farm 5 was excluded due to tissue processing and staining errors. In the histopathological examinations of BFs, varying degrees of tissue damage were detected in all vaccinated farms. Mild to severe atrophy and lymphoid depletion were determined in BF follicles. It was determined that reticular cells in the follicular became prominent. In addition to vacuoles and cystic spaces within the follicles, necrosis of varying severity was sometimes observed. In some cases, regenerative follicles were also observed. In the interfollicular region, mononuclear cell

infiltrations were seen, as well as an increase in fibrous connective tissue on occasion. There were cyst formations in the intraepithelial region, and edema in the subepithelial and interfollicular regions (Fig. 2A). Table 3 shows bursal lesion scores based on histopathological examination results of BFs examined across all farms. As a result, it was determined that the severity of lesions in Farm 1 and 5 samples taken on the 25th day decreased on the 38th day, but this was not statistically significant ($p>0.05$). In Farm-2, Farm-3, and Farm-4, it was revealed that the second samples showed a significant improvement (decrease) in bursal lesion score ($p<0.05$). The bursal lesion score in the second samples (38th day) was lower (27-61%) than in the first samples (25th day) in all farms, indicating that the bursal lesion score decreased over time (Fig. 2B-C).

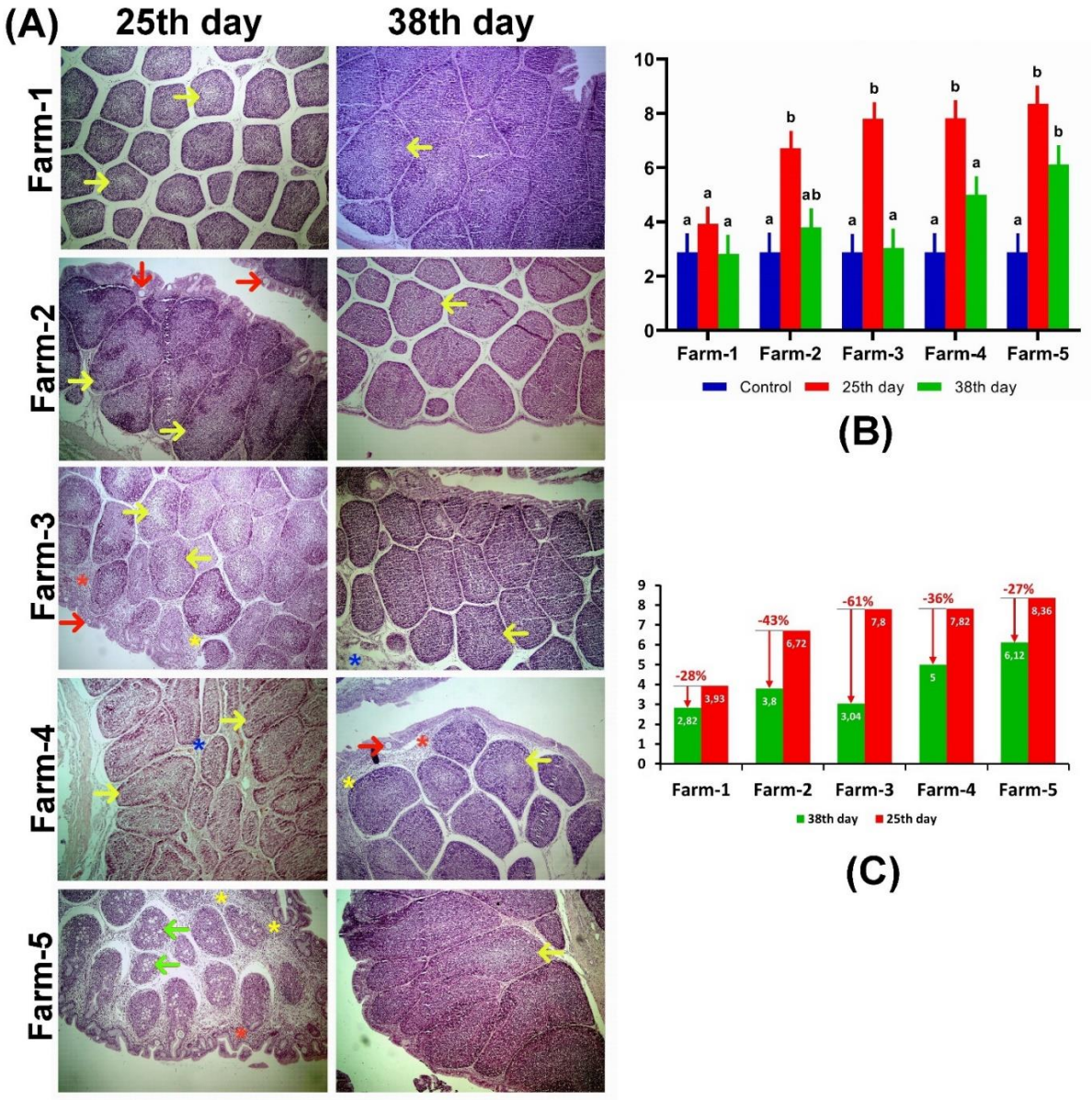

Figure 2. The effect of intermediate plus live IBD Vaccine on bursa Fabricius. (A) Representative photomicrographs of comparison of samples taken on days 25th and 38th days, bursa Fabricius, HE, 10X. Yellow arrows: Lymphoid depletion in follicle; Red arrows: Intraepithelial cysts; Green arrows: Vacuoles and cystic spaces within the follicles; Red asterisks: Inflammatory cell infiltration; Yellow asterisks: Increase in fibrous connective tissue, Blue asterisks: Edema in the subepithelial and interfollicular regions (B) Graphical representation of statistical results Changes in bursal lesion score in the 25th and 38th days. a,b The difference between the different superscripts compared to the Control group is significant ($p <0.05$, one-way ANOVA post hoc Duncan test). (C) Graphical representation of the changes in Bursal lesion scores at 25th and 38th days.

Table 3. Histopathological scoring and results

Replicate No	Control n:5	Farm-1		Farm-2		Farm-3		Farm-4		Farm-5	
		25th day n:4	38th day n:5	25th day n:5	38th day n:5	25th day n:5	38th day n:5	25th day n:5	38th day n:5	25th day n:5	38th day n:4
1	3,2	2,3	2,5	2,9	2,7	3,6	3,5	9,4	8,2	9,6	8,6
2	2,9	2,6	3,1	9,8	3,1	8,4	3,2	9	3,1	8,2	9,4
3	3	7,6	2,5	3,1	7	8,6	2,4	8,6	7	9	3,5
4	2,6	3,2	3	8,6	3,4	8,8	3	3,5	3,6	7	3
5	2,7		3	9,2	2,8	9,6	3,1	8,6	3,1	8	
Mean ±SEM	2,88 ±0,11[a]	3,93 ±1,23[a]	2,82 ±0,13[a]	6,72 ±1,53[b]	3,80± 0,80[ab]	7,80 ±1,07[b]	3,04 ±0,18[a]	7,82 ±1,09[b]	5,00 ±1,08[a]	8,36 ±0,44[b]	6,12 ±1,67[b]

[a,b]The difference between the different superscripts compared to the Control group is significant ($p < 0.05$, one-way ANOVA *post hoc* Duncan test).

