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# Morphometric analysis of long bones (Femur and Tibia) of hamdani crossbred sheep fetuses (*Ovis aries*) in the second and third trimester: 3D model

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## ABSTRACT

**Objectives:** In our study, it was aimed to obtain 3D models of femur and tibia bones using CT images in Hamdani crossbred sheep fetuses (second and third trimester) and to determine the developmental differences between sexes.

**Materials-Methods:** Fetuses of pregnant sheep slaughtered in Siirt province slaughterhouse were used. The gestational days of the fetuses were estimated using a previously determined formula. A total of 32 Hamdani crossbred sheep fetuses, 16 second trimester (8 females and 8 males) and 16 third trimester (8 females and 8 males) were used in the study. CT imaged the hind leg bones of the fetuses and the images were modeled in 3D Slicer software. Nine measurements were taken from the models. The measurements were evaluated statistically.

**Results:** In the second trimester, the FL parameter had a statistically significant difference between males and females ($p<0.01$). FDW parameter also showed a significant difference between males and females in this period ($p<0.05$). In the third trimester, the TPW measurement parameter had a highly significant difference between genders ($p<0.01$), and the ITDD parameter showed a significant difference between genders in the same period ($p<0.05$).

**Conclusions:** Osteometric studies are quite limited because bone development continues in the fetal period. In this sense, it is thought that the results of our research, which is the first 3D modeling study, will be useful in the fields of anatomy, zooarchaeology, taxonomy, obstetrics and gynecology, and surgery.

***Keywords:*** *Femur, Hamdani crossbred sheep, morphometry, tibia, 3D modeling*

## INTRODUCTION

Sheep breeding is an animal production activity with significant advantages in terms of cost and quality in Turkey (Aksoy and Yavuz, 2012). The short reproduction and adaptation periods of sheep, and their ability to utilize low-quality pastures effectively, reduce maintenance and feeding costs compared to cattle breeding (Dağıstan et al., 2008; Semerci and Çelik, 2016; Tamer and Sarıözkan, 2017). This situation provides sheep breeding to enterprises with small scale and low capital. In the eastern and southeastern provinces of Turkey (Hakkari, Van, Siirt, Batman, Bitlis) where different breeds are bred, the Hamdani sheep breed is a preferred breed (Örkiz et al., 1984). Considering the productivity characteristics of the breed, Hamdani crossbred sheep are preferred by the local people in Siirt province. Similar to general sheep breeds, the average gestation period in Hamdani crossbred ewes is 150 days (5 months), and growth and development are highest in the last 5 months of

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pregnancy (Harmeyer and Schlumbohm, 2006; Turgut et al., 2024).

Computed tomography is an imaging system in which cross-sectional images are obtained by rotating the detector and X-ray tube around the object (Adapınar, 2016). It is known that computed tomography images are converted into three-dimensional (3D) models through different programs. After the industry, medicine is also an area where 3D models are used (D'Urso et al., 1999). The accuracy and reliability of linear and angular measurements on 3D models have been clearly defined (Kim et al., 2012; Savio et al., 2016; Stull et al., 2014). There are studies in which morphometric characters of long bones were determined in sheep breeds that show great polymorphism within themselves (Alpak et al., 2009; Salami et al., 2011). In addition to these studies in adult animals, studies to be carried out in the fetal period are very important developmentally.

Most of the considered parameters such as shape, height, length, and size are easily accessible in the skeleton. This makes the skeletal system one of the body structures used in the characterization of both human and animal species (Watson, 1972; Guintard and Lallemand, 2003). Long bone structures, one of the parts of this skeletal system, can be used to determine interspecific differences and are also considered important in determining intraspecific differences (Rowley-Conwy, 1998). In previous studies, osteometric measurements were made on different bones and it was reported that the differences observed between the sexes would help determine gender (İşbilir and Güzel, 2023; Güzel and İşbilir 2024). In addition to the determination of sex characteristics, osteometric studies were carried out on different bones in different animal species, which will be useful for clinical sciences and zoo-archaeology (Dalga, 2021; Özüdoğru et al., 2023; Akçasız et al., 2024). For this reason, the osteometric properties of bones are of interest to zooarchaeologists.

In our study, we aimed to obtain 3D models of femur and tibia bones in Hamdani, crossbred sheep fetuses (second and third trimester) using CT images. The morphometric data obtained from these models will provide information about the developmental differences and will also be effective for the realization of statistical differences between sexes.

## MATERIALS and METHODS

A total of 32 Hamdani crossbred sheep fetuses, 16 second trimester (8 females and 8 males) and 16 third trimester (8 females and 8 males) were used in the study. The fetuses were obtained from pregnant ewes slaughtered in the Siirt province slaughterhouse. The fetuses were removed from the uterus. The fetuses with a single pregnancy were included in the study, while the fetuses with twin pregnancies were excluded from the study. Fetal weights were determined with a scale. The gestation period was determined using the formula X=2.1(Y+17) (X=gestation period in days, Y=crown-anus length) (Noakes et al., 2001; Singh et al., 2023). Two separate groups were formed in the second trimester and third trimester. The gestation days of the fetuses used in the second trimester were estimated as 80-96 days and 105-125 days in the third trimester. No deformation was observed in the fetal bones. The bones were scanned by computed tomography. Using a 64-detector MDCT (General Electric Revolution) device, 80 kV, 200 MA, 639 mGY scan dose and protocol, 0.625 mm slice thickness was scanned. The images obtained were saved in DICOM format. Reconstructions were performed using 3D Slicer (5.0.2) software. Measurements from the obtained models were performed with reference to Bakici et al. (2021).

Second-trimester and third-trimester femur and tibia 3D model images and morphometric measurement points are presented in Figure 1 and 2.

**The measurements taken were as follows:**

Internal femoral diaphysis diameter (IIFD): Transverse diameter of the medullary cavity at the middle of the femur

Femoral proximal width (FPW): The maximum distance between the femoral head to the greater trochanter

Femoral head diameter (FHD): The maximum diameter at the middle of the femoral head

Femoral distal width (FDW): The maximum distance across the femoral condyles

Femoral length (FL): The maximum length of the femur

Internal tibial diaphysis diameter (ITDD): Transverse diameter of the medullary cavity at the middle of the tibia

Tibial proximal width (TPW): The maximum distance at the proximal articular surface

Tibial length (TL): The maximum length of the tibia

The results of morphometric measurements were statistically analyzed by SPSS 22.0 software. Independent samples t-test was used for gender comparison in the fetuses in the same period.

This study was approved by the Siirt University Animal Experiments Local Ethics Committee (2024/06/38).

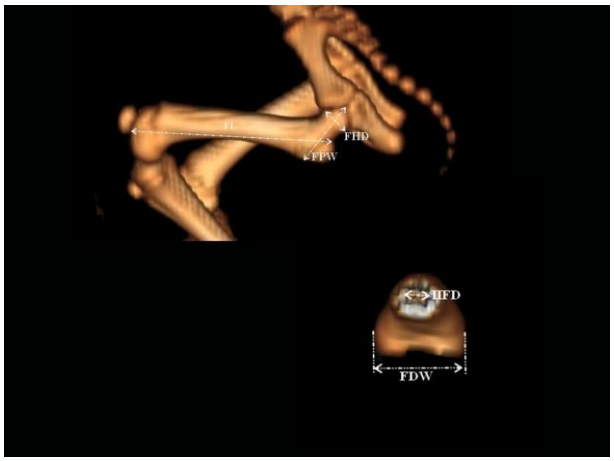


**Figure 1.** Osteometric measurement points of the femur in the sheep fetus.

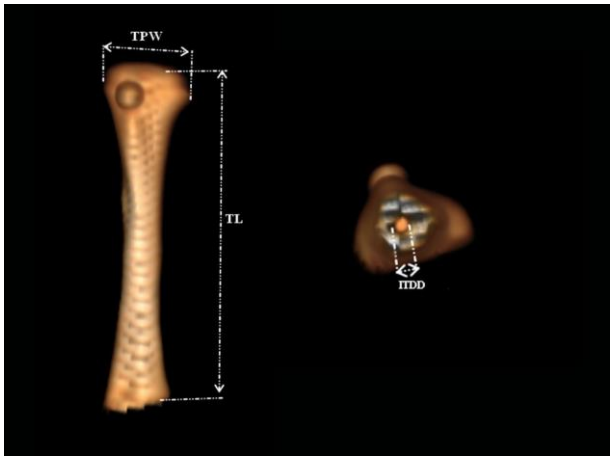


**Figure 2.** Osteometric measurement points of the tibia in the sheep fetus.

## RESULTS

The first data obtained from the fetuses was the weight parameter. This value was determined as 284.42±276.77 g in second-trimester female fetuses and 312.30±270.52 g in males; 2255.71±549.44 g in third-trimester female fetuses and 2372.12±552.03 g in males. Osteometric measurement data were evaluated separately in two trimesters. Mean, standard deviation, and p value results of the measurement data according to gender are presented in Tables 1 and 2.

**Table 1**. Second trimester fetal femur and tibia osteometric measurement results (mm)

| | Gender | n | Mean | Standard Deviation | P |
|---|---|---|---|---|---|
| IIFD | Male | 8 | 0.719 | 0.580 | NS |
| | Female | 8 | 0.611 | 0.640 | |
| FHD | Male | 8 | 1.180 | 0.032 | NS |
| | Female | 8 | 1.085 | 0.041 | |
| FPW | Male | 8 | 1.896 | 0.052 | NS |
| | Female | 8 | 1.579 | 0.075 | |
| FDW | Male | 8 | 2.219 | 0.101 | * |
| | Female | 8 | 2.082 | 0.034 | |
| FL | Male | 8 | 7.942 | 0.061 | ** |
| | Female | 8 | 7.744 | 0.177 | |
| TL | Male | 8 | 8.840 | 0.168 | NS |
| | Female | 8 | 8.281 | 0.123 | |
| TPW | Male | 8 | 1.921 | 0.071 | NS |
| | Female | 8 | 1.769 | 0.060 | |
| ITDD | Male | 8 | 0.357 | 0.034 | NS |
| | Female | 8 | 0.354 | 0.038 | |

NS: no significant; *: p<0.05; **: p<0.01

**Table 2**. Third trimester fetal femur and tibia osteometric measurement results (mm)

| | Gender | n | Mean | Standard Deviation | P |
|---|---|---|---|---|---|
| IIFD | Male | 8 | 0.863 | 0.331 | NS |
| | Female | 8 | 0.796 | 0.442 | |
| FHD | Male | 8 | 1.452 | 0.097 | NS |
| | Female | 8 | 1.267 | 0.049 | |
| FPW | Male | 8 | 2.339 | 0.128 | NS |
| | Female | 8 | 2.164 | 0.038 | |
| FDW | Male | 8 | 2.444 | 0.076 | NS |
| | Female | 8 | 2.312 | 0.106 | |
| FL | Male | 8 | 8.454 | 0.169 | NS |
| | Female | 8 | 8.292 | 0.074 | |
| TL | Male | 8 | 9.270 | 0.126 | NS |
| | Female | 8 | 9.184 | 0.096 | |
| TPW | Male | 8 | 2.419 | 0.056 | ** |
| | Female | 8 | 2.222 | 0.101 | |
| ITDD | Male | 8 | 0.482 | 0.061 | * |
| | Female | 8 | 0.440 | 0.034 | |

In Table 2, it was observed that males had higher values than females in terms of femur and tibia bone lengths (TL and FL) in the third trimester, but the difference was not statistically significant. In the second trimester, the FL parameter had a

statistically significant difference between males and females ($p<0.01$). FDW parameter also showed a significant difference between males and females in this period ($p<0.05$). In the third trimester, the TPW measurement parameter had a highly significant difference between genders ($p<0.01$), and the ITDD parameter showed a significant difference between genders in the same period ($p<0.05$). In the second and third trimesters, all of the statistically different values were found to be higher in male fetuses than in female fetuses.

## DISCUSSION

Sheep and goats have become standard models for investigating biological responses during healing, understanding the mechanical conditions following injury, and investigating surgical treatments such as meniscal healing, ligament reconstruction, and cartilage repair (Bosch and Kasperczyk, 1992; Guisasola et al., 2002; Weiler et al., 2002; Field et al., 2003; Mora et al., 2003; Evans et al., 2004; von Rechenberg et al., 2004). For this purpose, there are studies in which long bone structures were examined in different animal species and with different techniques (Alpak et al., 2009; Kartal and Alpak, 2022; Özkadif and Halıgür, 2022; Marouf et al., 2024). However, no study was found in the literature on the fetal period.

In adult ewes, the FL parameter was analyzed by 3 different methods and reported as 204 mm (Marouf et al., 2024). The same value was reported as 188.15±4.720 mm in Morkaraman ewes (Alpak et al., 2009). In the measurements made in lambs, it was reported that the FL parameter was 134.5 mm in 1st week, 174.8 mm in 4th week, and 201.2 mm in the 8th week after birth (Eck et al., 2019). In our study, the FL parameter had higher values in male fetuses than in females in both gestational periods. While the FL parameter had a statistical difference between males and females in the second trimester, no statistical difference was determined in the third trimester.

Although distal femur width showed significant differences between genders in studies conducted in rabbits, no statistical differences were reported between laterality (dexter, sinister) and gender (Pazvant and Kahvecioğlu, 2009; Bakici et al., 2021). In addition, no statistical difference was reported between the right and left sides of New Zealand rabbits (Selçuk, 2023). In dogs, the FDW parameter was reported as 361.9±152.0 mm in males and 340.3±141.1 mm in females, while no statistical difference was observed between the sexes (Kartal and Alpak, 2022). In our study, it was determined that the FDW parameter was statistically different between the sexes in the second trimester ($p<0.05$). The distal width of the femur was higher in male fetuses than in female fetuses.

The tibial proximal width (TPW) value was found to be larger in male fetuses than in female fetuses in the third trimester. While this value was reported as 47.03±1.096 mm in the Morkaraman sheep breed (Alpak et al., 2009), 21.49±0.16 mm in Yankasa sheep and 19.10±0.29 mm in Red Sokoto goats, it was mentioned that there was a statistical difference between these two animal species (Salami et al., 2011). In our study, the ITDD parameter showed a statistically significant difference between male and female fetuses in the third trimester ($p<0.05$).

According to the results of the study, statistical differences were observed in the femur in second trimester fetuses and the tibia in third trimester fetuses. In the second trimester of pregnancy, the length and distal width of the femur were greater in male fetuses than in female fetuses. In addition, the proximal width of the tibia and the diameter of the internal tibial diaphysis were larger in male fetuses in the last period of pregnancy.

## CONCLUSION

In conclusion, our study revealed the similarities and differences between the sexes in the morphometric measurements of femur and tibia in second and third trimester fetuses. The study was based on the principle of determining osteometric data by 3D modelling method from CT images. Although there are studies on this subject in adult animals, there are almost no studies in the fetal period. The data of the study will be a pioneer for developmental studies since the osteometric properties of the long bones of the lambs in the fetal period will be revealed. It is thought that the findings will contribute to studies in the field of obstetrics gynaecology and ultrasonography.

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# Investigation of cartilage development in sea bream (*Sparus aurata*) larvae at 22 °C by double staining technique in the first 20 days after hatching

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## ABSTRACT

**Objective:** To investigate the normal cartilage development of the caudal fin system in gilthead sea bream (*Sparus aurata*) larvae under intensive aquaculture conditions was investigated up to age 20 days. This study was carried out because of the increasing incidence of skeletal malformations, especially those related to the columna vertebralis, in bream farming and because the cause and progression of such skeletal malformations are uncertain and there are very few studies on the cartilage-osteological development.

**Materials and Methods:** Approximately 400 *Sparus aurata* larvae were used to determine the development. The materials were stained with Alcian Blue for cartilage staining and Alizarin Red for bone staining. 300 mg alcian blue and 100 ml 70% ethanol solution were mixed with 100 mg alizarin red and 100 mg 95% ethanol solution. A solution was prepared by adding 100 ml of glacial acetic acid and 1700 ml of 70% ethanol to this mixture. The larvae were kept in this solution in the oven at the appropriate temperature for 4 days and washed in running water for 2 hours after being removed from the oven. The prepared specimens were examined under stereo microscope.

**Results:** It was observed that the cartilage formation in the caudal fin of the larvae started at 13 days of age. In 13-day-old larvae, hypural 1 (ventral extensions of the vertebrae) was measured as 0.035 mm on average. At 20-day old larvae, hypural 1, hypural 2, hypural 3, hypural 4, hp parhypural and hs (heamal spine) were detected and measured. Hypural 1 and hypural 4 were found to be quite thickened. no ossification was observed in 20-day-old material. In this study, the development up to 20 days was analysed since cartilage development was especially considered.

**Conclusion:** The results of skeletal development in *Sparus aurata* larvae showed similarities when compared with the findings in other fish species. Additionally, cartilage anomalies were observed at a rate of 13%. The observed anomalies were detected as tail curl.

***Keywords:*** *Larval rearing, Cartilaginous development, Caudal fin, Sparus aurata*

## INTRODUCTION

No osteological structure was detected on the spine of bream larvae at hatching. In the study, cartilage formation in the caudal fin was not observed in 1-12 days old bream larvae. The first structure formed in the caudal tail on the spine was Hy1 in the ventral region with an average length of 0.035 mm in 13-day-old larvae (Figure 2). This marks the beginning of cartilage development in the caudal fin of the larvae. During the 14–20-day period, hy 1, hy2, hy3, hy4, hp and hs structures gradually developed and increased in size. While the lengths increased, transverse enlargement and thickening were

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observed on the 16th and 20th days depending on the days. hy2 was observed for the first time in 15-day-old larvae (Figure 3). Thickening was observed to be more effective on hyl and hy4. In 16-day-old larvae, hy3 was observed for the first time, while hyl and hy2 continued their development. It was found that hy 1, which was larger than the others from the 16th day onwards, continued to grow transversely from the 18th day onwards, and the other protrusions took the shape of a rectangle rather than a transverse rod. In 17 days, old sea bream larvae (Figure 4), no new cartilage was observed, growth continued. On the 18th day, hs cartilage was detected. On the 19th day (Figure 5), hyl, hy2, hy3, hy4, hy4, hy5, prh and hs were observed. On the 20th day (Figure 6), the development continued. These structures increased with the increase in the age of the fish, the increase in number was completed and the increase in length and width continued. On the 20th day, it was determined that the caudo ventral end of hy4 expanded more and resembled a triangular shape.

In all periods of development of the caudal fin complex, especially in cartilage formation, no changes were observed between parhypural, hypural 1, 2, 3 and 4 in the form of cartilage fusion and then separation.

In study, fifty-two larvae with abnormal tails were observed. Cartilage Anomalies were observed with a rate of 13%. These anomalies were observed as tail curling. Figure 7 shows the tail anomaly pictures of the 13th, 15th, and 16th days.

## MATERIALS and METHODS

This study was carried out with sea bream larvae obtained dead from the hatchery of a private aquaculture facility in Muğla and reared at 22°C.

### *Larval Production and Investigation Group Organization*

In larval production, black coloured polyester tanks were preferred and each tank had a volume of 1 $m^3$. When incubation was completed, 80 prelarvae/litre were stocked in the tanks. After 3 days of prelarval stage in dark environment, larvae passed to postlarval stage on the 4th day with mouth opening. In the tanks where they were kept, water ph:7.25 and temperature values varied between 20-24°C on average. Larvae of known age were used by the producer.

In the study, an average of 20 dead larvae were sampled from day 1 to day 20 from a fixed tank containing sea bream larvae. The samples were preserved in 10% formaldehyde.

### *Cartilage Structure Analyses*

Alizarin red and Alcian blue staining method was applied for the developmental analysis of caudal fin cartilage structure of *Sparus aurata* larvae. *Sparus aurata* larvae stored in 10% formol were washed with distilled water and placed in containers containing 95% ethanol. 300 mg alcian blue and 100 ml 70% ethanol solution and 100 mg alizarin red and 100 mg 95% ethanol solution were mixed. A solution was prepared by adding 100 ml of glacial acetic acid and 1700 ml of 70% ethanol to this mixture. The larvae were kept in this solution in the oven at the appropriate temperature for 4 days and washed in running water for 2 hours after being removed from the oven. The prepared specimens were examined under stereo microscope.

The procedures applied in this study were approved by Balıkesir University Experimental Animals Application and Research Center with the ethics committee report numbered 2024/6 at the meeting dated 04.07.2024.

## RESULTS

Dead fish samples collected from the same tanks on different days were preserved under appropriate conditions and after staining. Hypural (hy 1, hy2, hy3 and hy4), paryhypural (prh) and heamal spine (hs) cartilage development in caudal fin were examined under stereo microscope (Figure 2, 3). *Sparus aurata* larvae were examined from the first day (Figure 1) until the twentieth day by taking 20 different samples from each. No bone development was observed in any fish during the twenty-day period, but cartilage development was measured. No cartilage formation was observed in the caudal fin of the larvae analysed from one day to twelve days. It was observed that cartilage formation in the caudal fin of the larvae started at thirteen days (Figure 2).

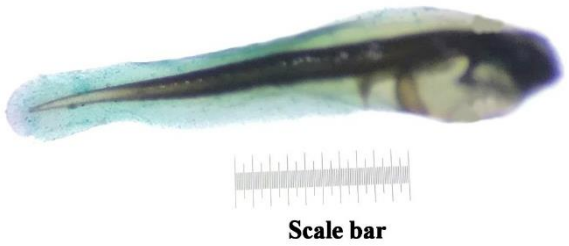


**Figure 1**. One-day-old sea bream larva. (Scale bar 1 mm).

**Table 1.** Hs, PrH (Parhipural), Hy1, Hy2, Hy3 and Hy4 measurements in *Sparus aurata* larvae.

| | HS | PrH, Parhipural, mm | Hy1, mm | Hy2, mm | Hy3, mm | Hy4, mm |
|---|---|---|---|---|---|---|
| Day 13 | - | - | 0.03 | - | - | - |
| Day 14 | - | - | 0.055 | - | - | - |
| Day 15 | - | 0.02 (only in 2 items) | 0.11 | 0.07 | - | - |
| Day 16 | - | 0.06 | 0.125 | 0.09 | 0.035 | - |
| Day 17 | - | 0.075 | 0.14 | 0.11 | 0.08 | - |
| Day 18 | - | 0.15 | 0.21 | 0.15 | 0.14 | 0.09 |
| Day 19 | 0.14-0.11 | 0.21 | 0.24 | 0.17 | 0.15 | 0.12 |
| Day 20 | 0.23 | 0.3 | 0.28 | 0.19 | 0.17 | 0.15 |

Development measurements of caudal fin in *Sparus aurata* larvae are described in Table 1.

No osteological structure was detected on the spine of bream larvae at hatching. In the study, cartilage formation in the caudal fin was not observed in 1-12 days old bream larvae. The first structure formed in the caudal tail on the spine was Hy1 in the ventral region with an average length of 0.035 mm in 13-day-old larvae (Figure 2).

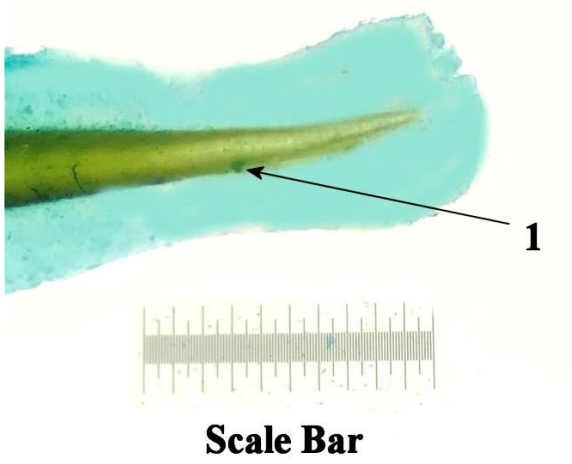


**Figure 2.** 13-day-old sea bream larva. 1- Hy1 (Scale bar 1 mm).

This marks the beginning of cartilage development in the caudal fin of the larvae. During the 14–20-day period, hy 1, hy2, hy3, hy4, hp and hs structures gradually developed and increased in size. While the lengths increased, transverse enlargement and thickening were observed on the 16th and 20th days depending on the days. hy2 was observed for the first time in 15-day-old larvae (Figure 3).

Thickening was observed to be more effective on hyl and hy4. In 16-day-old larvae, hy3 was observed for the first time, while hyl and hy2 continued their development. It was found that hy 1, which was larger than the others from the 16th day onwards, continued to grow transversely from the 18th day onwards, and the other protrusions took the shape of a rectangle rather than a transverse rod. In 17 days, old sea bream larvae (Figure 4), no new cartilage was observed, growth continued.

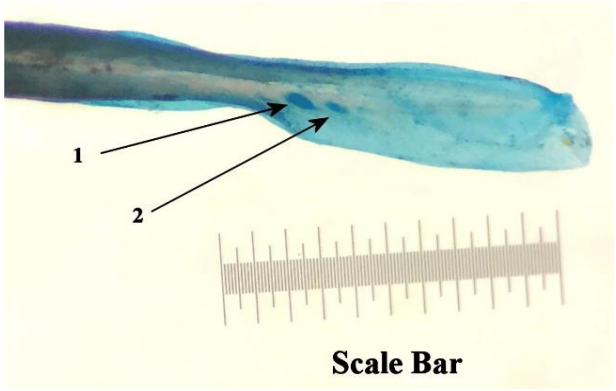


**Figure 3.** 15-day-old sea bream larva. 1- Hy1, 2- Hy2 (Scale bar 1 mm).

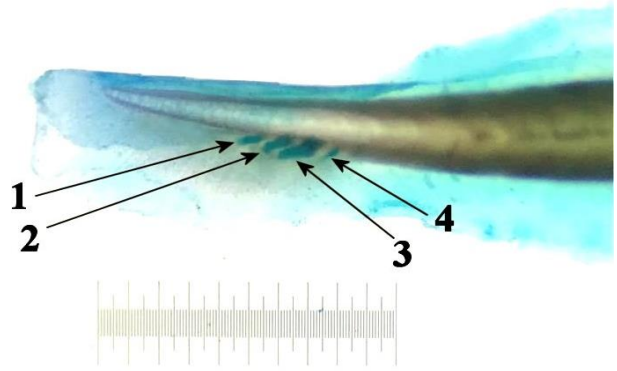


**Figure 4**. 17-day-old sea bream larva. 1- Hy 3, 2- Hy 2, 3- Hy 1, 4-Prh (Scale bar 1 mm).

On the 18th day, hs cartilage was detected. On the 19th day (Figure 5), hyl, hy2, hy3, hy4, hy4, hy5, prh and hs were observed. On the 20th day (Figure 6), the development continued. These structures increased with the increase in the age of the fish, the

increase in number was completed and the increase in length and width continued. On the 20th day, it was determined that the caudo ventral end of hy4 expanded more and resembled a triangular shape.

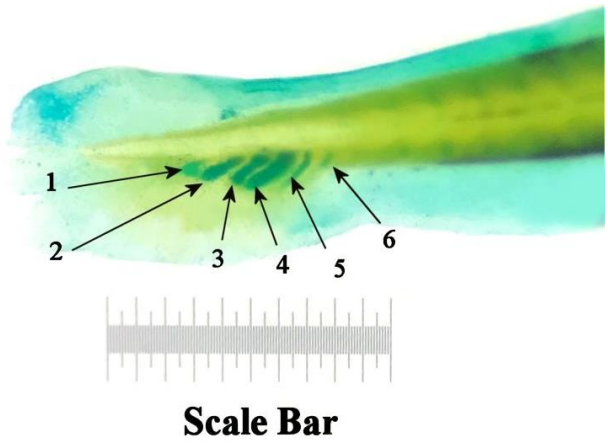


**Figure 5**. 19-day-old sea bream larva, 1-Hy4, 2-Hy3, 3-Hy2, 4-Hy1, 5- Prh,6- Hs (Scale bar 1 mm).

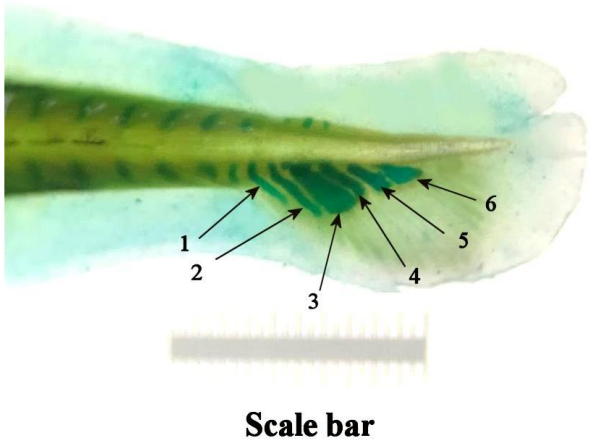


**Figure 6.** 20-day-old sea bream larva, 1-Hs, 2-PrH, 3-Hy1, 4-Hy2, 5-Hy3, 6-Hy4 (Scale bar 1 mm).

**Figure 7**. Examples of tail anomalies on the 13th, 15th and 16th days (Scale bar 1 mm).

In all periods of development of the caudal fin complex, especially in cartilage formation, no changes were observed between parhypural, hypural 1, 2, 3 and 4 in the form of cartilage fusion and then separation.

In study, fifty-two larvae with abnormal tails were observed. Cartilage Anomalies were observed with a rate of 13%. These anomalies were observed as tail curling. Figure 7 shows the tail anomaly pictures of the 13th, 15th, and 16th days.

## DISCUSSION

This study is one of the rare studies in which cartilage development in cultured larvae of *Sparus aurata* was stained with alizarin red and alcian blue and real pictures were given periodically as development.

Normal and abnormal cartilage and osteological development of the caudal fin in *Sparus aurata* larvae is important in fish farming. Because this level of development affects the morphology, growth and survival of the fish and changes the quality of the fish produced

Normal and abnormal cartilage and osteological development of the caudal fin in *Sparus aurata* larvae is important in fish farming. Because this level of development affects the morphology, growth and survival of the fish and changes the quality of the fish produced (Koumoundouros et al., 1997). These developmental disorders are mostly caused by larval and juvenile stages as a result of abiotic conditions. Examples of abiotic factors in aquaculture include water, sunlight, tides, pH, temperature, minerals, volcanic eruptions and storms. (Bolla and Holmeord, 1988; Wiegand et al., 1989).

Early in the development of the caudal fin complex, no cartilaginous fusion between the parhypural, hypural 1 and 2 has been reported to be observed, which is consistent with our study (Faustino and Power, 1998). Later in development, when the larva is about 5.1 mm in size, hypural 1 and 2 have been reported to be joined proximally by a temporary cartilaginous bridge and then fused with the parhypural at about 7.1 mm in size (Faustino and Power, 1998). These fusions were not seen in our study. Since only cartilage development up to day 20 was found in our study, it was predicted that such fusion might occur at later periods. It was reported by the same author that when ossification was completed, all hypurals were once again separated from each other and from the parhypural. This kind of separation was not observed in our study.

It has been reported that some cartilaginous centres in the caudal fin were reduced in size or triangular in shape and the associated arches and spines were observed as double, twisted, fused or broken without any specific abnormality, and an extra hypoplasmic fin was found on the dorsal part (Koumoundouros et al, 1997). In our study, such a double, fused or broken cartilaginous formation was not observed. Only a small proportion showed a bent tail as shown in the picture (Figure 7). It was reported to be caused by lateral bending of the plate formed by deformed and fused Hy elements. However, despite these anomalies, it was reported that the swimming behaviour of the specimens did not change, only the cartilage development was different (Koumoundouros et al., 1997). In our study, such bending was detected.

Barahona-Femandes (1982) reported that abnormalities occurring in *Dicentriochus labrax* in the first 40 days after hatching are mostly lethal, but those occurring after 40 days (post larval stage) are not lethal.

In *Sparus auruta*, Papema (1978) described a curvature of the caudal peduncle. Malformations of the caudal skeleton have also been reported in other species such as Pagrus major and *Dicentrzothus labrax* under rearing conditions (Matsuoka, 1985; Daoulas et al., 1991; Boglione et al., 1993; Marino et al., 1993). In cultured specimens of *Dicentrurchus labrax*, a higher rate (34%) of bone abnormality was observed in the caudal fin (Marino et al., 1993). In our study, cartilage anomalies were observed at a rate of 13%. The observed anomalies were detected as tail curling. In previous studies (Matsuoka, 1985; Daoulas et al., 1991; Boglione et al., 1993; Marino et al., 1993), tail anomalies were also observed as curling. However, the rate in our study is quite low. It is thought that this low rate is not related to the rearing of larvae at 22°C.

Determination of growth stages in terms of cartilage development is important in terms of species breeding and fisheries biology (Koumoundouros et al., 1999). However, knowing the normal development of the species is very important for the successful production of that species (Çoban et al., 2009). Especially in commercial production enterprises, deformations cause economic losses up to 100% and 60% of these deformations occur on the spine (Koumoundouros et al., 1997).

## CONCLUSION

Cartilage and osteological development studies are of great importance in preventing deformations under aquaculture conditions, eliminating them in advance and removing them from the system. In future studies, the genetic and environmental effects on the development of bone and cartilage structures should be studied.

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# Investigation of the presence of antibodies to SARS-CoV-2 in cats owned by people with COVID-19

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## ABSTRACT

**Objective:** Coronaviruses are viral agents with zoonotic potential that can cause disease in many animal species. Recently a novel coronavirus agent excited pandemia in humans. It was named SARS-COV-2. In addition to causing pandemia, it was reported that the agent has been detected in pets. Although it has reduced, its effectiveness since the pandemic, it continues to exist without losing its importance due to its potential. In this study, we aimed to research the presence of SARS CoV 2 Ig G specific antibodies in domestic cats whose owners were diagnosed with COVID 19.

**Materials and Methods:** SARS-COV-2 specific Ig G antibodies were searched by receptor-binding domain-ELISA (RBD ELISA) method in 88 domestic cats whose owners were diagnosed at most 3 months ago and approved by The Ministry of Health of the Republic of Türkiye and have positive COVID-19 results.

**Results:** As a result of the study, the seroprevalence of COVID-19 in cats was 2.27%. In the literature reviews, it was seen that there were a limited number of studies in our country.

**Conclusion:** This study is the first article conducted in Central Anatolia. The results show that cats can carry the potential for anthropozoonotic potential.