DISCUSSION

Infectious bursal disease, also known as Gumboro disease, is a highly contagious viral infection that reduces the immunity of young chickens and can lead to their death at 3 - 6 weeks of age (Berg, 2000). Control of the disease is tried to be ensured with strict hygiene measures and vaccination protocols. However, epidemics that occur from time to time can cause very important economic losses in the poultry industry (Dey et al., 2019). On the other hand, the fact that the vaccines, which play a key role in this struggle, can cause immunosuppression, may lead to a decrease in the effectiveness of other vaccinations and indirect losses due to increased sensitivity to other diseases (Müller et al., 2012). This can be even more devastating in broilers reared under high yield pressure in a very short time. One of the desirable features of the vaccine to be used is that the degenerative effects on the bursa Fabricius are minimal and that regeneration takes place over time. For this reason, in this study, we focused on the histopathological investigation of the effects of commercial vaccines (intermediate plus) originating from WF2512 on bursa Fabricius in enterprises where traditional care, feeding and vaccination programs are carried out.

The histopathological bursal lesion score is one of the tests used to assess the vaccine's efficacy (Commission, 2002). However, the bursal lesion scoring scale has some drawbacks, including being too short, subjective, and difficult to optimize sampling time (Butter et al., 2003). With the development of previously used scoring methods, the bursal lesion scoring used in this study was modified. The fact that the parameters to be evaluated in the previously used methods were not very clear was considered as an important shortcoming. The current study was based on scoring the severity of different degenerative findings in randomly selected follicles during the examination of BFs. On the other hand, it was revealed that the numerical value obtained as a result of giving a general score according to the extent of these degenerative follicles and multiplying the two data as a result allows a more quantitative measurement. In fact, while degenerative changes are prevalent in some of the follicles examined in BFs, normal or even regenerative changes can be seen in others. The most important point is to determine the severity of the lesion within the degenerative follicles, as well as the extent to which these degenerative follicles have diffused.

The histopathological findings were found to be similar to those seen in prior experimental trials (Hair-Bejo et al., 2004; Henry et al., 1980; Thornton & Pattison, 1975). The degenerative alterations are caused by the vaccine's mechanism of action. Vaccine viruses almost mimic the disease and replicate in B cells, which are in the active division stage.

Meanwhile, it causes necrosis and/or apoptosis in B cells, and heterophile granulocyte and inflammatory cell infiltrations in the BF (Dey et al., 2019). Thus, an immunosuppression develops, characterized by bursal damage because of inflammation and a decrease in immunocompetent B cells, particularly affecting the humoral immune system. Some intermediate and the majority of hot vaccines, have been shown to cause severe bursal lesions, similar to those seen in IBD outbreaks (Hair-Bejo et al., 2004). However, mild and even some intermediate vaccines are known to be less effective than other vaccines at breaking a certain level of maternal antibody and protecting against the very virulent form of IBDV (vv-IBDV) (Dey et al., 2019; Müller et al., 2012). Maternal antibodies help protect offspring until the adaptive immune response is fully effective. The necessity to protect chicks in the first weeks after hatching and the high infection pressure make vaccination inevitable despite strict hygienic measures (Müller et al., 2012). Considering that immunity obtained with highly attenuated vaccine strains (mild and intermediate vaccines) cannot control outbreaks caused by vv-IBDV strains, the use of low attenuated vaccine strains (intermediate plus/hot vaccines) may become necessary in high-risk situations (Müller et al., 2012) . All these reasons complicate the selection of vaccines that are formulated to provide both minimal bursal tissue damage and optimal protection.

In a study conducted in broiler chickens, the bursa lesion scoring that occurred as a result of live intermediate vaccination on the 14th day changed from mild to moderate on the 28th and 35th days, but returned to mild on the 42nd day (Hair-Bejo et al., 2004). Similarly, both some literatures and vaccine manufacturers report that intermediate plus vaccines can temporarily interrupt lymphoid depletion and normal B cell development in bursa follicles, but this is usually followed by B cell repopulation and histological regeneration (Castro et al., 2009; Iván et al., 2001). Ezeokoli et al. (1990) reported that the IBD vaccine, of which they did not explain the type, caused serious lesions in the bursa in 3-7 days, but the bursa tissue was completely healed after 15 days, and there was no difference between the vaccinated group and the control group. In addition, another study reported that necrosis in the follicles partially disappeared, and the B lymphocyte population was recovered by 40-80% in 7 weeks (Kim et al., 1999). In the current study, it is observed that the bursa fabricius lesion score is mild to moderate in the first sampling after vaccination (Table 3, Figure 2). In the second samplings after vaccination, however, all groups revealed a decrease in bursal lesion scores (27-61 %). This was interpreted as an indication of lymphoid depletion decrease and histological regeneration in bursal follicles. With this improvement, which was observed at different levels in five different farms, it was thought that restoration of the immune response could be contributed as a result of the normalization of bursal functions, which provides a necessary micro-environment for the diversification of B cells with their immunoglobulin genes. Because the duration of immunosuppression and restoration of the humoral immune response are reported to be associated with the regeneration of BF after vaccination (Castro et al., 2009). In the study investigating the relationship between IBDV-induced bursal damage and the humoral immune response against Brucella abortus in SPF chickens, it was emphasized that the probability of being immunocompetent is low until more than half of the bursal repopulation rate is achieved (Edwards et al., 1982). In another study, it was reported that the alleviation of bursal lesions and the increase of B cell repopulation were faster in chickens inoculated with the vaccine strain than the virulent strain, and bursal damage caused by both applications resulted in a decrease in antibody synthesis (Kim et al., 1999). Based on this information, a positive correlation emerges

between the alleviation of bursal lesions and the humoral immune response. Therefore, in the present study, it was commented that regeneration in bursal histological architecture may have positive effects on the immune system.

CONCLUSION

In this study, it was concluded that the bursal lesion score resulting from the administration of the commercial intermediate plus vaccine originating from WF2512 did not rise to very high levels, and the lymphoid depletion decreased while the regenerative changes increased in the bursal follicles over time. It was also noted that the regression in vaccine-related bursal lesions in all farms was statistically significant when it was 30% or more. In the field, very virulent strains characterized by the continuous development of antigenicity and virulence of IBDV are seen as the cause of high mortality and economic losses due to long-term and severe suppression of the immune system. In order to prevent this, it may become inevitable to fight with less attenuated intermediate plus or hot vaccines by considering farms where IBD outbreaks occur as endemic. In such cases, the degree of bursal damage, the time it takes for lesions to heal, and the length of the rearing period should all be considered when choosing a vaccine.