***Keywords:*** *COVID-19, Cat, ELISA, Seroprevalence, Antibody*

## INTRODUCTION

In 2019, a novel coronavirus agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was discovered in human in China (Wu et al., 2020). This originally zoonotic virus caused a pandemic all over the world and it was named SARS-CoV 2 and sparked off COVID 19 pandemic (Zhou et al., 2020). The novel coronavirus disease (SARS-CoV-2, COVID-19) induced over 4.5 million death and over 220 million cases in humans until September 2021 and SARS-CoV-2 has cost social and economic impacts global extensity (Murphy and Ly, 2021). SARS-CoV-2 is a member of genus Betocoronavirus and has single stranded RNA, as an enveloped virus (Yılmaz et al., 2021). SARS-CoV-2 is closely in sight with severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle Eastern respiratory syndrome virus (MERS-CoV) (Murphy and Ly, 2021). Between 2002-2013 years the zoonotic severe acute respiratory syndrome-CoV (SARS-CoV) and the Middle East respiratory syndrome-CoV (MERS-CoV) caused significant outbreaks in humans and they are broadly determined (Bennett et al., 2019). SARS CoV 2, SARS CoV and MERS CoV are members of genus Betocoronavirus and also SARS CoV 2 has amino acid sequences at the rate of %80 like SARS CoV that caused the 2003 SARS

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outbreak with almost %10 fatality rate. Although SARS-Cov 2 has the same nucleotide level in proportion to %96.2 with the coronavirus RaTG13-which was identified in Yunnan province, China, in 2013 at horseshoe bats (Zhou et al., 2020). SARS-CoV and MERS-CoV are thought to derive from bats and transmit through intermediate hosts, such as camels and civet cats to humans (Wan et al., 2020). For coronaviruses are thought to spread to humans through animal hosts but for SARS CoV 2 the potential intermediate host(s) are still unknown (Ye et al., 2020).

Coronaviruses (CoVs) are grouped in four different genera according to their genetic characters and evolutionary structures (Masters and Pearlman, 2013). Genera diversity is grouped under the headings of alpha, beta, gamma and delta (Stout et al., 2020). Coronaviruses have enveloped RNA with single stranded genome that with 4 structural proteins (spike S, envelope E, membrane M, nucleocapsid N), several accessory proteins and 16 non-structural proteins (nsp1-nsp16) are encoded (V'kovski et al., 2020). Coronaviruses can cause disease both animals and humans. In humans, coronavirus can cause variable disease manifestations, ranging from common cold lesions to acute respiratory disease. For animals, SARS-CoV 2 has been detected, especially in ferrets, dromedario camels, cats and dogs, in line with the studies conducted in the last five years. In the clinical findings caused by SARS CoV 2 in the aforementioned animals, are various; as follows animals can show upper and lower respiratory system infection findings and digestive system disorders (Shi and Hu, 2008; Schulz et al., 2021). At the same time SARS can be asymptomatic in animals (Barrs et al., 2020). Both SARS CoV and CoV 2 agents originate from bats. They can use palm civets/racoon dogs and dromedary camels as an intermediate host (Song et al., 2019).

It has been reported that infected people with Coronavirus transmit the agent to their pets (Fritz et al., 2021; Zhao et al., 2021). It is still unclear for animals how they play role as hosts for the SARS CoV 2 virus. The SARS CoV 2 agent can infect animals both naturally and experimentally (Rowe et al., 2004). Detection of the prevalence of the agent in terms of the potential for human-to-animal and animal-to-human transmission has an important place in disease control policies. For this reason, in this study, the presence of SARS CoV 2 IgG specific antibodies among domestic cats, whose owners were diagnosed with COVID 19 and contact with them, was investigated via quantitative ELISA method.

## MATERIALS and METHODS

### Study Population

The study involved cats admitted to Animal Hospital, Veterinary Faculty, Kırıkkale University for clinical examinations. In line with anamnesis, cats of both gender and any breed owned by people had COVID-19 in the last 3 months diagnosed using PCR by the Turkish Ministry of Health were included in the study. All cats were older than 6 months age and were household cats with no access to the outdoors. In total 88 cats met the criteria.

### Sampling

Blood sampled were taken from vena cephalica antebrachiums into 2 ml serum tubes. Blood samples were centrifuged at 5000 rpm/10 min to obtain serum. Serum samples were stored at -20°C until analyses.

### Detection of antibodies with ELISA

We used quantitative ELISA kits to detect Ig G antibodies against S1 antigen (inc. RBD) of SARS-CoV-2 (Anti-SARS-CoV-2 Quantivac ELISA Ig G, Germany) in the Laboratory of Infectious Diseases and Clinical Microbiology of Medical Faculty, Kırıkkale University. The assay was run in accordance with the recommendations of the manufacturer. The RBD-ELISA prove high sensitivity (100%) and specificity (98.23%) with the OD cut off value of 0.493. The test includes a 6-point calibration curve. According to the manufacturer's instructions, high results of relative units per milliliter were considered positive with up to a reader value of 0.337 optic density (OD). OD values above the cutoff value were taken as positive. Also, for the RU unit, results above 10 RU/ml were assessed as positive, and the binding antibody units per milliliter (BAU/ml) level was >3.2 BAU/ml.

### Statistical analyses

Descriptive statistical analyses were made to determine the prevalence of COVID-19 in cats using SPSS Statistics 23.

### Ethical statement

The study was approved by the Animal Experiments Local Ethics Committee of Kırıkkale University (Decision Number: 2021/04/20)

## RESULTS

In total, 88 cats were evaluated with the RBD-ELISA. Two cat had positive results including all criteria (OD, RU/ml, BAU/ml) (Table 1). Positive cats OD levels were over 0.337 (0.389, 0.851 OD value). For RU levels, positive two cats had higher results than 10 RU/ml (11.79, 28.78 RU/ml). Also, these cats' BAU results were above positive criteria levels (>3.2) (37.74, 92.11 BAU/ml). SARS-CoV-2 seroprevalence was 3.41 % in cats in this study (Table 2).

**Table 1.** SARS-CoV-2 Ig G antibodies results with ELISA.

| Sample | O.D. Value[1] | RU/ml Value[2] | BAU/ml Value[3] | Result |
|---|---|---|---|---|
| Cat 10 | 0.389* | 11.79 | 37.74 | Positive |
| Cat 68 | 0.851* | 28.78 | 92.11 | Positive |

*Cause of positivity above cut off value OD (0.337)
[1]Optic Density, [2]Relative Unit, [3]Binding Antibody Unit

**Table 2.** Prevalance of COVID 19 results in cats.

| Sample | Number | Ratio (%) |
|---|---|---|
| Negative | 85 | 96.59 |
| Positive | 2 | 3.41 |
| Total | 88 | 100 |

## DISCUSSION

It has been reported that COVID 19 can infect many animal species. SARS-CoV were experimentally isolated from masked palm civets, monkeys, mice, pigs and chickens, guinea pigs, golden syrian hamsters and ferrets (Hosie et al., 2021; Hossain et al., 2021). SARS-CoV prevalance was high in masked palm civets. Although these animals are consumed by humans, it is determined that animals develop neutralized antibodies (Wang et al., 2005; Sun et al., 2020). Palm civets were identified accidental hosts at temporary period (Kuiken et al., 2003; Wang et al., 2005) and it was thought that they can be occasional direct sources of human infections (Shi and Hu, 2008). Ferrets had quite high prevalance to SARS- CoV-2 after experimental inoculation and they could transmit the virus other ferrets (Shi et al., 2020). Dogs can be infected experimentally but they cannot spread the agents (Schlottau et al., 2020). There are several studies that were reported COVID 19, were detected cats whose owners had positive result with COVID 19 (Hossain et al., 2021). First SARS spread it was stated that natural infection was seen in cats (Rowe et al., 2004; He et al., 2013). In a study conducted in domestic cat in an apartment block where 100 people with SARS-CoV positivity, it was reported the agent was found in eight cats. However, it is stated that there are deficiencies in the direction of this transmission from humans to animals (Martina et al., 2003). In studies that in experimentally and natural infected cats, it was notified that human can be reservoir for animal infections (Hossain et al., 2021). Cat-human transmission can possible theorically but there is not enough evidence to demonstrate this situation (Barrs et al., 2020). In this study 3 cats were positive and the both of them lived same house. In line with this information, we thought that the positive cases in our study contributed to literature in terms of contamination.

Within the scope of One Health, determining the natural and intermediate hosts of COVID 19 plays a key role in identifying the spread potential of the COVID 19 and establishing control policies. It is still unclear where cats are as a host and what role they play in case of transmission (Leroy et al., 2020). Bessière et al. (2021) examined five cats whose owners had COVID 19 positive results. They isolated the viral agent in 2 cats and it was seen that 1 cat had COVID 19 antibody (Bessière et al., 2021). Similarly, Hosie et al. (2021), researched 2 COVID 19 positive cats thought to be transmitted from humans to cats, reported that it is not clear what role cats play in the epidemiology of COVID 19, but they emphasized the need to clarify this point in the one health concept (Hosie et al., 2021). In this study, the presence of COVID 19 antibodies was determined in cats in contact with humans. It is recommended that more comprehensive studies are required, especially in terms of the anthropozoonotic feature of COVID 19, as well as its epidemiological importance.

There are several studies reported that serological surveys of COVID-19 pet populations rates ranging from 0% - 15.8% in cats and dogs (Chen et al., 2020; Deng et al., 2020). In Europe, canine coronavirus prevalence is lower than feline coronavirus (Stavisky et al., 2010). Feline coronavirus prevalence is 50% in healthy Swiss cats and 37% in Japan (Taharaguchi et al., 2012). Barrs et al. (2020) stated that the prevalence of SARS-CoV-2 was 12 % of 50 cats in Hong Kong at in their study (Barrs et al., 2020). Zhang et al. (2020) reported that, 15 of 102 (14.7%) cats were positive about SARS-CoV-2 for positive antibodies that recognized by ELISA (Zhang et al., 2020). During the first COVID-19

wave in Europe, a study was organized on identifying SARS-CoV-2 specific antibodies in domestic cats, and in this study SARS-CoV-2 seroprevalence was found 4,2 % in Germany. Also, the ratio was about COVID-19 announced 3.3% in the UK, 4.2% in Italy and 6.4% in Spain (Schulz et al., 2021). There are different epidemiological studies have been conducted that have taken samples on routine veterinary checks, where the dispersion of COVID-19 was not known at people. A study of 919 companion animals, 5.8% of cats was shown that had neutralizing antibodies (Patterson et al., 2020). Along these lines, in another study demonstrated that only 0.69% of samples contained antibodies at Germany (Michelitsch et al., 2020). Furthermore, in the study which was realized in 20 veterinary community students, 18 of them displayed COVID-19 symptoms and 2 students had positive COVID-19 results, was reported that none of these students' cats and dogs developed antibodies (Wu et al., 2020). In this study we found the prevalence of COVID-19 ratio as 3.41 % in 88 cats. We took to the study only the cats which had had COVID-19 positive results with their owner in the last three months. Our results are close to the data at Europe. This closeness in the results was considered significant in terms of the similarity of our living conditions, cats' similar environmental status, nutritional habits and routine veterinary practices, geographical proximity and the application of similar control policies during the COVID-19 pandemic.

## CONCLUSION

As a result, the transmission from human to animals and animals to humans have not been clarified in the spread and control policies of COVID 19 which still continues to exist. Determining how the cats play a role in transmission remains an important point in controlling COVID 19. Certainly, we believe that studies on the presence of COVID 19 in cats, increasing the data reservoir on the spread and transmission potential of the disease will help identify what role cats play in COVID 19. In addition, we thought that, we contribute veterinarians by answering the question of what kind of path they should follow for controlling COVID 19 both human and animal care by informing patient owners.

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# The efficacy of human recombinant luteinizing hormone for in vitro embryo production in sheep

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## ABSTRACT

**Objective:** This study was carried out to assess the ability of recombinant human luteinizing hormone (r-LH) to be used instead of sheep hypophyseal luteinizing hormone (h-LH) in the maturation of sheep oocytes and its influence on embryonic development and quality.

**Materials and Methods:** The oocytes were obtained from slaughtered sheep ovaries. For oocyte maturation, grade 1 oocytes were incubated at 38.5°C with 5% $CO_2$ for 24 hours. The maturation medium for the hypophyseal LH (h-LH) group was supplemented with 0.1 IU/mL LH derived from sheep pituitary LH (Sigma®), while the medium for the recombinant LH (r-LH) group contained 0.1 IU/mL recombinant LH (Luveris® 75 IU, Serono). In vitro fertilization (20h) and embryo culture were performed at 5% $CO_2$, 5% $O_2$ and 38.5°C incubation conditions. The maturation rates were reported based on the MII stage chromosomal formation and the existence of first polar body by bisBenzimide (Hoechst 33342). Embryonic developments were controlled on the 3rd and 8th day of in vitro culture. For the embryonic cell count and determination of inner cell mass (ICM) and trophectoderm cell (TC), the differential staining technique was used with Hoechst 33342 + Propidium Iodide (PI).

**Results:** The proportion of cleavage (%), the rate of embryos developing the morula (%) and blastocyst stage (%), and the ICM, TC, and total cell numbers of the embryos were found to be statistically similar in the h-LH and r-LH groups ($p>0.05$).

**Conclusion:** It was concluded that r-LH could be used as an alternative LH source instead of hypophyseal LH.

***Keywords:*** *Embryo development, Hypophyseal LH, In vitro maturation, Recombinant human LH, Sheep oocyte*

## INTRODUCTION

Currently, in vitro embryo production procedures are increasingly used in sheep with high breeding values in veterinary practice and biotechnological research (Wani, 2002). So far, researchers have tried various culture systems, different hormone sources and media to increase the in vitro success of embryo production (Zhu et al., 2018). Nevertheless, the success rate of oocytes to mature and reach to blastocyst stage in vitro has been lower than those produced in vivo (Moor and Trounson, 1977; Rizos

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et al., 2002; Birler et al., 2003; Paramio and Izquierda, 2016; Zhu et al., 2018).

In vivo cytoplasmic and nuclear maturation of oocytes are achieved by luteinizing hormone (LH) and follicle stimulating hormone (FSH) as a part of gonadal-pituitary axis and local cellular factors (Wani, 2001). Hormones, serum, and various growth factors added to maturation media increase the rates of mammalian oocytes reaching the blastocyst stage.

In vitro semi-defined culture systems use gonadotropins derived from pituitary or recombinant proteins for oocyte maturation. However, hypophyseal gonadotrophins that participate in the media may cause different contaminants which enter into the culture systems (Vanroose et al., 2001).

There are some problems such as; non-standard hormone activity in every production batch and transmission of undesired hormones (e.g., TSH) (De Koning et al., 1994; Kanitz et al., 2002; Baruselli et al., 2023) or diseases (Hesser et al., 2011), low efficiency and high cost of the method (Rosano and Ceccarelli, 2014; Baruselli et al., 2023).

Gonadotropic hormones obtained from the pituitary gland are widely used in the field of reproductive biotechnology (Demir et al., 2019; Baruselli et al., 2023). There are recent works obtained various results by using different types of recombinant gonadotropins (Törnell et al., 1995; Andresiez et al., 2000; Accardo et al., 2004; Sha et al., 2010; Tharasanit et al., 2014; Gifre et al. 2017; Arıcı et al., 2022).

This study aims to compare the effect of recombinant human luteinizing hormone (r-LH) and hypophyseal luteinizing hormone (h-LH) on ovine oocyte maturation, blastocyst ratios and total cell counts of blastocysts.

## MATERIALS and METHODS

Unless otherwise noted, all of the chemicals used in the current research were purchased from Sigma (St. Louis, MO, USA).

### Oocyte collection

A total of 80 ovaries from adult ewes were obtained from a local abattoir and conveyed to the laboratory within 3 hours in Dulbecco's phosphate buffered saline (DPBS) solution at approximately 35-37°C. After washing with fresh DPBS, ovaries were sliced and the follicle contents were flushed in TCM-199 (M5017) medium with 25 mM HEPES (H6147), 50 IU/ml penicillin (P4687), 50 µg/ml streptomycin (S1277), and 0.4% Bovine Serum Albumin (BSA) (A8806). A total of 370 cumulus-oocyte complexes (COCs) were retrieved. Out of these, 270 COCs with three to ten layers of cumulus cells and homogeneous cytoplasm were selected for in vitro maturation (Arıcı et al., 2022).

### In vitro maturation (IVM)

COCs obtained from ovaries were divided into two separate maturation groups. Basic maturation media consisting of bicarbonate-buffered TCM199 (M5017) with 0.4% BSA (A8806), 2 mM L-glutamine (G7513), 0.3 mM sodium pyruvate (P4562), 1 µg/ml estradiol-17β (E2758), 100 IU/ml penicillin (P4687), 100 µg/ml streptomycin (S1277), 0.1 IU/ml FSH (Folltropin-V®) addition (Birler et al., 2002). The maturation media for the hypophyseal LH (h-LH) group was supplemented with 0.1 IU/ml LH (from sheep Piturity LH, Sigma, L5269) and the Recombinant LH (r-LH) group was supplemented with 0.1 IU/ml LH (Luveris® 75 IU, Serono).

Oocytes were incubated in four-well plates (500 µL per well), with 35-40 oocytes per well, at 38.5°C, 5% CO2 for 24 h. Subsequent to maturation, the cumulus cells surrounding the oocytes were denuded by vortexing for 2-3 min. After a complete denudation, oocytes were incubated in 5µg/ml Hoechst 33342 added DPBS for 15 minutes.

Following incubation, a total of 40 oocytes were examined for each group under an epifluorescence microscope (IX 70, Olympus, Japan) with a 365 nm excitation filter and a 410 nm barrier filter. Each oocyte was evaluated in conformity with chromatin structure. Evaluation of maturation was made according to Metaphase II (MII) or the other stages of meiosis respectively.

### In vitro fertilization (IVF)

Mature COCs were then washed three times in ovine fertilization medium (bSOF) [3 mg/mL BSA-fraction V (A3311), 0.72 mg/mL D-glucose (G7021), 0.06 mg/mL sodium pyruvate (P4562), 0.25 mg/mL L-glutamine (G7513), 0.12 mg/mL kanamycin monosulfate (K1377), 0.075 mg/mL penicillin (P4687), and 0.05 mg/mL streptomycin (S1277)] at 38.5°C with 2% estrous sheep serum. After maturation, oocytes of the study groups were transferred to a four-well plate (Nunc, Thermo Scientific, USA) containing 500 µl fertilization medium (Birler et al., 2002).

Fresh sperm from a Kivircik ram was prepared for IVF using a modified swim-up technique based on the protocol described by Shirazi et al. (2010). A

volume of 100 µL of semen was placed under 1 mL bSOF in a 15 mL conical tube and incubated at 37°C for up to 1 h. The supernatant of each tube (one third of the volume) was aspirated and mixed with h-SOF then it was centrifuged at 1700 rpm for 5 minutes.

After centrifugation, the spermatozoa concentration was calculated by haemocytometer. The spermatozoa concentration was adjusted to $0.8\times10^6$ spermatozoa/ml, washed and centrifuged (200×g for 5 min) twice in h-SOF, and added to the fertilization medium.

Maturated oocytes (35-40 per well) and spermatozoa were carried out co-incubated at 38.5°C, 5% $CO_2$ for 24 h.

**In vitro culture (IVC)**

At the 20th hour post-fertilization, the pipetting technique was used to remove the cumulus cells and spermatozoa residues. Then, cumulus-free cells of all groups were cultured by placing 12-15 in a 50 µl droplet of synthetic oviduct fluid (SOF) supplemented with 1% (v/v) essential amino acids, 1% (v/v) MEM-nonessential amino acids (M7145), 1mM glutamine and 10% fetal bovine serum (FBS) (F2442). They were incubated with 5% $O_2$, 5% $CO_2$ and 90% $N_2$ at 38.5°C.

Embryos were transferred into 15 mM glucose (G7021) including SOF medium on day 4 post-fertilization and culturing continued until the 8th day.

Embryonic developmental stages were evaluated by inverted microscope. The quality of blastocysts from h-LH and r-LH maturation media were determined with a differential staining of blastocysts depicted by Thouas et al. (2001) with modifications. Embryos were held in PBS with 1% (v/v) Triton X-100 and 100 µg/ml propidium iodide (PI) for approximately 15-20 seconds. During this treatment trophectoderm's colour turning to red were transferred to 500 µl fixative solution consisted of 25 µg/ml bisBenzimide (Hoechst 33342) and absolute alcohol and incubated overnight at 4°C in dark. Then, the blastocysts were put in a glycerol droplet on a glass slide to be covered with a coverslip.

Samples were examined by using a fluorescent microscope (IX70, Olympus, Japan) equipped with an UV filter. By Hoechst 33342 and PI, the inner cell mass (ICM) was observed blue, and trophoblastic cells (TC) nuclei as pink to red. The total cell count, ICM, and TC numbers were assessed in 17 blastocysts from the h-LH group and 23 blastocysts from the r-LH group, respectively.

**Statistical analysis**

The chi-square test was used to compare the effect of LH type added to the culture in the growth of cultured oocytes up to MII, blastocyst stage and total cell of blastocysts. All analyses were performed with the SPSS Version 20. Each experiment was repeated at least five times. Differences were considered significant at $p<0.05$.

**Ethical approval**

This study was approved by the Experimental Animals Local Ethics Committee at the Istanbul University-Cerrahpaşa with decision number 2017-295820.

## RESULTS

**In vitro maturation**

No statistically significant difference was determined between the groups in the maturation medium when h-FSH and h-LH (67.50%) or h-FSH and r-LH (65.00%) were compared to the MII stage ($p>0.05$) (Table 1).

**Table 1.** Effect of different LH sources on oocyte maturation.

| Group | No. of oocytes examined (n) | Metaphase II (%) |
|---|---|---|
| **h-LH** | 40 | 27 (67.50)[a] |
| **r-LH** | 40 | 26 (65.00)[a] |

Different letters indicate statistical difference within each column ($p<0.05$).

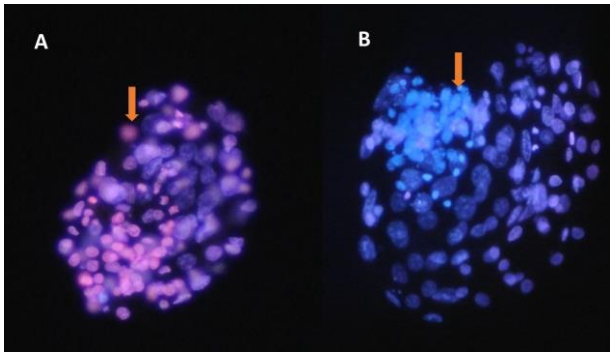


**Figure 1.** Image of inner cell mass (ICM) and trophectoderm cells (TC) of blastocysts. Since the membranes of TC are lysed, their nuclei appear dark pink in colour (A). The cytoplasm is blue / pink with the effect of flurochrome dyes of propidium iodine and bisbenzimide. However, as the membranes of ICM cells are intact, only the bisbenzimide dye can pass through and stain the nucleus making the cells to be seen blue (B).

**Embryo development**

Oocytes were maturated for 24 hours in maturation medium containing different types of LH, fertilized and transferred to culture. For the evaluation of embryonic development after IVC, 90 oocytes were used in the h-LH group and 108 oocytes in the r-LH group. Cleavage, morula, and blastocyst rates were determined as 76.66%, 45.55%, and 18.88% in the h-LH group, and 84.25%, 50.92%, and 21.29% in the r-LH group, respectively (Table 2). There was no statistically significant difference in the cleavage, morulae, and blastocyst rates of cultured oocytes ($p>0.05$).

**Embryonic cell evaluation**

Although, ICM, trophectoderm and total cell numbers of embryos reaching the blastocyst stage in culture medium containing h-LH were superior to another group, there was no statistical difference between the two groups ($p>0.05$) (Table 3). Images of ICM and TC of blastocysts belonging to LH groups are given in Figure 1.

**Table 2.** Embryonic development rates of recombinant and pituitary LH groups.

| Group | Oocytes (n) | Cleavage (%) | Morulae* (%) | Blastocyst*(%) |
|---|---|---|---|---|
| **h-LH** | 90 | 69 (76.66)[a] | 41 (45.55)[a] | 17 (18.88)[a] |
| **r-LH** | 108 | 91 (84.25)[a] | 55 (50.92)[a] | 23 (21.29)[a] |

Different letters indicate statistical difference within each column ($p<0.05$). * Morulae and blastocyst rates / oocytes.

**Table 3.** Means of inner cell mass (ICM), trophectoderm (TC) and total cell numbers of blastocysts.

| Group | Blastocyst (n) | Cell number (mean±SEM) | | | ICM:TC ratio |
|---|---|---|---|---|---|
| | | Total | ICM | TC | |
| **h-LH** | 17 | 210.33±71.90[a] | 75.33±26.02[a] | 135.00±47.96[a] | 1:1.79 |
| **r-LH** | 23 | 171.60±27.43[a] | 58.80±10.42[a] | 112.80±17.39[a] | 1:1.90 |

Different letters indicate statistical difference within each column ($p<0.05$).

## DISCUSSION

Adding gonadotropins to the in vitro maturation media, provides nuclear and cytoplasmic maturation of oocytes and increases the number of embryos developing from in vitro fertilized oocytes (Moor and Trounson, 1977; Sha et al., 2010; Demir et al., 2019).

In vitro nuclear maturation of oocytes is the sequence of events that oocyte proceeds to meiotic divisions and progressing to the metaphase stage of the second meiosis and can easily be detected by observing discard of the first polar body.

When the polar body extrusion of matured oocytes in maturation media containing h-LH and r-LH were compared, a similar rate of MII development were observed ($p>0.05$). This indicates that LH coming from both sources can support nuclear maturation.

Pituitary-derived FSH and LH play an important role in the cytoplasmic and nuclear development of oocytes in vivo. Hormones used in the in vitro development stage are usually extracted from the pituitary glands of slaughtered animals. During this extraction, hormones are contaminated with various contaminants. This affects the purity of the products obtained and probably the fate of in vitro studies. In in vivo development process, meiosis takes place with the effect of LH before ovulation. It has been observed that r-LH and h-LH added to the maturation medium support oocytes to continue meiotic divisions at a similar rate with in vitro conditions. These results are consistent with the results obtained by Accardo et al. (2004).

In farm animals, embryos are produced using IVM, IVF, and IVC techniques, followed by embryo transfer. However, the success rates for embryo development and pregnancies achieved through these methods are notably lower compared to in vivo conditions. This discrepancy is primarily due to the inability to replicate the favorable conditions of the in vivo environment in vitro. Despite some progress in enhancing oocyte quality, achieving significant, large-scale improvements in in vitro oocyte maturation remains a considerable challenge. As a result, the application of assisted reproductive technologies to improve reproductive efficiency in both agricultural and biomedical fields

has faced limitations. To overcome these challenges, extensive research has focused on the influence of the in vitro environment on oocyte quality. Key components that impact oocyte developmental potential include proteins, energy substrates, hormones, growth factors, pH, oocyte-secreted factors, and antioxidants. Each of these elements plays a critical role in regulating the developmental competence of oocytes, emphasizing the complexity and importance of optimizing in vitro conditions to enhance reproductive outcomes (Lonergan et al., 2006; Krisher, 2013).

Oocyte meiotic maturation is an essential process for oocyte development, facilitating the progression from meiotic arrest to full maturation. The luteinizing hormone surge plays a pivotal role in this process by releasing oocytes from meiotic prophase arrest and triggering the resumption of meiosis, culminating in the completion of the first meiotic division (Mehlmann, 2005). This maturation process is initiated by the generation of an LH signal within the ovarian follicle, which activates key molecular pathways necessary for oocyte development. Luteinizing hormone binds to luteinizing hormone receptors (LHR) located on mural granulosa cells, initiating a cascade of intracellular events through G protein activation and subsequent cyclic adenosine monophosphate (cAMP) signaling. This LH-mediated signaling affects both the follicular compartment and the oocyte, orchestrating the regulation of oocyte meiotic maturation. The primary molecular targets of LH signaling within the ovarian follicle include the C-type natriuretic peptide (CNP)/natriuretic peptide receptor 2 (NPR2) system, the epidermal growth factor (EGF) network, and intercellular communication through gap junctions (Conti et al., 2012; Jaffe and Egbert, 2017). In the oocyte, the primary target of the LH signal is the maturation-promoting factor (MPF) (Adhikari and Liu, 2014). Upon activation, MPF phosphorylates several critical systems, including the spindle assembly checkpoint (SAC) and the anaphase-promoting complex/cyclosome (APC/C), as well as other downstream proteins. This cascade drives the progression of meiosis by inducing key cellular events such as germinal vesicle breakdown (GVBD), chromosome condensation, and chromosome segregation, thereby facilitating oocyte maturation (Arroyo et al., 2020).

Although the chromosomal development in the maturation of oocytes is demonstrated by detection of the first polar body, cytoplasmic maturation is not adequately demonstrated. Therefore, it is important to examine the effects of r-LH and h-LH used in maturation media on embryo development.

No statistically significant difference was found between groups in terms of cleavage, morulae and blastocyst after in vitro fertilization with h-LH and r-LH ($p>0.05$). These results are similar to previous studies (Andresiez et al., 2000; Accardo et al., 2004) suggesting the use of recombinant LH in sheep oocyte maturation.

The number of ICM and TC in blastocysts that develop in vivo or in vitro is an important indicator of embryo quality (Leppens et al., 1996; De la Fuente and King, 1997). When the cell numbers of blastocysts developed in Recombinant and Pituitary LH groups were compared, no statistically significant difference was observed ($p>0.05$).

## CONCLUSION

Based on the results, it has been demonstrated that human recombinant luteinizing hormone is a suitable option for use in sheep in vitro embryo production and transfer programs.

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# Three-dimensional (3D) geometric morphometrics of the carnivora axis: shape variation, allometry

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## ABSTRACT

**Objective**: The *axis* is an important bone due to its adjacency to various structures and its contribution to the bony structure of the neck, as well as its responsibility for supporting the neck. Given the absence of similar studies in the literature, we sought to investigate the three-dimensional geometric morphometry of the *axis*. For this purpose, 17 *axis* bones from 8 different domestic species belonging to the carnivora family were analysed.

**Materials and Methods**: CT images of the bones were converted into three-dimensional (3D) bone surfaces. A total of 102 landmarks were applied to the images recorded with the generalized Procrustes method. Principal Component Analysis (PCA) was performed with SlicerMorph programme. As a result of this analysis, shape variations of the bones were analysed. In addition, Procrustes Distance and Centroid size values of the bones were measured with the same programme. These values were processed into SPSS programme and the effect of age and weight on size was investigated by regression analysis.

**Results:** The shape changes of the bones were analysed from five different perspectives: cranial, caudal, lateral, dorsal, and ventral. These analyses revealed shape variations, which were subsequently explained. Principal component analysis (PCA) showed that PC1 accounted for 21.04% of the total variation, PC2 explained 13.6%, and PC3 contributed 12%. Furthermore, regression analysis demonstrated that age and weight were statistically significant factors influencing centroid size.

**Conclusion:** Principal component analysis revealed significant shape variations in the axis bones of carnivora, particularly in key anatomical features such as the processus transversus and dens, with notable bilateral differences. The allometric analysis showed a significant relationship between centroid size, shape, age and weight, emphasizing the influence of allometry on axis morphology. These findings enhance our understanding of the morphological diversity and functional adaptations of the axis bone in carnivorous mammals.

***Keywords:*** *Cat, Cervical, Computed tomography, Dog, Vertebra*

## INTRODUCTION

The axis is the second of the vertebrae cervicales and is an important bone in terms of its location, vital adjacent formations such as cranial nerves, spinal cord and vertebral arteries, and its anatomical shape (Şengül and Kadıoğlu, 2006). Since the specialized shape of the axis is unique, it

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is considered special, much like the first vertebrae cervicale, the atlas. (Gosavi and Swamy, 2012). Vertebrae cervicales form the bony structure of the neck and also carry the weight of the neck (Dursun, 2007). As a whole, the foramen vertebralis of the columna vertebralis merge to form a canal called canalis vertebralis. This canal contains the medulla spinalis (Dursun, 2007).

It articulates with the atlas, the first vertebrae cervicales, through its protrusion dens, which is conical shaped in carnivora, located in front of the axis. In addition, at the front of the corpus, where the corpus and the dens meet, there is a facies articularis cranialis covered with cartilago articularis, which articulates with the fovea articularis caudalis of the atlas (Dursun, 2007). Dorsal to the axis is the processus spinossus. This is higher than the processus spinossus of other vertebrae cervicales (Demiraslan and Dayan, 2021). On the ventral side of the axis, there is a long crest called crista ventralis that extends up to the dens, which is characteristic of carnivora (Dursun, 2007; Demiraslan and Dayan, 2021).

Axis is the longest of the vertebrae cervicales for carnivora. Nutritional and behavioural changes of animals belonging to the carnivora family group can cause anatomical changes in their bones (Van Valkenburgh, 1987). For this reason, the mobility of the axis is higher in carnivora than in other mammals (Ewer, 1998). These changes in mobility can lead to anatomical alterations in the bones, as the stress on the bone generates varying degrees of strain (Bertram and Biewner, 1990).

Geometric morphometric methods have developed a lot over the years and still continue to develop. In the past, these methods were limited to various statistical analyses and linear measurements. Recently, new statistical analyses and different measurement methods have been developed (Adams et al., 2004). Landmark placement measurements are among these developments. Adams et al. (2004) evaluated the contribution of this emerging measurement method to geometric morphometric methods in their study. When measurements are made using landmarks, various coordinates are created on 2D and 3D images (Adams et al., 2004). By superimposing these coordinates, it is easier to perform shape analyses and statistical analyses (Gündemir et al., 2020; Rolfe et al., 2021; Demiraslan et al., 2024). Geometric morphometric analysis methods performed in 3D differ from linear measurement in terms of examining and investigating the bone as a whole (Rolfe et al., 2021).

In the literature, geometric morphometry studies with linear measurements on the axis were found (Gosavi and Swamy, 2012; Singla et al., 2015). Similarly, 3D geometric morphometric studies on vertebrae thoracicae were also found (Kikuchi and Ogihara, 2021). Nevertheless, no 3D geometry morphometry study was found among species belonging to the carnivora family group.

In this study, specimens belonging to carnivora were used and it was aimed to determine the shape changes on axis bones collected from these specimens. Since carnivora contains many species, morphological differences could be seen (Gündemir et. al., 2023; Manuta et. al., 2023). Thus, in the present study, specimens belonging to domestic species were selected among the felis and canis genus of this family group.

## MATERIALS and METHODS

### Samples

In this study, 17 axis bones belonging to the carnivora family were used. The selected specimens belonged to 8 different breeds. Descriptions of species, age, weight and sex were given in Table 1. The images of axis bones used in this study were obtained from Istanbul University-Cerrahpaşa Veterinary Faculty, Research and Application Animal Hospital. These were healthy bones without any pathological problems.