ACKNOWLEDGMENT

Ethical approval:

The Ethics Committee of Experimental Animal Production and Research Center of the Faculty of Veterinary Medicine of Selcuk University (SÜVDAMEK) approved the study's ethical compliance (Approval No: 2021/ 144)

Conflict of interest: The authors have no conflict of interest.

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Investigation of some acute phase proteins and antioxidant/oxidant system in infected sheep with bluetongue virus disease

Research Article

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ABSTRACT

In this study, it was aimed to determine the level of some acute phase proteins and oxidative stress in sheep infected with bluetongue virus disease. Twenty five bluetongue virus-infected and 10 healthy sheep were used. Blood samples from V. jugularis of animals were taken into tubes without anticoagulant. Total antioxidant (TAC)/oxidant capacity (TOC), haptoglobin, serum amyloid A (SAA), ceruloplasmin and albumin levels were determined colorimetrically. Oxidative stress index (OSI) was calculated using the formula. As a result of the analysis, when sheep infected with bluetongue virus disease and healthy sheep were compared, it was determined that SAA, TOC and OSI concentrations increased, albumin and TAC values decreased. However haptoglobin and ceruloplasmin levels increased but were statistically insignificant. In conclusion, it was concluded that oxidative stress occurs in sheep infected with bluetongue virus disease and that acute phase proteins haptoglobin, SAA and ceruloplasmin can be used as inflammation markers.

Keywords: Acute phase proteins, bluetongue virus disease, oxidative stress index (OSI), sheep

INTRODUCTION

Bluetongue disease caused by Orbivirus is a viral disease of ruminants infected by flies of the genus Culicoides (Tabachnick 2004; Sperlova and Zendulkova 2011). There are symptoms in sheep such as high fever, drooling, mucopurulent nasal discharge, ulceration in the oral mucosa and necrosis. In addition, high morbidity and low mortality are observed in animals. Cyanosis occurs in the mouth lesions of heavily infected animals and the appearance of a dark blue tongue is the characteristic finding of the disease. The disease is on the list of notifiable diseases in the world (Sperlova and Zendulkova 2011; Maclachlan 2011).

Acute phase response (APR), which is a reaction of the organism against tissue damage, inflammation, infection, causes the production and release of certain proteins known as acute phase proteins (APP) produced in hepatocytes and peripheral tissues (Iliev and Georgieva, 2018). While the blood levels of some APPs increase, others decrease. While haptoglobin and serum amyloid A (SAA) are very important in ruminants, ceruloplasmin is a moderately important APP. The concentrations of these APPs in circulation are generally related to severity of the disorder and extent of the tissue damage.

Article info
Submission: 26-01-2022
Accepted: 12-05-2022
Online First: 27-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

How to cite this article
Merhan, O., Yılmaz, V., Bozukluhan, K., Maraşlı, Ş., Öğün M. (2022). Investigation of some acute phase proteins and antioxidant/oxidant system in infected sheep with bluetongue virus disease. *Journal of Advances in VetBio Science and Techniques, 7*(2), 259-264.
https://doi.org/10.31797/vetbio.1063406

Therefore, measurement of serum haptoglobin, SAA and ceruloplasmin concentrations in ruminants can be used for diagnostic and prognosis as well as evaluation of APR (Tothova et al., 2014; Kuru et al. 2015).

Free radicals produced by the mitochondria during normal oxygen use of the organism cause oxidative damage by creating changes in the structure of lipids, proteins and nucleic acids (Karabulut and Gülay, 2016). The system that works in order to prevent the damage caused by the free radicals is defined as antioxidant system and this system acts by preventing the radical production and/or eliminating the harmful effects of the formed radicals (Süleyman et al. 2018). Normally, while there is a balance between the oxidant and antioxidants in the organism; stress, chronic diseases and infections in the organism stimulate the immune system, as a result, tissue damage occurs due to the increase in the amount of free radicals (Atmaca et al., 2015; Karabulut and Gülay, 2016).

The studies have reported that the total oxidant/antioxidant capacity (TOC, TAC) and oxidative stress index (OSI) will be able to change in cases of the local and/or systemic inflammation or infection and can be used as non-invasive marker (Celi and Gabai, 2015; Aydoğdu et al., 2018).

In this study, it was aimed to determine the diagnostic importance of haptoglobin, SAA, ceruloplasmin, albumin, TAC, TOC and OSI levels in sheep diagnosed with the bluetongue virus disease.

MATERIAL and METHOD

The study was carried out in 25 bluetongue virus-infected and 10 healthy sheep raised in Kars and its districts. Blood samples taken from the Vena jugularis of the animals were taken into tubes with and without anticoagulant. Samples were stored at -20 °C until analysis. Both groups used in the study consisted of animals not vaccinated against bluetongue virus disease.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted from the samples using a High Pure Viral RNA Kit (Roche, Mannheim, Germany) and complementary DNA (cDNA) synthesis was performed using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, USA), according to the manufacturers'instructions. RT-PCR was performed using the method and primer (PP-1) described by Nikolakaki et al. (2005). The formation of PCR products of the expected size (822 bp) was analysed by DNA gel electrophoresis.

Biochemical analysis

The TOC and TAC levels have been determined by using a commercial test kit (Rel Assay Diagnostics, Turkey). The OSI has been calculated by using the formula [TOC (μmol H2O2 equivalents/L)/10 x TAC (mmol Trolox equivalents/L)] (Karababa et al., 2013). The serum haptoglobin concentration has been determined with determining the hemoglobin binding capacity described by Skinner et al. (1991). Ceruloplasmin has been determined by the colorimetric method based on the p-phenylenediamine oxidase activity described by Colombo and Richterich (1964). SAA has been determined by ELISA test kit (Tridelta phase range, Ireland), and albumin has been determined in accordance with the procedure with a commercial test kit (Biolabo, France).

Statistical analysis

SPSS for Windows 20.0. was used for the statistical analyses. The distribution of the data obtained from the groups were shown as normal distribution according to the Kolmogorov-Smirnov test. Therefore, Student-t test was used to compare sheep infected with bluetongue virus disease and control group.

RESULTS

RT-PCR: The amplification of bluetongue virus disease specific 822 bp fragment from RNA of samples and positive control were described as positive reaction (Figure 1).

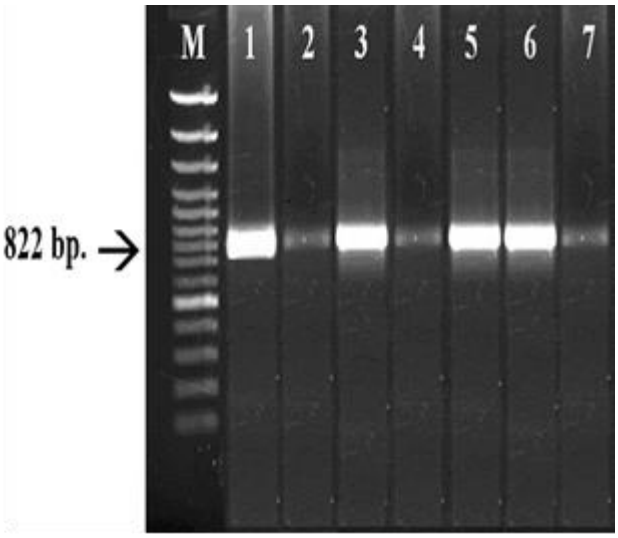

Figure 1. The result of bluetongue virus disease RT-PCR in serum samples. Line M: 100 bp DNA ladder, Line 1: Positive control (822 bp), Lines 2-7: Positive samples.