### Ethical statement

The required ethics committee report for the study was obtained from the Istanbul University-Cerrahpaşa Veterinary Faculty Ethics Committee (Report Number: 2022/38).

### Statistical analysis

Images were obtained with a Siemens SOMATOM brand 16-slice CT device. These images were converted into a three-dimensional (3D) bone surface. Afterwards, they were transferred to the computer in 'PLY' format. Landmark placement and geometric morphometric analyses were performed on these images using SlicerMorph program. A total of 102 landmarks were applied to the images. The landmarking process was visualized in SlicerMorph (Figure 1). Firstly, Generalized Procrustes Analysis was applied to the images transferred to the computer. Then Principal Component Analysis (PCA) was applied to explain the shape variations. PCA contains information

about how variations within a dataset are separated from each other and is numbered proportionally to the number of data points (e.g., PCA1, PCA2, PCA3, etc.).

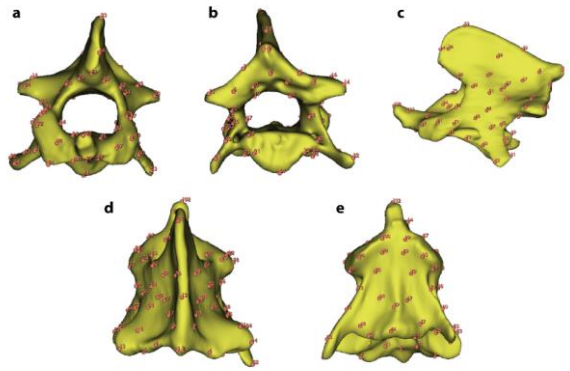


**Figure 1.** Landmarks on 3D models of *axis*.

The resulting PCAs explain certain percentages of the total variation based on the feature causing the variation. The sum of the variation percentages of these PCAs equals 100%, which corresponds to the total variation of the analyzed data (Aytek, 2016).

**Table 1**. Descriptions of species, age, weight and gender of specimens

| Sample No | Species | Age | Weight (kg) | Gender |
|---|---|---|---|---|
| 1 | British | 2 years | 2 | Male |
| 2 | British | 5 months | 1 | Male |
| 3 | British | 1.5 years | 3.3 | Female |
| 4 | British | 3 years | 4 | Female |
| 5 | British | 4.5 years | 4 | Male |
| 6 | British | 4 months | 1 | Male |
| 7 | Chihuahua | 9 months | 1.8 | Male |
| 8 | Chihuahua | 3 years | 3.3 | Female |
| 9 | Cocker | 8 years | 13 | Male |
| 10 | Cocker | 8 years | 18 | Male |
| 11 | Golden | 7 years | 40 | Male |
| 12 | Golden | 8 years | 33 | Male |
| 13 | Labrador | 6 years | 28 | Female |
| 14 | Pomerian | 2 years | 3.5 | Female |
| 15 | Scottish | 4 months | 1.2 | Male |
| 16 | Terrier | 4 years | 1 | Female |
| 17 | Terrier | 8 years | 18 | Male |

In this study, a total of 10 principal components were obtained. Of these, the first three principal components with the highest explanation of total variation were visualized and explained in the results section. In addition, the values of the 10 principal components with the highest explanation are given in Figure 2 and Figure 3.

The centroid size and Procrustes distances obtained from the SlicerMorph programme were processed into SPSS in order to make size-related evaluations. In this program, multivariate regression analysis was performed between centroid size and Procrustes distances. In addition, the effects of age and weight changes on the size of the animals were investigated using the same program. For this purpose, first multivariate regression analysis was performed for age values and centroid size values. Then the same steps were applied for weight values.

## RESULTS

### Shape variations

Principal component analyses revealed 10 different principal components (Figure 2). Of these principal components, the first three principal components with the highest explanation rate were visualized and shape variations were explained (Figures 2). PC1 explained 21.04% of the total variation, PC2 explained 13.6% and PC3 explained 12% (Figure 2).

The shape variations between the maximum and minimum values for PC1 were quite evident in the processus transversus and processus articularis caudalis on the right side of the animal. For the maximum value, the processus articularis caudalis on the right side was shorter. For the minimum value, the processus articularis caudalis on the right side was longer. On the other hand, for the maximum value, the processus transversus was longer on the right side, while for the minimum value it was shorter and travelled more steeply caudally. At the cranial edge of the processus spinosus, different shape variations were formed for maximum and minimum values. In addition, the dorsal side of the processus spinosus formed different shape variations for maximum and minimum values. Processus spinosus exhibited a curved appearance with maximum and minimum values forming angles towards different sides. It was observed that the position between the maximum and minimum values in the dens was different from each other. Due to the shape variations between the maximum and minimum values of the bony roof forming the foramen vertebrale, the images in this region were different from each other. For PC1, the crista ventralis formed an oblique shape by making an angle at the

minimum value, while it formed a straight line at the maximum value. In addition, when viewed from the lateral side, the axis bone in PC1 was dorsoventrally flattened compared to the maximum value of PC1.

Similar shape variations were observed in PC2 as in PC1. The processus articularis caudalis was shorter for the maximum value than for the minimum value, but unlike PC1, the processus articularis caudalis in PC2 was closer in distance for the maximum and minimum values. The cranial end of the processus spinosus showed a more flattened and curved shape variation at the maximum value. For the minimum value, it showed a longer and flatter shape variation compared to the maximum value. To explain this shape variation from the lateral side, the cranial end of the processus spinosus showed a more oval shape variation at the maximum value and a more angular shape variation at the minimum value. On the caudal side of the bone, the angle where the processus spinosus and processus articularis caudalis meet showed a flat junction in the maximum value, while a sharper angle was observed in the minimum value.

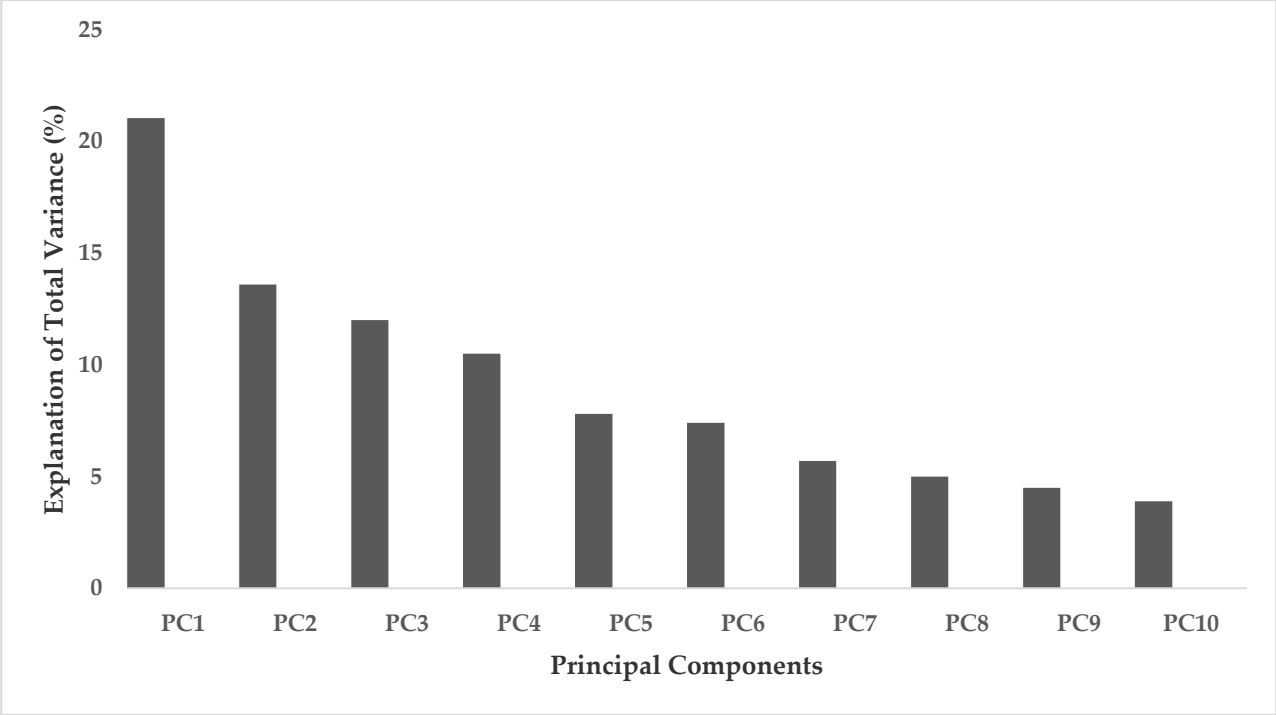


**Figure 2.** Percentage of Principal Components explaining total variation (%).

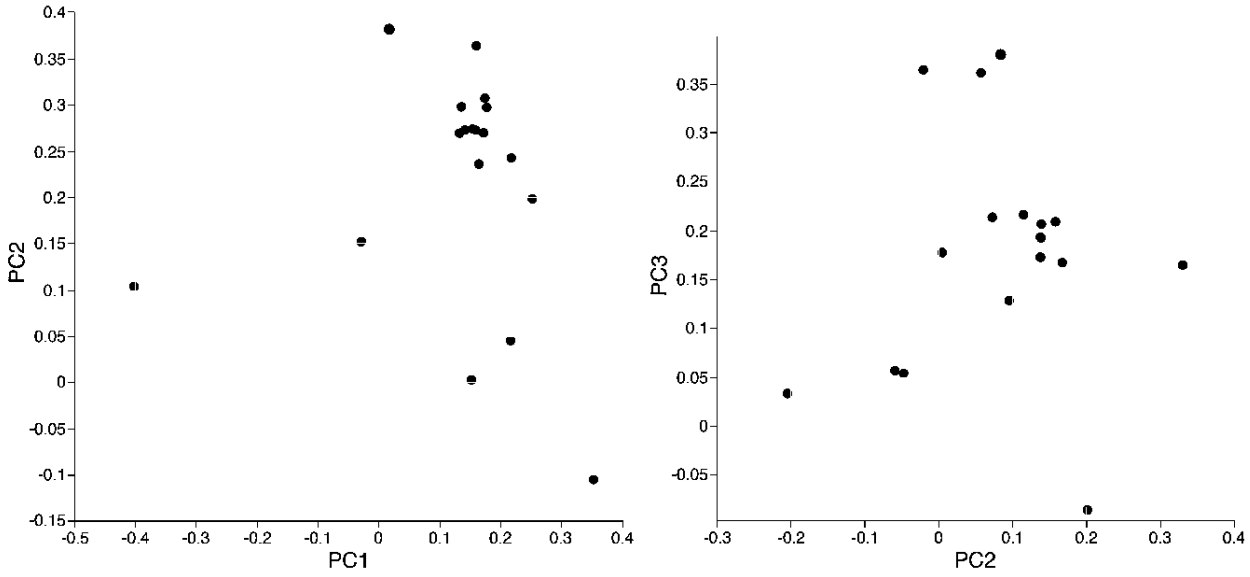


**Figure 3.** Scattter Plot based on the characteristics of the samples for PC1, PC2 and PC3 as a result of Principal Component Analysis.

Another change in PC2 was the processus transversus. This was more posterior and longer in the minimum value than in the maximum value. The dens was located in different positions at the minimum and maximum values of PC2. It was noticed in the ventral poses of PC2 that the dens was longer at the maximum value than at the minimum value. In PC2, the caudal end of the corpus vertebrae, the part where the fossa vertebrae is located, showed a flatter shape variation at the minimum value and a triangular shape variation at the maximum value. These shape variations were seen in both caudal and ventral poses. Additionally, the corpus vertebrae was shorter for the minimum value. It was observed that these differences in the corpus vertebrae also affected the crista ventralis. This triangular shape variation observed at the maximum value of the caudal end of the corpus vertebrae caused the crista ventralis to form an angular shape variation. At the minimum value, the crista ventralis was shorter due to the shorter corpus vertebrae. In addition, the crista ventralis showed a flatter shape variation due to the caudal end of the corpus vertebrae showing a minimum flat shape variation. The foramen vertebrale was larger at the maximum value than at the minimum value.

In PC3, the processus spinosus showed a shape variation in the form of an ellipse with a blunt cranial tip and a pointed caudal tip at the maximum value, while it showed a shape variation close to a rectangle at the minimum value. While the cranial tip of the processus spinosus showed an oblique shape variation for the maximum value, the shape variation occurred in an upright view for the minimum value. The caudal end of the processus spinosus showed a shape variation that extended to the caudal side for maximum value. However, on the contrary, for the minimum value, it formed a shape variation in the form of a triangle with its tip extending to the dorsal side. The processus transversus on the right side was longer and moved backwards for the maximum value. A similar shape variation was shaped on the left side for the minimum value. However, for the minimum value, the processus transversus on the left side extended obliquely forward. For the minimum value, the processus transversus on the right side showed a more horizontal shape variation. The left processus transversus, visualized at maximum value, was shorter than the right side. For PC3, the processus articularis caudalis showed a thicker and shorter shape variation than that seen in the other principal components for the maximum value and minimum value. The corpus vertebrae formed a longer and thinner shape variation at the maximum value than at the minimum value.

**Allometry**

The relationship between centroid size and Procrustes values was evaluated for allometric interpretation. A graphic plot of the results of the multivariate regression analysis is given in Figure 4. According to these results, allometry was statistically significant in carnivora axis bones ($p < 0.005$; Figure 4). Separately, another multivariate regression analysis was performed to investigate the effect of weight on size. The results of this analysis were statistically significant ($p < 0.005$).

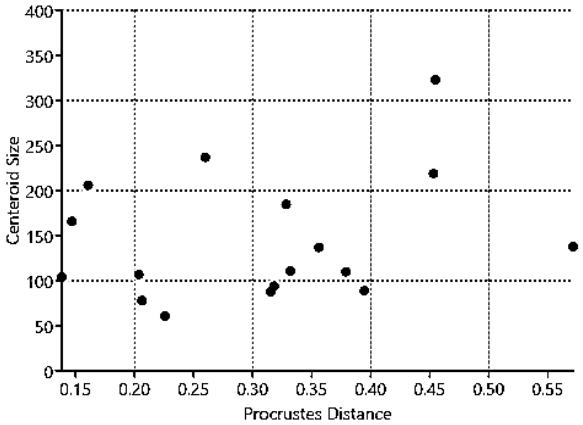


**Figure 4.** Scatter plot of the distribution of centroid size versus Procrustes distance of the samples, along with the relationship between the two variables.

## DISCUSSION

The axis is a special bone in terms of shape and is also important due to the structures adjacent to the bone (Gosavi and Swamy, 2012). Therefore, knowing its anatomy and geometric morphometric studies are also important in terms of surgical interventions (Doherty and Heggeness, 1995). Additionally, as surgical techniques in this region improve, anatomical knowledge also needs to improve (Xu et al., 1995; Şengül and Kadıoğlu, 2006).

Although the samples belong to the same family group, it is common for them to show different shape variations in geometric morphometry studies performed on the same bone (Gündemir et al., 2023). In addition, vertebrae cervicales undertake the task of carrying the neck. Especially the axis bone participates with structures such as muscles and ligaments as a result of this task (Dursun, 2007). Some of these structures are m. longus colli, m.

spinalis, m. multifidus, m. intertransversus, lig. nuchae (Zileli, 2002; Dursun, 2007). Shape variations occurring in bones associated with muscles and ligaments are also observed in other studies in the literature (Reno, 2014). A change can be observed in the intensity and use of the muscles in the neck area (Mayoux-Benhamou et al., 1989; Fortin et al. 2018). As observed in our study, the shape differences between the right and left halves of the bone are thought to vary due to the similar reasons mentioned above.

The shape variations occurring between the maximum and minimum values for PC1 showed a serious change on the right and left sides of the bone. A similar shape variation was also observed in geometric morphometry studies in the literature where linear measurements were made. In the study conducted by Singla et al. (2015) on human axis bones, various measurements were made from the right and left sides with a line drawn from the median line of the bones. In these measurements, changes were observed between the right and left sides (Singla et al., 2015). Similarly, in Gosavi and Swamy's study in 2012, found that the difference in the width of the pedicle between the right and left sides was found to be statistically significant. Considering the structures on the right and left sides of the bones, previous studies have reported differences between these structures, a finding further supported by our study. Additionally, we believe that the difference between humans and animals in terms of normal posture also influences the use of neck muscles. However, it seems that, despite the differences in the muscle groups used, the distinction between the right and left sides in both humans and animals develops throughout life due to certain habits in neck movements and genetic factors.

As a result of the regression analysis, the relationship between size and age was examined and the results were statistically significant ($p<0.05$). A similar situation was observed in the study conducted by Johnson et al. (1988) on the vertebrae thoracicae of mice. In this study, the relationship between size and shape changes of vertebrae and age was examined by regression testing, and the results showed that age had an effect on size (Johnson et al., 1988). Since this study focuses on vertebral analysis in animals, it shows a similarity to our research, and the results have also been found to be similar, as expected. Accordingly, it can be said that the dimensional development and change of the vertebrae is related to age, as can be observed in many other bones (Mosekilde, 2000; Havill et al., 2007). In a study conducted on 7420 lumbar vertebrae in humans, it is reported that the body parts of the vertebrae increase in both length and width with age (Mavrych et al., 2014). As is evident in many studies, it is clear that the size of bones increases with age. However, although there are different opinions regarding the cause of this, it is suggested that the growth of adjacent formations over time may be a contributing factor. (Gosavi and Swamy, 2012). In our study, the significant allometric relationship between centroid size and Procrustes distance can be explained by the development of ligament and muscle tissues around the axis over time, and it is also our perspective that this may be a contributing factor. Additionally, the relationship between weight and size of the bone in our study shows similar results to a study conducted on humans, which found that size increases with weight (Mølgaard et al., 1998).

The principal component analysis revealed significant shape variations in the axis bones of carnivora, particularly in the first three principal components (PC1, PC2, and PC3), which accounted for a substantial portion of the total variation. These variations were primarily observed in key anatomical features such as the processus transversus, processus articularis caudalis, processus spinosus, dens, crista ventralis, and corpus vertebrae. Differences in the shapes of these structures were evident between maximum and minimum values across components. The findings highlight significant bilateral differences and provide evidence for shape variation across cranial, caudal, and ventral perspectives. Additionally, the observed variations in the bony roof of the foramen vertebrale and the crista ventralis suggest functional or developmental implications. Furthermore, the results of the allometric analysis demonstrated a statistically significant relationship between centroid size and shape, as well as between size and weight, emphasizing the influence of allometry on the morphological characteristics of carnivora axis bones. These findings contribute to a deeper understanding of the morphological diversity and functional adaptations of the axis bone in carnivorous mammals.

## CONCLUSION

These findings contribute to a deeper understanding of the morphological diversity and functional adaptations of the axis bone in carnivorous mammals.

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# Retrospective analysis of the horse racing industry in Türkiye with a specific focus on population, earnings, and injury rates

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## ABSTRACT

**Objective:** The global horse racing industry is expanding in terms of horses and market value, but these growths may not always yield positive outcomes. The study aims to evaluate the current situation of horse racing on a Türkiye scale and provide information for the industry.

**Material and Methods:** This study conducts a retrospective analysis. Data from all horse races held across the country were used in the study. The data utilised came from the TJC software support team and the official website.

**Results:** The study observed a notable increase in the number of horses participating in races, and the number of races being held from these races over the years. However, it's important to note that while the number of young horses participating in races has seen a proportional increase, the ratio of horses in older age groups has seen a decrease. Furthermore, the average earnings of horses in the older age groups have seen a proportional decline, leading to a significant decrease in all age groups. The findings from this study suggest that over the years, horses in Türkiye have been tending to shorten their racing life.

**Conclusion:** The relative decrease in the number of horses participating in races in the senior age categories, along with the decrease in the amount of earnings within the total values, is predicted to have a negative impact on the future of the industry. The study proposes early identification and removal of horses with restricted athletic abilities. These limitations may arise from conformational defects, biomechanical predisposition, or developmental delays.

***Keywords:*** *Horse, Age, Injury, Thoroughbred, Arabian horse*

## INTRODUCTION

Horse flat races are among the most popular sports activities in Europe and Asia, especially in countries where the English language and culture prevail. The horse racing industry includes horse owners, breeders, veterinarians, jockeys, trainers, and related professional groups. In addition, it provides employment and income to people who provide feed, medicine, and materials for horses. The horse racing industry has a large employment and economic impact. In 2022, the market value of the horse racing industry worldwide was US $402 billion. The market value is expected to reach US $793 billion in 2030 (Global and Regional Industry Overview, Market Intelligence, Comprehensive Analysis, Historical Data, and Forecasts 2023 – 2030, 2023). In 2019, Australia's annual Thoroughbred export revenue was announced at US $1.9 billion. It is possible for countries to increase their export revenues through the sale of racing and breeding horses, in addition to their income from horse

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racing. For this, the horse racing industry must have a strong infrastructure (Hardy and Limoli, 2019).

The Türkiye Jockey Club (TJC) organizes Thoroughbred and Arabian flat racing events in 10 hippodromes. In 2022, 3789 Thoroughbred horses made 30968 starts, with a total bonus value of US $33 million. The number of Arabian horses was recorded as 3130, the number of starts as 29527, and the total bonus value was US $26 million. The races attracted 6919 horses, resulting in 60495 starts and US $59 million prize awards (Türkiye Jockey Club Detailed Statistics, 2023).

The number of racing horses competing in TJC-affiliated hippodromes, the number of races, the age and breed distribution of the horses, and their profits have all varied over time (Türkiye Jockey Club Detailed Statistics, 2023). Changes in these factors and their correlations may be examined to provide information about the horse racing business across the country. The information derived from the data collected for this purpose will be useful in presenting the industry's past and present, as well as estimating its future.

Genetics play a significant role in racehorses' natural physical skills and performance. Furthermore, there are two critical systems that influence racehorse performance. The first is nutrition/energy metabolism, or energy provision and consumption, and the second is conditioning, which includes gait mechanics, coordination, and muscle strength (Lawrence, 1996). Optimal care, feeding, and training for these characteristics, as well as providing proper environmental circumstances, all have a positive effect on horse racing success and performance (Yıldırım, 2014).

Customers who want to own a horse demand horses with high success and earning potential from breeders. Conformation-based studies aimed at revealing athletic ability through body structure analysis in racehorses have gained importance (Belloy and Bathe, 1996; Anderson et al., 2004; Love et al., 2006; Smith et al., 2006; Bakhtiari and Heshmat, 2009; Robert et al., 2013; Yıldırım and Erden, 2023). In addition, studies on the causes and treatments of injuries in racehorses continue intensively (Perkins et al., 2005a, 2005b; Cogger et al., 2006; Cheetham et al., 2010; Crawford et al., 2021; Morrice-West et al., 2021). Many horses depart the industry irreversibly due to injuries that end their racing lives (Perkins et al., 2005a, 2005b; Yıldırım, 2014). Ensuring that racehorses lead healthy lives both during and after their racing careers is essential for animal welfare and rights. Awareness of the health, injuries, and post-racing lives of racehorses has markedly increased over the past three decades (Perkins et al., 2005a). In racehorses, health can deteriorate as a result of injuries and diseases, sometimes even resulting in death (Perkins et al., 2005b). Horses, animals known for their close cultural and emotional connection to humans, are significantly affected by this shift in public awareness.

It is thought that these retrospective studies will provide useful information for the veterinary profession as well as other stakeholders in the industry. The purpose of this study is to review TJC's statistical data on horse races organised between 1997 and 2022, as well as to assess the industry's present position.

## MATERIALS and METHODS

Data from all horse races held across the country were used in the study. The data utilised came from the TJC software support team and the official website (Türkiye Jockey Club Detailed Statistics, 2023) with the permission of TJC on April 11, 2023. Data on the number of running horses between 1975 and 2022 was used in the study. In the evaluations made according to race-based age groups, data between 1997 and 2022 for the Thoroughbred horse races. Data between 2003 and 2022 for the Arabian horse races due to the change in race categories in 2003 were evaluated. To understand the changes in earnings over the years, the earnings data was examined by converting it into US $.

TJC hippodromes conduct flat racing events for both Thoroughbreds and Arabians, with each breed competing in distinct races. Thoroughbreds typically begin their racing careers at the age of two, with races organised into three age groups: two-year-olds, three-year-olds, and four-year-olds and above. Arabians, in contrast, commence racing at the age of three and are classified into three groups: three-year-olds, four-year-olds, and five-year-olds and above. This report presents data for both breeds according to the respective age groups in which they participate.

The injury records from the İzmir Şirinyer Hippodrome from 2018 to 2023 were utilised, with permission from TJC on March 29, 2023. This part of the study was carried out with the permission of Aydın Adnan Menderes University, Animal Experiments Local Ethics Committee, numbered 64583101/2023/44.

The study utilized descriptive statistics, including frequency and percentage, with graphs created in Microsoft Excel 2016® (Microsoft Corp., Redmond, Washington, USA). In the manuscript, the statistical data related to the racing industry are presented using line, bar, and pie charts.

## RESULTS

When the number of horses and the number of races are studied, both metrics have shown considerable growth over the past few decades (Figure 1). Between 1975 and 2022, the number of Thoroughbred horses increased 13.73 times, while the number of Arabian horses increased 7.35 times (Thoroughbreds 276, 3789; Arabians 426, 3130, respectively). The number of races grew by 8.03 times for Thoroughbreds and 3.68 times for Arabians between 1975 and 2022 (Thoroughbred races 437, 3509; Arabian races 811, 2981, respectively).

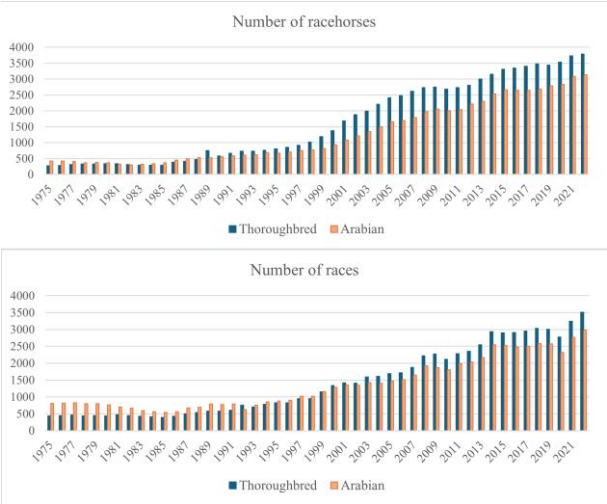


**Figure 1.** Number of racehorses and number of races by years in Türkiye

The numerical changes of Thoroughbred and Arabian horses participating in the races according to their age groups were evaluated. In the period until 2005, while the number of horses running in two age groups of the Thoroughbreds was lower compared to other age groups, it was determined that the number of horses running in the age group of four years old and older was higher compared to other age groups. This situation has changed since 2005, and it has been observed that the number of horses running in the three years old age group is higher than in other age groups, and the number of horses running in the four years old and older age group is mostly lower (Figure 2). When the change in the number of running horses of the Arabian race over the years was examined, it was seen that a similar situation existed, and since 2005, the number of running horses in the age group of five years old and older has been lower compared to other age groups.

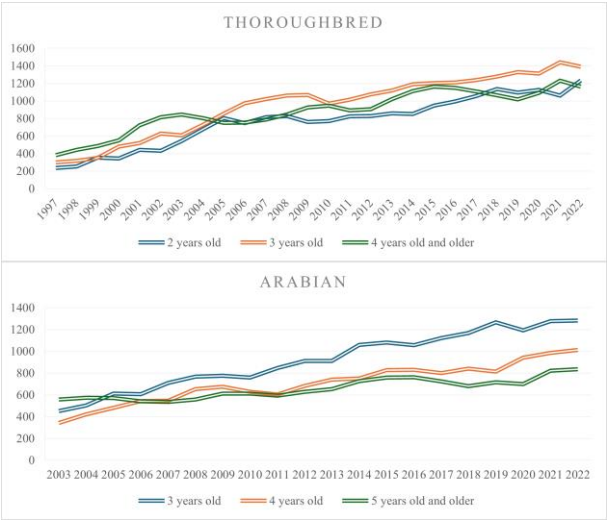


**Figure 2.** The number of Thoroughbred and Arabian racehorses according to age groups by years in Türkiye

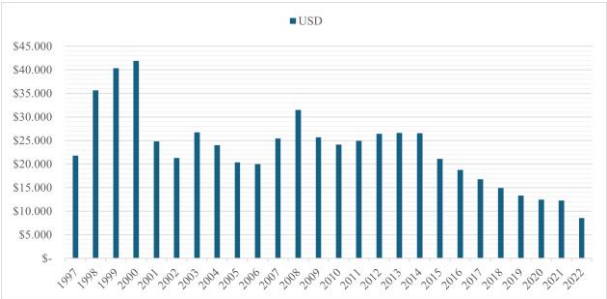


**Figure 3.** The average earnings per horse by year in Türkiye

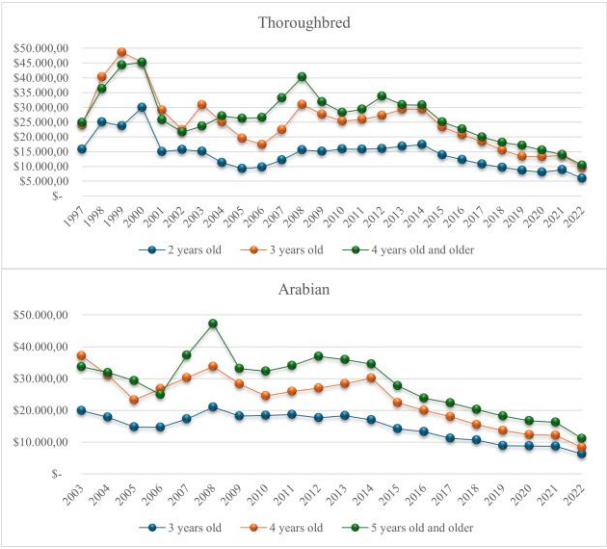


**Figure 4.** The average earnings (US $) per horse in the Thoroughbred and Arabian horses across age groups

Average earnings per horse from 1997 to 2022 are shown in US $ (Figure 3). The average earnings per horse go down.

Average earnings per horse in racehorses based on breed and age vary over the years. It has been observed that the average earnings per horse for both Thoroughbred and Arabian horses across age groups have approached each other over the years (Figure 4).

Race winning rates for Thoroughbred and Arabian horses in their age groups and the average number of wins per winning horse were evaluated. The lowest winning rate in Thoroughbred horses was found in the two age groups. In addition, it was determined that horses aged four years and older had the highest win rates per winning horse in Thoroughbreds (Figure 5).

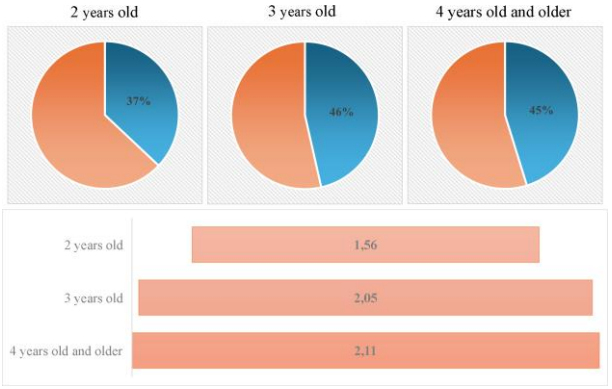


**Figure 5.** The average winning rates and the average wins per winning horse by age groups for Thoroughbred horses

It has been determined that the winning rates in Arabian horses are equal in the three and four age groups. In addition, it was seen that horses five years old and older had the highest number of wins per winning Arabian horse (Figure 6).

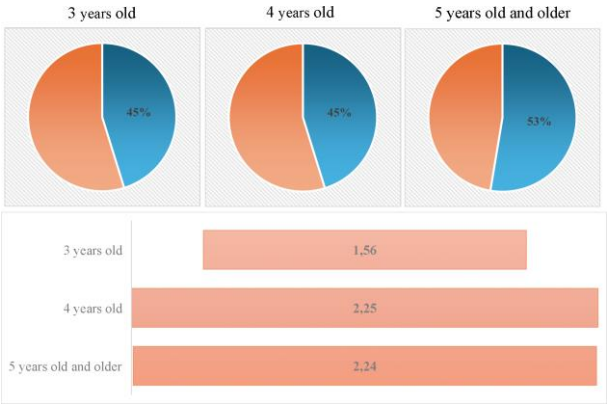


**Figure 6.** The average winning rates and the average wins per winning horse by age groups for Arabian horses

**Figure 7.** Injuries observed in Thoroughbred and Arabian horses at the Hippodrome Equine Hospital between 2018 and 2022

**Table 1.** Effects of injury categories on racing careers in Thoroughbreds and Arabian horses [f (%)].

| Category | Thoroughbred | | Arabian | |
|---|---|---|---|---|
| | Race carrier ending | Race carrier ongoing | Race carrier ending | Race carrier ongoing |
| Fracture | 128 (37.98) | 80 (23.74) | 33 (15.28) | 10 (4.63) |
| Bone fissure | 8 (2.37) | 17 (5.04) | 15 (6.94) | 14 (6.48) |
| Tendon injury | 50 (14.84) | 36 (10.68) | 51 (23.61) | 86 (39.82) |
| Ligament desmitis | 3 (0.89) | 2 (0.60) | 2 (0.93) | 2 (0.93) |
| Tendon rupture | 1 (0.30) | 0 (0.00) | 1 (0.46) | 1 (0.46) |
| Chip fracture | 3 (0.89) | 9 (2.67) | 0 (0.00) | 1 (0.46) |
| Total Injuries | 193 (57.27) | 144 (42.73) | 102 (47.22) | 114 (52.78) |

Data from TJC Equine Hospital records was utilised to assess the association between injury cases and the age distribution of running horses, and their consequences on their racing careers. The rates of injuries in Thoroughbred and Arabian horses were determined by age group. It was seen that injuries to Thoroughbred horses occurred at equal rates in all age groups. On the other hand, injuries in Arabian horses were seen at a greater rate in the upper age groups (Figure 7).

In both breeds, the percentage of injuries that ended a racer's career increased proportionally with age. When evaluating the impact of injuries on race careers, it was observed that fractures were more prevalent in Thoroughbreds, whereas tendon injuries were more common in Arabian horses (Table 1). In Thoroughbreds, fractures represented the most frequent career-ending injuries, while in Arabian horses, tendon injuries were noted to have a higher likelihood of allowing horses to continue their race careers. When the distribution of injuries across age groups was examined between the two breeds, differences were observed (Table 2). While fracture cases in Thoroughbreds remained the most prevalent across all age groups, tendon injuries in Arabian horses peaked at the age of three and showed a decreasing trend over the following years.