When comparing sheep infected with bluetongue virus disease to healthy sheep, it has been determined that the concentration of SAA, TOC and OSI (P<0.001) increased, albumin (P<0.05) and TAC value (P<0.01) decreased compared to the control group, in addition, haptoglobin and ceruloplasmin levels increased, however, has been found to be statistically insignificant (Table 1).

Table 1. Some acute phase proteins, TAC&TOC levels in healthy sheep and infected with bluetongue virus disease. Data are presented as mean±standard error (X±SEM).

Parameters	Control (n=10)	Infected (n=25)	P
Haptoglobin (mg/L)	161±17	177±9	NS
SAA (mg/L)	21.87±1.77	67.99±3.57	P<0.001
Ceruloplasmin (mg/dL)	12.74±0.75	14.12±0.61	NS
Albumin (g/dL)	3.39±0.13	3.04±0.07	P<0.05
TAC (mmol Trolox Equiv/L)	0.92±0.06	0.65±0.04	P<0.01
TOC (µmol H_2O_2 Equiv/L)	21.83±2.56	36.24±1.71	P<0.001
OSI (Arbitrary Unit)	2.42±0.28	6.02±0.44	P<0.001

DISCUSSION

In the event of chronic disease, stress or infection in the organism, the amount of free radicals increases by the cells making phagocytosis in the immune system and tissue damage occurs (Aydoğdu et al., 2018). Although there are many methods for determining the oxidative stress, which plays a role in the pathogenesis of many diseases and inflammatory conditions, these methods are complex and expensive methods that require long time and effort and allow the measurement of oxidant/antioxidant molecules one by one to evaluate only for the molecule being measured. For this reason, it has been reported that the measurement of TAC and TOC is easier than the measurement of individual oxidant/antioxidant in order to determine the oxidant/antioxidant balance. The OSI, which is defined as the ratio of TOC level to TAC level, is an indicator of the oxidative stress level (Erel 2004; Erel 2005). It has been stated that the oxidative stress develops in the viral, bacterial and parasitic diseases such as foot-and-mouth disease (Deveci et al., 2018), brucella (Merhan et al., 2017a), hypodermosis (Merhan et al., 2017b) and cryptosporidiosis (Çenesiz et al., 2017) in cattle and pox virus infected sheep (Bozukluhan et al., 2018), the antioxidant level decreases while the oxidant level increases. It has been stated that the cell and tissue damages occur with increasing the amount of free radicals in the organs and tissues (Küçük 2021).

In our study we conducted that TOC and OSI (P<0.001) values increased and TAC (P<0.01) values decreased in sheep infected with the bluetongue virus disease. Therefore, it is seen that the findings we have obtained are compatible with the above-mentioned studies.

While the serum level of positive APPs increased in the liver as a result of the inflammation, tissue damage and infection; it is stated that the negative APPs decrease. Haptoglobin has many functions such as forming stable complexes with the free hemoglobin and thus creating a bacteriostatic effect by preventing iron loss, as well as regulating the lipid metabolism and stimulating the immune system as an immunomodulator (Petersen et al., 2004). Haptoglobin, which is a positive APP, has been reported to increase in bacterial (Bozukluhan et al., 2016), viral (Merhan et al., 2017c) and parasitic (Bozukluhan et al., 2017; Merhan et al., 2017b) diseases. It has been reported that SAA, another positive APP, will be able to be used in determining the severity and prevalence of inflammatory events, prognosis and evaluating the success of the treatment applied (Tothova et al., 2014). The SAA, which has functions such as transporting cholesterol to hepatocytes, preventing oxidative destruction of neutrophils, stimulating calcium release from monocytes; has been reported to increase in bacterial (Kaya et al., 2016), viral (Merhan et al., 2017c) and parasitic infections (Merhan et al., 2017b). In a study conducted in sheep infected with the bluetongue (Aytekin et al., 2015), it has been reported that the sialic acid levels increased, while the albumin and ceruloplasmin levels decreased. In another study conducted in sheep infected with the bluetongue, it has been reported that the haptoglobin, SAA and ceruloplasmin levels increased, while the albumin levels decreased (Sanchez-Cordon et al., 2013). In this study, it has been determined that while the SAA (P<0.001) and haptoglobin levels, which are major APPs, increased in ruminants, the albumin (P<0.05) levels decreased, and it is thought that this situation will be able to be related to the tissue destruction.

The ceruloplasmin, an α-2 globulin, has functions such as copper transport, oxidation of the toxic iron to the non-toxic iron, and antioxidant effect. Copper increases the immune function by affecting on various enzyme levels that mediate the antioxidant system. The ceruloplasmin mediates for the copper transport to lysyl oxidase and copper-zinc superoxide dismutase enzymes by involving in the tissue repair and plays a role in the antioxidant system. It also protects the cells against the oxidative damage. If the serum ceruloplasmin level decreases, the phagocytosis and antimicrobial activity also decrease. Therefore, the need for this enzyme increases in the inflammatory conditions (Cerone et al., 2000). Studies have reported that its concentration increases in bacterial (Bozukluhan et al., 2016), viral (Merhan et al., 2016) and parasitic diseases (Nisbet et al., 2008; Bozukluhan et al., 2020). In the study, although the ceruloplasmin level increased in sheep infected with the bluetongue, it was found to be statistically insignificant. The reason for the increase in the ceruloplasmin concentration is thought to be due to the increase in the antimicrobial and phagocytolytic effects of the cells in the defense system.

CONCLUSION

In conclusion, sheep infected with bluetongue virus disease was detected to cause important changes in the oxidative-antioxidative capacity and APP levels in cattle. It has been concluded that the SAA will be able to be used as an inflammation marker in sheep infected with the bluetongue.

ACKNOWLEDGMENT

This study was presented in "1st International Congress of The Turkic World on Health and Natural Sciences", 21-23 April, 2019, Osh, Kyrgyzstan, as oral presentation.

Ethical approval:

Studies were performed by Erciyes University Animal Testing Local Ethics Council (ERU-2013/102).

Conflict of interest: No potential conflict of interest was reported by the author.

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Tracheal adenocarcinoma and surgical treatment in a cat

Case Report

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Article info
Submission: 20-10-2021
Accepted: 05-03-2022
Online First: 20-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

Primary tracheal malignant neoplasms are rare in dogs and cats. Mostly seen in middle-aged to older cats. Clinical findings associated with airway obstruction are observed such as shortness of breath, wheezing, cough, and cyanosis.The material of this report is a 12-year-old, 6.3 kg, castrated male cat brought to Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Surgery due to with symptoms of cough and difficulty breathing. In the anamnesis, it was learned that the patient's tracheal mass was removed by operation 8 months ago in another clinic. As a result of the clinical and radiographic examinations, it was observed that the tracheal mass recurred. The mass was removed by tracheal ring resection. The histopathological result of tracheal mass was determined as adenocarcinoma.