**Table 2.** Distribution of injuries by age groups in Thoroughbreds and Arabian horses [f (%)].

| Thoroughbred[1] | | | | | | |
|---|---|---|---|---|---|---|
| **Age** | **Fracture** | **Bone fissure** | **Tendon injury** | **Ligament desmitis** | **Tendon rupture** | **Chip fracture** |
| 2 years | 54 (16.12) | 10 (2.98) | 37 (11.05) | 0 (0.00) | 0 (0.00) | 5 (1.49) |
| 3 years | 85 (25.37) | 8 (2.39) | 24 (7.16) | 2 (0.60) | 0 (0.00) | 5 (1.49) |
| 4 years and older | 67 (20.00) | 7 (2.09) | 25 (7.46) | 3 (0.89) | 1 (0.30) | 2 (0.60) |
| Total Injuries | 206 (61.49) | 25 (7.46) | 86 (25.67) | 5 (1.49) | 1 (0.30) | 12 (3.58) |
| **Arabian[2]** | | | | | | |
| **Age** | **Fracture** | **Bone fissure** | **Tendon injury** | **Ligament desmitis** | **Tendon rupture** | **Chip fracture** |
| 3 years | 15 (6.98) | 5 (2.32) | 78 (36.28) | 2 (0.93) | 2 (0.93) | 1 (0.46) |
| 4 years | 14 (6.51) | 11 (5.12) | 47 (21.86) | 1 (0.46) | 0 (0.00) | 0 (0.00) |
| 5 years and older | 13 (6.05) | 13 (6.05) | 12 (5.58) | 1 (0.46) | 0 (0.00) | 0 (0.00) |
| Total Injuries | 42 (19.54) | 29 (13.49) | 137 (63.72) | 4 (1.86) | 2 (0.93) | 1 (0.46) |

[1] The total number of injuries of Thoroughbred horses in the hospital records is 337 (1 years: 2 fracture).
[2] The total number of injuries of Arabian horses in the hospital records is 216 (2 years: 1 fracture).

## DISCUSSION

As in all countries where horse racing culture is widespread, the racehorse industry continues to grow in Türkiye. The number of horses running, the number of races, and the earnings values provide data in this direction. However, numerical increases may not always be an indicator of a positive trend. Some problems can be seen because of a detailed evaluation of the data. By recognising these problems at an early stage, it may be possible to take the necessary regulations and precautions.

Although the Thoroughbred is the most popular racing breed in the world, several other breeds are bred and utilised for this purpose. Thoroughbred and Arabian horses are racehorses in Türkiye, competing in breed-specific races. While Arabian horses accounted for 61 percent of all horses running in 1975, Thoroughbred horses accounted for 39 percent. However, this ratio has shifted in favour of Arabian horses over time. Thoroughbred horses had a 61 percent rate from 2001 to 2003. By 2022, it will be composed of 45 percent Arabian and 55 percent Thoroughbred horses. The proportionate growth of Thoroughbred horses, the world's most popular racing breed, is a normal and expected outcome. However, given their biologic, historical and geographical significance, the presence of Arabian horses in the racing business must be monitored and taken care of.

The number of Thoroughbred and Arabian horses participating in races and the number of races is increasing. While the number of Thoroughbred horses was 276 and the number of races was 437 in 1975, the number of Thoroughbred horses and 3509 races were reached in 2022, respectively. While the number of Arabian horses was 426 and the number of races was 811 in 1975, the number of horses and races reached 3130 and 2981 races in 2022. When the process is examined, it is seen that the increase in the number of running horses is greater than the increase in the number of races. As a result, it is noteworthy that the number of horses running today in both races is more than the number of races organised. The increase in the number of running horses is a positive situation for the development of the racehorse industry. However, it is not a sufficient parameter on its own for the development of the racing industry. In addition to this increase, it should be ensured that the racing lives of the horses

continue uninterrupted for many years. At this point, the change in the numbers and rates of running horses according to their age groups over the years provides important information about the racing lives of the horses. Especially in the first year of racing, the injury rate and the recurrence rate of these horses' injuries are higher (Perkins et al., 2005b). For this reason, injuries of young horses are an important problem for their racing continuity in older age groups. It is desired that racehorses have a successful and long racing career without being interrupted by injuries (Stover, 2003). In the presence of this situation, among the total number of horses that started their racing career, the number of those that could not continue their racing life due to injury or similar reasons would be less than the other half. As a result, the proportion of age groups within the total number of running horses increases from young to older age groups. To reveal the existence of this situation, which is seen as positive, the numbers and rates of the number of Thoroughbred and Arabian horses running according to age groups were examined over the years. Until 2005, the number and rates of running horses were higher in the age group of four years and older. In the same period, it was observed that the number of two years old horses was proportionally less, while the number of three years old horses was at an intermediate level, and there was a balanced age distribution. On the other hand, it was noted that since 2005, the rate of horses aged four and over has never exceeded that of three years old horses and has even gone below the rate of two years old horses since 2018. In addition to the rapid increase in the number of horses that have just started racing, the increase in the number of horses in the upper age group is proportionally behind, which is also true for Arabian horses. These findings, which are valid for both races, indicate that young horses participating in races have problems reaching the upper age groups, meaning their racing lives are shortened. The most critical finding in the study is the decrease in the number of racehorses reaching the upper age group. In order to concretize this finding, the distribution of injury data obtained from the Hippodrome Equine Hospital records between 2018-2023 according to breed and age groups was used. Detailed data on the distribution of injuries due to racing and training have been published previously (Yildirim et al., 2025). While 32 percent of injuries to Thoroughbred horses occur at the age of two, 52.83 percent of these injuries end their racing career. In Arabian horses, 48 percent of the total injuries were seen in the first year of racing life, and 43.69 percent of these injuries were of a nature that would end their racing life. While fractures remained the most prevalent injury in Thoroughbreds across all age groups, tendon injuries in Arabians peaked at age three and showed a declining trend in subsequent years. These variations are likely influenced by differences in conformation between the two breeds, as well as the age at which they commence their racing careers (Yıldırım, 2014; Yıldırım and Erden, 2023). The breeding quality and preferability of horses decrease when horses with superior performance that are interrupted by injuries and have a short racing life are used in production. As a result, although there is an increase in the number of young horses, the proportion of horses running in the upper age groups decreases. In this case, the horse racing industry suffers a loss in earnings for both the running horse and the breeder.

In order to raise horses that are not interrupted by injuries and have a successful and long racing life, in addition to race performance, conformation, that is, the selection of horses with ideal structure, is of critical importance. In addition to increasing the number of horses with ideal body structures in the sector, feeding, training, and race planning should be made in accordance with the individual athletic characteristics of the horses. The racehorse industry worldwide has a long-standing reputation for feeding and care strategies based on traditional methods. This situation is further strengthened by strong family ties and generational influences. While younger generations are becoming successful trainers, care-feeding strategies are strongly influenced by previous generations, with little emphasis often being placed on feeding and care techniques that have developed in line with the latest research on horse health and performance (Wood et al., 2020). It is thought that the use of more advanced techniques in line with scientific developments in the care-management conditions and nutrition of racehorse breeding will make significant contributions to improving the current situation.

When the average winning rates of horses running in different age groups were examined, the winning rates of Thoroughbred horses were 37 percent, 46 percent, and 45 percent, respectively, in the two, three, four, and older age groups between 1997 and 2022. In Arabian horses, between 2003 and 2022, these rates were calculated as 45 percent, 45 percent, and 53 percent in the three, four, five, and older age

groups. When the average number of wins per horse in different age groups was calculated at the same year intervals, it was seen that the number of wins per horse increased with increasing age.

The increase in the market value of the racehorse industry is a positive development for all related sectors, as well as racehorse breeding. It is thought that increasing the share of horse owners from this economic development will increase the efforts to obtain more successful racehorses. In this context, the earnings per running horse of horses registered with TJC between 1997 and 2022 were evaluated. When the average earnings per horse are examined, it is observed that the purchasing power has decreased. This decrease may cause the deterioration of the care and feeding conditions of horses, a decrease in breeders' interest in the sector, and a decline in breeding. This may result in a decrease in the quality of racehorses. For the process to be more positive in racehorses and to increase the number of high-quality and successful horses, the average earnings per horse should be increased. In addition to increasing the average earnings per horse, the focus should be on increasing the number of successful horses and their ratio within the total number of horses. It is thought that if the average earnings per horse are higher in the upper age groups, breeders and horse owners will turn to horses that will maintain their superior performance for many years. This trend results in an increase in the quality of running horses, a decrease in economic losses due to injuries and similar reasons, and progress in animal welfare and rights. In other words, orientation towards the ideal is encouraged within this industry.

Earnings of racehorses by age group are an important metric. In this context, assessing earnings by age group is necessary for a more thorough review. For this reason, the average earnings per horse at TJC hippodromes were calculated based on breed and age groupings. In 2008, the prices for Thoroughbred horses aged two, three, four, and older were US $15.567, US $30.994, and US $40.296, respectively. It is important to note that by 2022, earnings will have reduced to US $5.930, US $9.596, and US $10.362, in that sequence. In 2008, the earnings of Arabian horses aged three, four, five, and older were US $21.033, US $33.834, and US $47.267, respectively. By 2022, earnings had reduced to US $6.215, US $8.275, and US $11.092, respectively. As a result, the corresponding shift in average earnings per horse in the higher age group relative to total earnings was determined. In 2008, the four years and older age group accounted for 46.39 percent of total Thoroughbred horse earnings, whereas it will account for 40.02 percent by 2022. In Arabian horses aged five years and older, the figure was 46.27 percent in 2008 and 43.35 percent in 2022. The reduced earnings of horses running in the upper age categories are seen to constitute a risk to meeting the production aim of durable horses with extended racing careers and outstanding success.

*Limitation:* In this study, age, race, and income data were evaluated at certain intervals in the Türkiye sample. The relationship between disability, age, race, and earning was evaluated using data from a hospital where approximately 20% of the total racehorses are affiliated. More comprehensive evaluations and results can be obtained in further studies with larger groups from many countries.

## CONCLUSION

This study provides summary information about the numerical change of Thoroughbred and Arabian racehorses in the example of Türkiye, the age distributions of the horses participating in the races, as well as their earnings status. It is thought that these results, supported by analysis-based determinations and comparisons as well as graphics and descriptive statistics, will contribute to the definition of the current situation of the racing industry and the problems that may arise in the future. When the data between 1975 and 2023 is evaluated, the findings indicate that the racehorse industry has achieved significant growth in the number of running horses. However, in terms of the development of the sector, it is thought that the increase in the number of running horses should be accompanied by an uninterrupted and long racing life. The decrease in the ratio of horses competing in the upper age groups within the total number of horses and in earnings is also an important issue and poses a threat to the future of the sector. To improve decreasing earnings, horses with developmental delays due to breeding and feeding and limited athletic ability due to conformation defects should be considered early and eliminated before starting racing life. Because there is an important relationship between susceptibility to injury and horse's conformation and its athletic ability. It will be possible to raise horses with ideal conformation who do not experience injuries and have a successful and lengthy racing career. It will also be possible to raise horses with high levels of health and racing performance by using the right care and feeding techniques. It is thought that

increasing the number of studies on the mentioned subjects will benefit the development of the racing industry.

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# Effect of agomelatine on ischemic damage in experimental head trauma model in rats

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## ABSTRACT

**Objective:** This study aims to investigate the effect of agomelatine on ischemic injury in an experimentally created head trauma model in rats.

**Materials and Methods:** Groups: 1) control (C) incisions were made to the coronal and lambdoid sutures and a steel disc was placed without creating a controlled impact acceleration model; 2) head trauma (HT) did not receive any treatment after head trauma with a controlled impact acceleration model; 3) agomelatine (A) 20 mg/kg oral agomelatine given for 7 days after incisions were made to the coronal and lambdoid sutures and a steel disc was placed without creating a controlled impact acceleration model; 4) head trauma+agomelatine (HT-A) 20 mg/kg oral agomelatine given for 7 d after head trauma with a controlled impact acceleration model. The rats were humanely euthanized after the study by ethical protocols, and blood and tissue samples were taken. Biochemical (tumor necrosis factor [TNF]-$\alpha$ and interleukin [IL]-6) and histopathological analyses (terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL]) were conducted from these samples.

**Results:** A significant difference was found in TNF-$\alpha$ between the HT and HT-A groups ($p<0.05$). There was a statistically significant increase in IL-6 in the HT group compared to that in the HT-A group ($p<0.01$). In the histopathological analyses, a decrease in TUNEL-positive cells was observed in the HT-A group compared to that in the HT group

**Conclusion:** As a result, a decrease in both apoptotic cells and inflammatory responses was observed in the HT-A group. Therefore, more studies should be included.

***Keywords:*** *Agomelatine, Head Trauma, IL-6, Rat, TNF-α*

## INTRODUCTION

Head trauma is the most common type that is immediately admitted to the hospital and is related to long-term morbidity and mortality. Head trauma is most frequently caused by car crashes, falls, gunshot wounds, blunt object attacks, sports injuries, and leisure activities (Hardman and Manoukian, 2002). These types of injuries are most common in people between the ages of 15 and 24 and are more common in males. Conditions that increase the harmful effects of trauma include alcohol, previous head trauma, and a history of meningitis, epilepsy, mental retardation, and psychiatric disorders (Adams et al., 1980; Hardman and Manoukian, 2002).

A blow to the head can cause fractures or cracking of the calvaria bones. These fractures can be linear, star, segmental, or collapse, and the intracranial soft tissues receive the impact force. Within milliseconds following the impact, the brain tissue

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is subject to deformation, tears, and crushing. During this severe trauma, the surface of the brain strikes the irregular bony interior of the skull. The areas of the brain damaged by head trauma are usually both adjacent (impact) and opposite (opposite), and the impact results in immediate damage to the neuronal cell bodies, intracranial vasculature, axons, and glial tissue (Kinnunen et al., 2010; Sundman et al., 2015). It is believed that the shock impulse generated by the impact depolarizes the cells through membrane mechanoreceptors. A severe concussion can cause a temporary coma due to impact-induced neuronal depolarization that can spread throughout the cortex. An increase in intracellular calcium concentration, initiated by mechanical depolarization, occurs in the axons, causing cessation of axoplasmic flow and resulting in the death of the distal axon within hours to days (Bayir et al., 2003; MacKay, 2004).

Head trauma is also characterized by other injuries, such as cerebral hypotension, hypoxia, ischemia, excitotoxicity, physiological hormone disruption, and inflammation (Chesnut et al., 1993; Schmidt et al., 2005; Wagner et al., 2012; Kumar et al., 2015). The molecular mechanisms associated with post-traumatic injury cascades are not fully known; however, the activation of neuroinflammation resulting from traumatic brain injury is triggered by the central nervous system (CNS) and peripheral inflammatory responses (Schmidt et al., 2005). These inflammatory responses include proinflammatory cytokines, chemokines, and cell-adhesion molecules (Feuerstein et al., 1998; Ghirnikar et al., 1998; Kumar et al., 2015).

In addition, cytokines exert a neuroprotective effect by reducing angiogenic, neurotrophic, and CNS damage (Morganti-Kossmann et al., 2010; Jeong et al., 2013). Although controlled by physiological mechanisms, chronic post-traumatic disorders and an increase in inflammation may cause blood-brain barrier and neuronal cell dysfunction or death (Shlosberg et al., 2010; Kumar et al., 2015). In addition, previous studies have reported that inflammation and depression are related results (Raison et al., 2006; Dantzer et al., 2011; Haroon et al., 2012). In particular, it is known that depression accompanies both immune suppression and immune activation (Irwin and Miller, 2007). The literature also reports that after depression the concentration of interleukin (IL)-6 and tumor necrosis factor (TNF)-$\alpha$ and proinflammatory cytokines increase in blood and cerebrospinal fluid (Molteni et al., 2013).

A melatonin receptor agonist, agomelatine is a new antidepressant with anti-inflammatory, antioxidant, and antiapoptotic properties (Molteni et al., 2013). A study reported that it may be a new drug candidate against acute neuronal damage secondary to septic disease, as it appears to have antioxidative (Nesterowicz et al., 2023) and anti-inflammatory (Yang et al., 2022) effects through deactivation of NF-kB and increasing SIRT-1 levels (Savran et al., 2020).

A synthetic melatonin analog called agomelatine has drawn interest due to possible neuroprotective benefits, especially when ischemia damage is present. Its antioxidant qualities and function as an agonist of melatonin receptors (MT1 and MT2) are the main explanations for its modes of action. Agomelatine has been shown to reduce ischemia-reperfusion injury, which is important in myocardial infarction and stroke (Hong et al., 2021; Yao et al., 2019; Chumboatong et al., 2017).

This study investigates the effect of agomelatine (N-[2-(7-methoxynaphthalen-1-yl) ethyl] acetamide), whose formula is $C_{15}H_{17}NO_2$, known as an anti-depressant, on inflammation and apoptosis in experimentally induced head trauma. Our working hypothesis is to answer the question of whether agomelatine can be an alternative treatment agent for head trauma.