Keywords: Cat, recurrence, tracheal adenocarcinoma, tracheal ring resection

INTRODUCTION

Primary malignant tumors of the trachea are rarely seen in cats and dogs (Dugas et al., 2011). Lymphosarcoma, adenosarcoma, squamous cell carcinoma, carcinoma, seromucinous carcinoma are among the tracheal tumors observed in cats. (Brown et al., 2003; Miller et al. 2020). The most common of these tumors is lymphosarcoma (Dugas et al., 2011). The long-term prognosis of cats with tracheal masses is not well known (Green et al., 2012).

Generally, tracheal tumors grow slowly and are not clinically evident (Howard et al., 2017). In cats with tracheal tumors, nonspecific respiratory tract findings such as breathing difficulties, wheezing, exercise intolerance, and cough are observed (Howard et al., 2017). Other clinical findings include lethargy, weight loss, intermittent cyanosis, and collapse (Azevedo et al., 2017). Most of the primary tracheal tumors in cats are seen radiographically as prominent solitary intratracheal masses (Dugas et al., 2011).

Within limited knowledge, tracheal tumors appear to be tumors with low metastasis that require aggressive local treatment (Green et al., 2012)

How to cite this article
Çerkez, EE., Özer, K., Kurt, T., Karabağlı M. (2022). Tracheal adenocarcinoma and surgical treatment in a cat. *Journal of Advances in VetBio Science and Techniques*, *7*(2), 265-268.
https://doi.org/10.31797/vetbio.1011695

CASE REPORT

Twelve years old, 6.3 kg weight, castrated male cat was brought Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Surgery due to cough and respiratory distress that has been present for 2 weeks. In the anamnesis, it was learned that the patient was taken to another clinic with a similar complaint 8 months ago and operated due to a tracheal mass.

Physical examination revealed that the color of the mucosal membrane was normal, the body temperature was 38.1 ° C, the capillary filling time was less than 2 seconds and there was no pain on abdominal palpation. During the blood draw, cyanosis was observed in the patient. No remarkable differences were seen in routine hemogram and biochemistry examinations of blood.

The X-ray examination of patient revealed a mass irregular borders at C4 level and narrowing of the tracheal lumen. When compared with the X-ray of the patient 8 months ago, it was understood that there was a recurrence at the same location (Figure 1A and B).

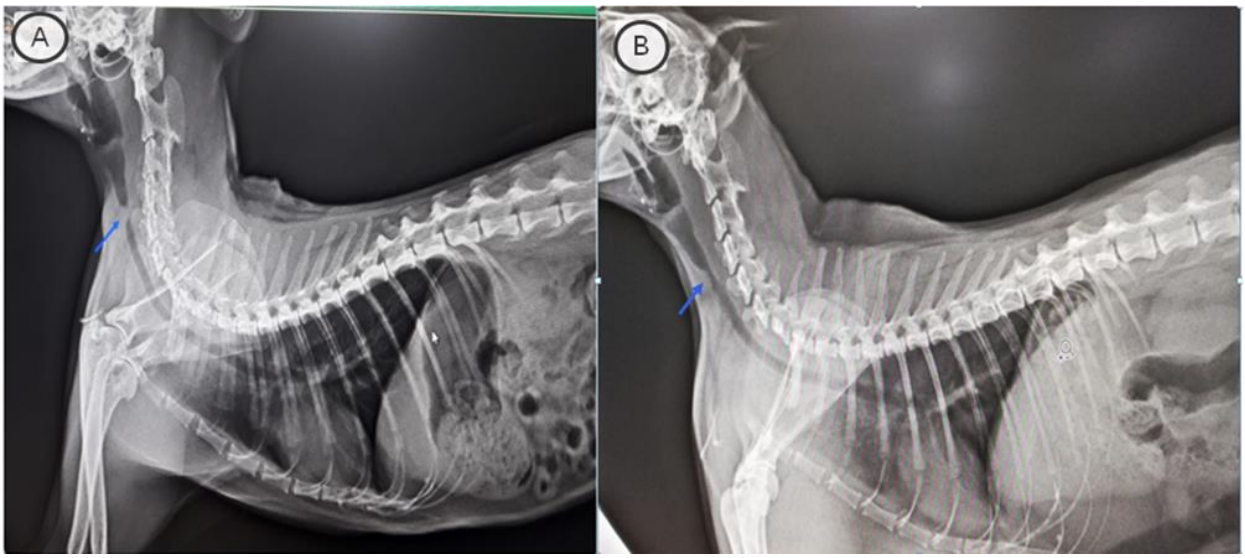

Figure 1. A. X-ray before the first operation, the mass indicated by arrow. B. X-ray taken when the patient was brought to our clinic, the appearance of the recurrent mass was observed in the same area.

Magnetic resonance imaging (MRI) was performed to determine the exact location and boundaries of the mass and the operative planning. According to the MRI examination, it was seen that the mass was 12.9 mm caudal to the larynx, 6.6 mm in width and 7.9 mm in length (Figure 2). It was decided to surgically remove the mass by tracheal ring resection.

The patient was anesthetized with 7 mg/kg propofol (Propofol-Lipuro 1%®, B. Braun, Germany). Tracheal intubation was performed with a size 3.5 intubation tube. Anesthesia was continued with 100% oxygen and isoflurane (Isoflurane®, Adeka, Turkey) by an anesthetic device. Ceftriaxone (Novosef®, 30 mg/kg, Sanofi İlaç, Turkey) administered intravenously and meloxicam (Melox®, 0.2 mg/kg, Nobel İlaç, Turkey) subcutaneously. Isotonic serum (Polifleks®,, Polifarma İlaç, Turkey) at a dose of 10ml / kg / hour was given during the operation.

The patient was positioned dorsally and the trachea was reached by making a skin incision through the caudal side of the larynx. After that, the intubation tube was removed and anesthesia was maintained with propofol. The mass, whose location was determined before the operation, was removed by tracheal ring resection. After

the anastomosis, the patient was intubated again and anesthesia was continued with isoflurane.

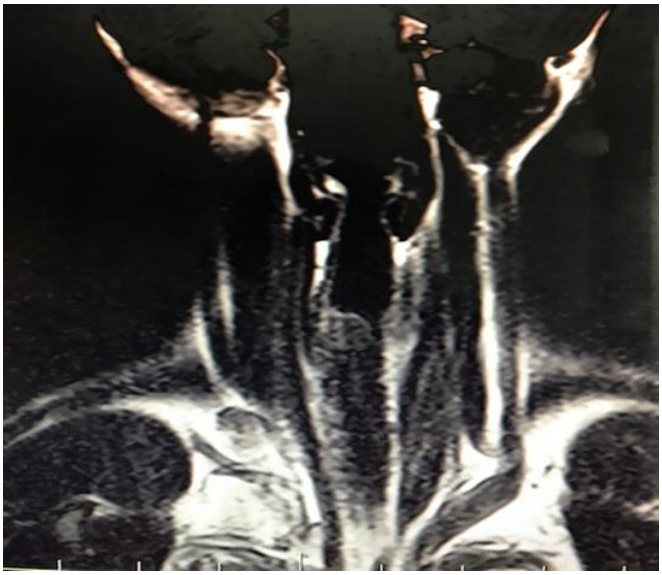

Figure 2. MR image of Case. The mass in the tracheal lumen, caudal to the larynx in the coronal plane is indicated by the arrow.

Postoperatively, meloxicam (Melox®, Nobel İlaç, Turkey) at a dose of 0.2 mg/kg for the first 2 days and ceftriaxone (Novosef®, Sanofi İlaç, Turkey) at a dose of 30 mg/kg for 6 days was used. The clinical examination findings were normal on the 3rd and 10th postoperative days. No postoperative complications were observed.

The mass was reported to be adenocarcinoma after histopathological examination. In the histopathological examination, proliferation in cystic and atypical gland structures in the trachea, marked pleomorphism of neoplastic epithelial cells, cystic and dilatative changes in the glands was reported (Figure 3A and B).

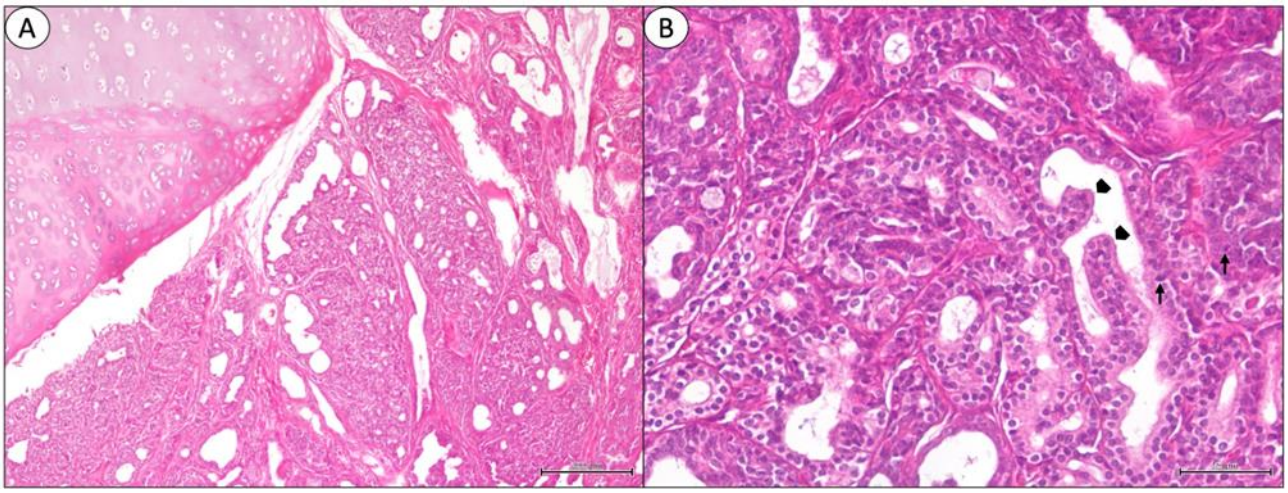

Figure 3. Histopathological appeareance of case. A. Proliferation of cystic and atypical gland structures. H&E. bar = 200 µm. B. Marked pleomorphism of neoplastic epithelial cells, cystic and dilatative changes of the glands, papillary proliferations (arrowheads), and mitotic figures (arrows) are visible. H&E. bar= 50 µm.

ACKNOWLEDGMENT

Ethical approval: Permission was obtained from the patient owner on 20.10.2019 with a "treatment and information consent form"

Conflict of interest: There is no conflict of interest between the authors.

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Clinicopathological evaluation of vaginal leiomyoma and ovarian luteoma in a bitch

ABSTRACT

A 7-years-old female Golden Retriever dog was presented to the clinic with a multilobular mass protruding from the vulva. On clinical examination, the mass was observed on the circumference of the vaginal canal. On vaginal cytology, parabasal cells, intermediate cells, neutrophil infiltration and clumps of bacteria were determined. Mild anemia, severe leukocytosis and high mean platelet volume (MPV) value were detected. As a treatment, vaginal mass was totally extirpated by partial vaginectomy and subsequently ovariohysterectomy were performed to avoid the re-occurrence of the vaginal tumor. Both the extirpated vaginal mass and the genital organs removed by ovariohysterectomy were examined histopathologically. The tissue samples were fixed in 10% neutral buffered formalin. After routinely processed they were embedded in parafin. Sections cut at 4 µm in thickness were stained with hematoxylin and eosin (H&E) stain to be evaluated by light microscopy. Vaginal mass was capsulated and non-invasive. Densely packed spindle cells with eosinophilic cytoplasm and elongated nuclei were determined on the vaginal tumor. Polyhedral tumoral cells with abundant, finely vacuolated cytoplasm that contain lipid droplets separated by thin fibrovascular stroma were detected on the ovarian mass. The nuclei were centrally located, small and oval. Mild to moderate anisocytosis and anisokaryosis were noted. Benign tumors were diagnosed which were vaginal leiomyoma and bilateral ovarian luteoma. Nutritional disorders, use of exogenous steroid hormones, hormone irregularities, genetic predisposition, exposure to neoplastic agents are effective to development of gynecological tumors. It was concluded that histopathological analysis of every surgically removed tissue should be performed in order not to miss a tumorous condition even if it does not show any symptoms.

Keywords: Bitch, ovarian luteoma, vaginal leiomyoma.

INTRODUCTION

Neoplasms in genital tract are more common in bitches. Leiomyoma is benign tumor which originates from any organ containing the smooth muscle cells. Vaginal leiomyoma can be oval or round shape, usually as well defined, capsulated, a single or multiple structures (Singh et al., 2014). Diagnosis of leiomyoma has to be made by histopathology. The most common treatment choice is surgical intervention with extirpation of the mass throughout episiotomy (Nelissen et al., 2012). To avoid the recurrence of the vaginal tumors, ovariohysterectomy must be performed (MacLachlan and Kennedy, 2002).

Ovarian tumors in domestic animals are relatively uncommon (Moulton, 1990). Luteoma is the rarest ovarian neoplasm which originates from sex-cord stromal tumors (McEntee, 2002). Luteoma is a term used for tumors of yellow brown color, solitary structure, which can reach quite large sizes, consisting of luteinized cells resembling the corpus luteum (Moris and Dobson, 2001).

How to cite this article
Kula, H., Günay Uçmak, Z., Kırşan, İ., Yüzbaşıoğlu Öztürk, G., Gülçubuk A. (2022). Clinicopathological evaluation of vaginal leiomyoma and ovarian luteoma in a bitch. *Journal of Advances in VetBio Science and Techniques, 7*(2), 269-273.
https://doi.org/10.31797/vetbio.1068008

Case Report

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Article info
Submission: 03-02-2022
Accepted: 18-04-2022
Online First: 25-08-2022
Publication: 31-08-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

Ovarian luteoma usually appear unilaterally and and regresses after birth (Ichimura et al., 2010; Yamini et al., 1997; Yılmaz et al., 2017). Based on clinicopathological, histopathological and immunohistochemical characteristics, ovarian luteoma is diagnosed (Namazi et al., 2015).

In this case report, clinicopathological evaluation of a dog with vaginal leiomyoma and ovarian luteoma, surgical interventions for diagnosed gynecological tumors and preventive approaches are presented.

CASE REPORT

A 7-years-old female Golden Retriever dog was presented to the clinic with a multilobular mass protruding from the vulva. There was no evidence of urinary incontinence. On clinical examination, the mass was observed on the circumference of the vaginal canal. On vaginal cytology, parabasal cells, intermediate cells, neutrophil infiltration and clumps of bacteria were determined. The pro-estrous bleeding went undetected for over a year. Total blood count and some biochemical parameters were evaluated. Mild anemia, severe leukocytosis and high mean platelet volume (MPV) value were detected (Table 1).

Table 1. Results of total blood count and some biochemical parameters.

Parameters	Results (Referance ranges)	Parameters	Results (Referance ranges)
RBC (M/µL)	6.23 (5.65 - 8.87)	**PLT (K/µL)**	156 (148 – 484)
HCT (%)	34.8 (37.3 - 61.7)	**MPV (fL)**	16.9 (8.7 - 13.2)
HGB (g/dL)	12.7 (13.1 - 20.5)	**PCT (%)**	0.26 (0.14 - 0.46)
MCV ((pg)	55.9 (61.6 - 73.5)	**GLU (mg/dL)**	110 (70-143)
MCH (pg)	20.4 (21.2 - 25.9)	**CREA (mg/dL)**	1.2 (0.5-1.8)
RDW (%)	28.1 (13.6 - 21.7)	**BUN (mg/dL)**	14 (7-27)
RETIC (K/µL)	77.3 (10 - 110)	**TP (g/dL)**	7.5 (5.2-8.2)
WBC (K/µL)	51.75 (5.05 - 16.76)	**ALB (g/dL)**	3.1 (2.2-3.9)
NEU (K/µL)	40.66 (2.95 - 11.64)	**GLOB (g/dL)**	3.5 (2.5-4.5)
LYMP (K/µL)	8.26 (1.05 - 5.10)	**ALT (U/L)**	45 (10-125)
MONO (K/µL)	2.75 (0.16 - 1.12)	**AST (U/L)**	22 (5-55)

(RBC:Red blood cell, HCT:Hematocrit, HGB:Hemoglobin, MCV:Mean corpuscular volume, MCH:Mean corpuscular hemoglobin, RDW: Red cell distribution width, RETIC:Reticulocyte, WBC:White blood cell, NEU:Neutrophil, LYMP: Lymphocyte, MONO:Monocyte, PLT:Platelet, MPV:Mean platelet volume, PCT:Plateletcrit, GLU:Glucose, CREA:Creatinine, BUN: Blood urea nitrogen, TP:Total protein, ALB:Albumin, GLOB: Globulin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase).

As a treatment, vaginal mass was totally extirpated by partial vaginectomy and subsequently ovariohysterectomy were performed to avoid the re-occurrence of the vaginal tumor. Also, quarterly routine check-up was recommended for the follow-up of possible distant metastases. Anesthesia was maintained as described by Çoban et al. (2021). An absorbable suture material was used for all sutures (Monocryl, No: 2/0, Medeks, Turkey).

Both the extirpated vaginal mass and the genital organs removed by ovariohysterectomy were examined histopathologically. The tissue samples were fixed in 10% neutral buffered formalin. After routinely processed they were embedded in parafin. Sections cut at 4 µm in thickness were stained with hematoxylin and eosin (H&E) stain to be evaluated by light microscopy. Grossly both ovaries were multilobulated and distended (Figure 1A). The tumor sizes were measured in the vagina 2 cm, 1 cm and 0.5 cm (Figure 1B).

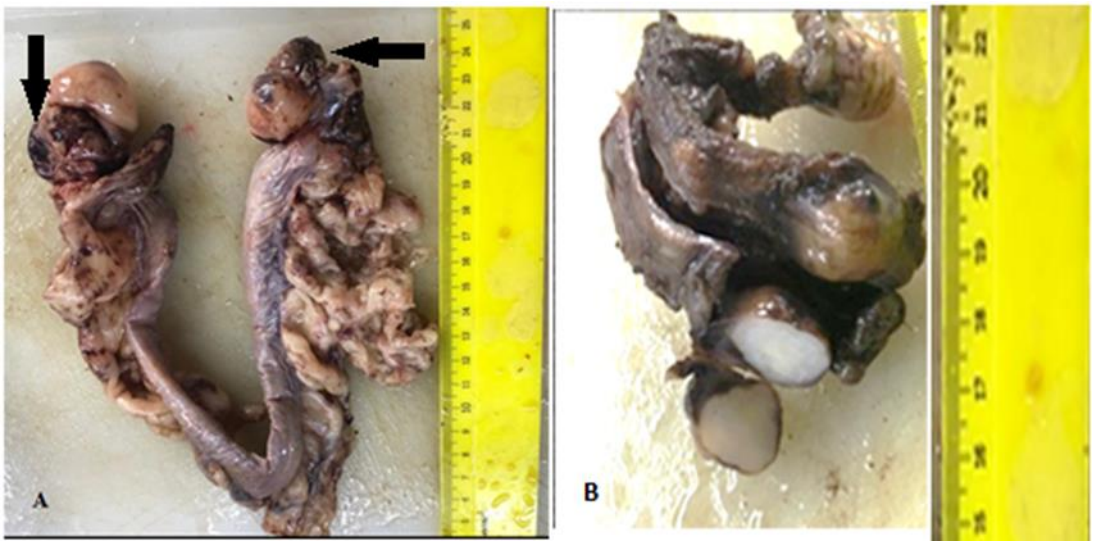

Figure 1.A. Macroscopic image of ovariohysterectomy material. Tumoral tissue in both ovaries (black arrows). B. Macroscopic image of multilobular vaginal mass. The masses were firm, solid and white in section.

Histological examination of both ovaries and the vaginal mass were revealed luteoma and leiomyoma, respectively. The luteoma was separated from surrounded tissue with thin fibrous capsula (Fig 2A). It composed of polyhedral tumoral cells with abundant, finely vacuolated cytoplasm that contain lipid droplets separated by thin fibrovascular stroma (Fig 2B). The nuclei were centrally located, small and oval. Mild to moderate anisocytosis and anisokaryosis were noted. Normal ovarian tissue with follicles in different stages of development was also observed. Vaginal mass was capsulated and non-invasive (Fig 2C). Densely packed spindle cells with eosinophilic cytoplasm and elongated nuclei (Fig 2D).

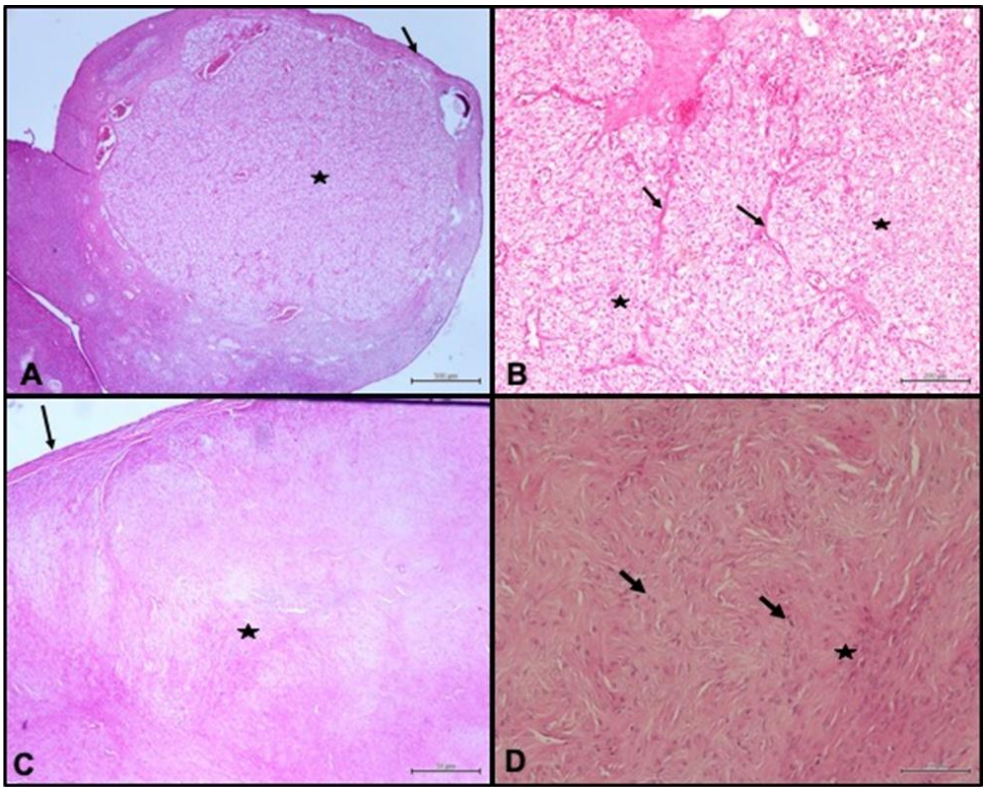

Figure 2. H&E. A. Tumoral mass (star) enclosed by fibrous capsula (arrow), Bar=500µm. B. Polyhedral cells with vacuolated cytoplasm and round nuclei (star), seperated by thin fibrovascular stroma (arrow), Bar=200µm. C. Microphotograph showing leiomyoma (star) with the overlying fibrous capsule (arrow), Bar=500µm. D. Well differentiated smooth muscle cells (arrow) arranged in streams or interlacing bundles (star), Bar=100µm

DISCUSSION

Benign neoplasms were determined in a 7 years old female Golden Retriever in this case. Vaginal leiomyoma and ovarian luteoma in bitches was reported in older ages (Ichimura et al., 2010; Salomon et al., 2004; Singh et al., 2014; Yılmaz et al., 2017). In contrast with the previous reports, the bitch in this case was in middle age. Yamini et al. (1997) diagnosed an ovarian luteoma associated with hyperadrenocorticism in a 6.5 years old Rottweiler bitch. However, there wasn't an additional systemic disease in this case, the age of the affected bitch was similar with Yamini et al. (1997) reported. Although mild anemia, severe leukocytosis and high MPV were determined, some biochemical parameters were within reference ranges in this case. Guner et al. (2020) reported blood parameters were within the reference range except the hemoglobin concentration in a bitch with vaginal lipoma. While the vaginal neoplasm in this case was benign, as reported by Guner et al. (2020), the total blood count results in the current case were not within the reference range contradictly with the researchers (Guner et al., 2020). Erythrocytosis, anemia, neutrophilic leukocytosis, and thrombocytosis are defined as the paraneoplastic syndrome that occurs in neoplastic diseases (Aydın et al., 2011). Because multi-neoplastic cases were diagnosed, mild anemia, severe leukocytosis and high MPV were observed in this case similar with the previous report as a paraneoplastic syndrome.

As a treatment of the vaginal neoplasm in this case partial vaginectomy was performed and ovariohysterectomy applied to avoid the recurrence of the disease. The researchers reported that ovariohysterectomy and antiprogestin treatment provided regression of vaginal leiomyoma (Ferré-Dolcet et al., 2020; Sathya et al., 2014). Besides, ovariohysterectomy is recommended to avoid the recurrence of the vaginal tumors (MacLachlan and Kennedy, 2002). In contrast with the researchers (Ferré-Dolcet et al., 2020; Sathya et al., 2014), partial vaginectomy was performed as a treatment of the vaginal leiomyoma but in line with MacLachlan and Kennedy (2002), ovariohysterectomy was performed to avoid the recurrence of this neoplasm.

The pro-estrous bleeding went undetected for over a year in the present case. Also, the affected ovaries were multilobulated and distended. Distant metastases caused by the gynecological neoplasms were not observed in the present case. Ovarian luteoma usually appear unilaterally (Ichimura et al., 2010; Yamini et al., 1997; Yılmaz et al., 2017). In contrast with the previous reports, both ovaries were affected with luteoma in this case. Although canine ovarian tumors with epithelial origin are usually bilateral, to the best of our knowledge, no literature data has been found about the formation of bilateral ovarian luteoma in dogs. Luteoma is a term used for tumors composed of luteinized cells resembling the corpus luteum. Histologically, they originate from granulosa cells and consist of luteal cells. The tumor, which is observed quite rarely, grows limitedly and does not metastasize (Moris and Dobson, 2001). Similarly, ovarian luteoma was not in huge sizes and non-metastatic in this case. Moderate virilization (masculine behavior) may be seen in the animal, depending on the tumor (MacLachlan et al., 2002; McEntee, 2002). Prolonged anestrous and undetected proestrus bleeding in this case is thought to be due to the moderate virilization as the previously reported.

In this case, it is emphasized that ovarian luteoma, which is a rare tumor in dogs, can progress together with vaginal leiomyoma. It was concluded that histopathological analysis of every surgically removed tissue should be performed in order not to miss a tumorous

condition even if it does not show any clinical symptoms. In cases with multiple gynecological pathologies, it should be recommended to investigate tumor biomarkers or blood hormone levels in order to decide on alternative treatment methods in addition to surgical treatment.

ACKNOWLEDGMENT

Ethical approval: Permission was obtained from the patient owner on 29.11.2021 with a "treatment and information consent form"

Conflict of interest: The authors declared that there is no conflict of interest.

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