## MATERIALS and METHODS

Four-month-old male Sprague Dawley rats (n=24) weighing between 200-250 grams apiece were used in the investigation. Rats were obtained from Kafkas University Experimental Animals Application and Research Center. Four groups of six rats each were used to categorize the rats:

1. Control (C) incisions were made to the coronal and lambdoid sutures and a steel disc was placed without creating a controlled-impact acceleration model.
2. Head trauma (HT) did not receive any treatment after head trauma with a controlled-impact acceleration model (Albert-Weissenberger and Sirén 2010).
3. Agomelatine (A) given 20 mg/kg oral agomelatine (Les Laboratoires Servier, France) for 7 day after incisions on coronal and lambdoid sutures and steel disc placement without creating a controlled-impact acceleration model (Albert-Weissenberger and Sirén 2010).

4. Head trauma + agomelatine (HT-A) given 20 mg/kg oral agomelatine for 7 day after head trauma with a controlled–impact acceleration model (Albert-Weissenberger and Sirén 2010).

Before the procedure, all rats were anesthetized using intramuscular (i.m.) delivery of 75 mg/kg ketamine hydrochloride (Pfizer, Istanbul, Türkiye) and 15 mg/kg xylazine (BioVeta, Ankara, Türkiye). After anesthesia, the rats were placed on a flat surface in the prone position and a skin incision was made in the coronal and lambdoid sutures. A 10-mm-diameter and 3-mm-thick steel disc was placed on the skull to prevent compression fractures. A head injury model was then created with a free fall of 300 g mass from a height of 1 m. The incisions were primarily sutured.

At the final step of the study (7 days), the rats were euthanized under anesthesia with 75 mg/kg ketamine hydrochloride and 15 mg/kg xylazine IM by the conditions. Samples of tissue (Brain) and blood (intracardiac) were obtained from the rats. The serum from the blood samples was kept at -20 °C until the analyses were completed after they were centrifuged for five minutes at 3000 rpm. The tissue samples taken for histopathological analyses were stored in a 10% formaldehyde solution.

**Biochemical analysis**

The Enzyme-Linked ImmunoSorbent Assay (ELISA-Elabscience®, Houston, TX, USA) manufacturer's methods were utilized to ascertain the amounts of serum TNF-$\alpha$ and IL-6.

**Histopathological analysis**

For the histopathological examination of brain tissue in all groups, the tissue was stained after being kept in 10% formaldehyde for 72 h. The samples were dehydrated by putting them through a series of escalating alcohol concentrations after being cleaned in running water for four hours. They were then made transparent by passing then through xylene, infiltrated by passing then through a series of paraffin, and embedded into paraffin blocks (5μm). The tissues were stained using hematoxylin-eosin (H&E) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

**TUNEL staining**

The TUNEL apoptosis test kit (HRP-DAP) (Elabscience; Cat no: E-CK-A331) was utilized in accordance with the manufacturer's instructions to identify cellular apoptosis in the brain tissues from all groups. From each slide, five fields were chosen at random, and the number of TUNEL-positive cells in each field was counted semiquantitatively. TUNEL staining was characterized by dark staining of apoptotic cells. The ratio of apoptotic cells was evaluated by dividing the number of apoptotic cells by the number of all cells in that area by selecting a 10x10 $mm^2$ area using the Image J program (Wu et al., 2020).

An Olympus BX43 (Evident Corporation Tokyo, Japan) microscope was used to analyze the TUNEL-positive cells, and an Olympus DP21 (Evident Corporation Tokyo, Japan) camera was used to take pictures of them.

**Statistical analysis**

Before the study, a power analysis was performed using G-Power 3.1.9.7 (Heinrich Heine University Düsseldorf, Germany). Based on the analyses, the sample size (at least six samples) was decided according to the test power of 0.96 and the significance level of 0.05. A one-way analysis of variance was conducted on all Histopathological parameters to test whether there was a difference among the four groups. Tukey's test was used for mean discrimination among the groups. Hence, $p<0.05$ was used to classify them as statistically significant. The software GraphPad 8.1 (GraphPad Software San Diego, CA, USA) was used for all biochemical analyses. Using SPSS version 22 (IBM Corp. Armonk, NY, USA), Tukey's test of one-way analysis of variance was used to count the TUNEL-positive cells in each group.

**Ethical approval**

The present study received permission from Kafkas University Animal Experimental Animal Ethics Committee (2020/72).

## RESULTS

**Biochemical analysis results**

In the biochemical analyses, TNF-$\alpha$ and IL-6 parameters were measured to determine inflammation. Within this context, a statistically significant increase in TNF-$\alpha$ was observed in the HT group when the C ($p<0.001$), A ($p<0.0001$), and HT-A ($p<0.05$) groups were compared with the HT group. Additionally, a significant difference ($p<0.05$) was seen between the A and HT-A groups. Between the C and A groups, there was no discernible difference ($p>0.05$) (Figure 1).

A significant increase in IL-6 was seen in the HT group compared to that in the C ($p<0.05$), A ($p<0.01$), and HT-A groups ($p<0.01$). Between the C,

A, and HT-A groups, there was no statistical difference (p>0.05) (Figure 1).

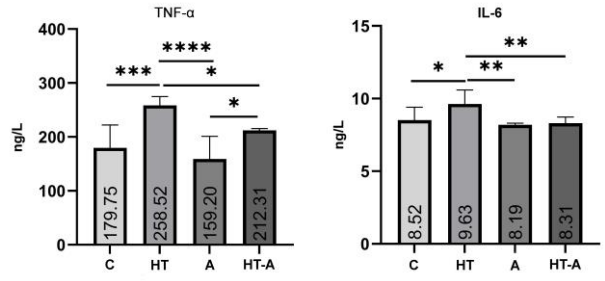


**Figure 1.** Means and Standard Deviation for biochemical parameters among the four groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Histopathological analysis results**

In the H&E staining, local lesioned areas were seen both in the HT group and in the HT-A group. No lesion areas were observed in C and A groups (Figure 2).

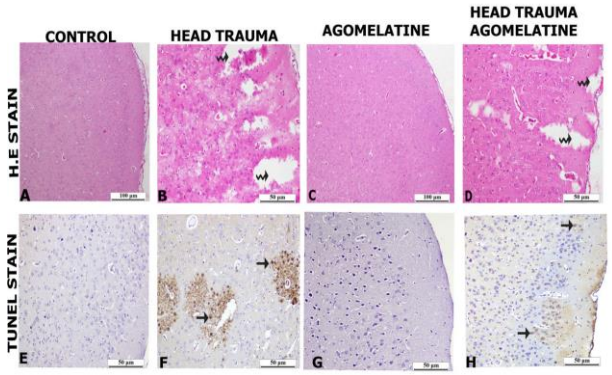


**Figure 2.** A) Control group hematoxylin and eosin (H&E) staining, 10x. B) Head trauma (HT) group normal arrow: brain defect, 20x. C) A without damage, 10x. D) Head trauma and given agomelatine (HT-A) normal arrow: brain defects, 20x. E) Control group terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, 20x. F) HT curved arrow: TUNEL-positive cells, 20x. G) A without damage, 20x. H) HT-A curved arrow: TUNEL-positive cells, 20x.

Tunnel staining to determine apoptosis showed that apoptotic cells increased in both HT and HT-A groups. However, these cells increased more in HT group than HT-A group. There was no statistical difference between the C and A groups, according to the statistical results (p>0.05). When comparing the HT group to the C, A and HT-A groups, there was a significant difference (p<0.05). There was statistical difference between the C and HT-A groups, according to the statistical results (p<0.05). Additionally, a significant difference (p<0.05) was seen between the HT and HT-A groups (Figure 3).

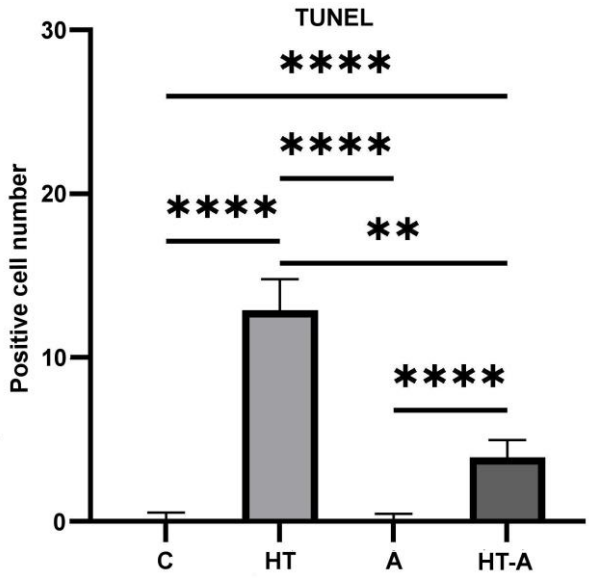


**Figure 3.** Means and Standard Deviation for the number of positive cells in tunnel staining among the four groups. **p<0.01, ****p<0.0001

## DISCUSSION

In both developed and developing nations, head trauma is the leading cause of morbidity and mortality for individuals under 40 years of age. 10% is thought to be the death rate for moderate trauma and 50% for severe trauma. Worldwide, the prevalence of head trauma is reported to be approximately 13/100,000 (Roozenbeek et al., 2013; Mirzaei et al., 2016; Salehpour et al., 2018; Sabouri et al., 2020).

There are two stages of post-traumatic injury. In the first stage, injury occurs in the brain, which causes cerebral microhemorrhage resulting from mechanical damage (Byrnes et al., 2012; Roozenbeek et al., 2013). A decline in cerebral blood flow and oxygenation, harm to the brain's vascular architecture and autoregulation, cytoskeletal disorder, edema, ischemia reperfusion injury, oxidative cell damage, metabolic dysfunction, and ionic hemostasis problem all occur in the second stage (Bramlett and Dietrich, 2002; Sabouri et al., 2020). These conditions cause inflammation and activation of endothelial cells. Additionally, they cause inflammatory cytokines and adhesion factors to rise. The ensuing inflammation speeds up neurodegeneration and neuroinflammation, stimulates glial cells and leukocytes, and causes the production of inflammatory mediators (Davalos et al., 2005; Mirzaei et al., 2016; Salehpour et al., 2018).

Pro- and anti-inflammatory cytokines and chemokines are activated in head trauma. After

head trauma, there is a significant increase in IL-6, IL-1β, and TNF-α astrocytosis and neuroinflammation. The resulting increase occurs approximately 1 h after neurotrauma and increases for up to three weeks after trauma (Liu et al., 2013; Tompkins et al., 2013).

Astrocytosis is the process by which astrocytes, the main glial cells in the central nervous system, become activated in reaction to inflammation or damage. Pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are frequently released in conjunction with this activation, which can worsen neuroinflammation and cause neuronal injury (Fei et al., 2021; Hu et al., 2021). Fei et al. (2021), for example, noted that enhanced release of these cytokines after neuroinflammation after intracerebral hemorrhage exacerbates early brain injury. Similarly, Hu et al. (2021) observed that increased levels of IL-1β, IL-6, and TNF-α contribute to the inflammatory response, and that traumatic brain injury intensifies neuroinflammation. Like our study, an increase in IL-6 and TNF-α levels was observed in the HT group.

Studies have found that depression increases the level of cytokines (Gałecki et al., 2018) and that inflammation plays a very important role in the pathophysiology of major depression (Maes, 1995; Maes et al., 2011; Leonard, 2014). It is also known that there is a relationship between the serum levels of proinflammatory cytokines IL-1 and IL-6, interferon-gamma, and TNF-α and symptoms of depression. In addition, it has been reported that the administration of antidepressants to patients increases the concentration of anti-inflammatory cytokines such as interleukin-10, while the negative effects of pro-inflammatory cytokines decrease (Colin et al., 2003).

Agomelatine was synthesized during the studies to develop more effective and safer antidepressant drugs in the mid-2000s. It is a synthetic analog of the hormone melatonin, which is synthesized in and released from the pineal gland (Uzbay, 2011). Agomelatine is an agonist of melatonergic MT1 and MT2 receptors as well as an antagonist of the 5-HT2C receptor (Popoli, 2009). Melatonin is a hormone with many regulatory functions that have important effects on the central nervous system. It has remarkable properties as an anti-inflammatory, antioxidant, and anti-apoptotic agent (Yahyavi-Firouz-Abadi et al., 2007). Studies have shown that melatonin regulates its anti-inflammatory effects on both pro- and anti-inflammatory cytokines in various diseases (Park et al., 2007; Mauriz et al., 2013; Habtemariam et al., 2016; Favero et al., 2017; Yu et al., 2017). Carrillo-Vico et al. (2003) have reported that the presence of melatonin receptors regulates the anti-inflammatory pathway by inhibiting TNF-α release. Similarly, Mahmood et al. (2010) have demonstrated the anti-inflammatory effect of melatonin at different doses in chronic inflammation that they created experimentally. It is known that chronic inflammation occurs in multiple sclerosis, and in the initial pathogenesis of this disease, a strong inflammatory-demyelinating process develops. Kang et al. (2009) have reported that melatonin supplements given externally during the inflammatory-demyelinating process improve the myelin status of nerve fibers. In the present study, it is thought that agomelatine, a melatonin agonist, exhibited an anti-inflammatory effect and showed an effect like the study of Kang et al. (2009).

Agomelatine inhibits 5-HT2c receptors in the frontal cortex, which causes an indirect increase in dopamine and norepinephrine (Karamustafalıoğlu and Baran, 2012). Dopamine plays a role in controlling apoptosis in cells that are not neurons as well as neurons. Additionally, dopamine concentrations stimulate the synthesis of anti-inflammatory mediators and prevent the overexpression of adhesion molecules, cytokines, and chemokines brought on by inflammation (Beck et al., 2004). In the present study, both cytokines and anti-inflammation were revealed, and the mechanism of apoptosis was revealed histopathologically. This evidence is believed to reveal that agomelatine exerts its effects by increasing dopamine levels.

It is known that norepinephrine together with cortisone has an anti-inflammatory effect by increasing intracellular CAMP, protein kinase A, glucocorticoid receptors, and β-adrenoreceptors (Straub et al., 2002). By increasing norepinephrine, agomelatine also indirectly increases the anti-inflammatory effect. In this study, the anti-inflammatory effect was observed in the HT-A group.

## CONCLUSION

A positive effect was obtained in the HT-A rats. This is thought to be because agomelatine is an antagonist of the 5-HT2C receptor and an agonist of the melatonergic MT1 and MT2 receptors. It is believed that agomelatine, a melatonin agonist, exhibits its therapeutic effect against trauma as an

anti-inflammatory. Since it is an antagonist of the 5-HT2C receptor, it is also thought that, by indirectly raising norepinephrine and dopamine in the frontal brain, it may also exercise its benefits through the anti-inflammatory and anti-apoptotic properties of both dopamine and norepinephrine.

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# Detection of *Mycoplasma agalactiae* in small ruminants in eastern of Türkiye

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## ABSTRACT

**Objective:** In this study, it was aimed to investigate the presence of *Mycoplasma* (*M.*) *agalactiae* in small ruminants with clinical symptoms such as milk withdrawal, milk reduction, and discoloration by molecular techniques in the Bitlis region.

**Materials and Methods:** For this purpose, 120 samples were collected from milk, blood, eye fluid, ear fluid, and joint fluid from animals, and the clinical symptoms were shown in the Bitlis Centre and Districts. DNA isolation of the collected samples was performed according to commercial kit protocols. Then, a Polymerase Chain Reaction (PCR) step was performed with primer pairs (*uvrC*) synthesized specifically for *M. agalactia*.

**Results:** For the *uvrC* gene region, 36.55% (34/93) of the milk samples and 28.30% of the whole samples were positive. It was determined that 33 (%32.4) of the positive samples originated from goat milk and 1 (%5,6) from sheep milk sample. As a result, the positivity rate determined by the molecular method in this study was higher than the classical methods (isolation, identification). This study is the first study investigating the presence of *M. agalactia* in the Bitlis region.

**Conclusion:** Further studies are needed to determine the prevalence of the agent in the region. In addition, further studies are required to control and eradicate the circulating agent in the area.

***Keywords:*** *Small Ruminant, Mycoplasma, PCR, Mastitis*

## INTRODUCTION

Infectious agalactia in sheep and goats is a disease that has been known for about two centuries (WOAH, 2018). The infection is seen in many world regions, especially in the Mediterranean basin (Tardy et al., 2012; WOAH, 2018). Morbidity and mortality rates can reach up to 100% in infectious agalactia infections (Yatoo et al., 2018). Infectious agalactosis occurs seasonally in an enzootic fashion and primarily affects sheep and goats. It usually causes mastitis in lactating sheep and goats, while it usually manifests itself with arthritis, keratoconjunctivitis, and respiratory system problems in male animals and offspring. Severe respiratory system infections may cause the death of the offspring. Diagnosis of infectious agalactia agents is based on isolation and identification by conventional culture method. The disease is combated with antibiotic treatment and vaccination in the regions affected by the disease (Leonovich, 2024). Infectious agalactia primarily affects sheep and goats. The causative agents of the disease include *M. agalactiae*, *M. capricolum* subsp. *capricolum* (Mcc) and *M. mycoides* subsp. *capri* (Mmc) (CFSPH, 2018; Dawood et al., 2022; Heller et al., 2015). In addition, due to the similar clinical picture, especially in goats, Mmc, Mcc, and Mp infections have been recognised as disease agents for about 30 years. However, due to the acceptance of *M.*

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*agalactiae* as the causative agent of the disease in national and international animal disease regulations, it was proposed to remove the other 3 agents from the OIE list in 2021, except for *M. agalactiae* (Migliore et al., 2021).

Considering the rugged geographical structure, varying climatic conditions, and socio-economic structure in different regions of our country, ovine breeding has come to the forefront. Although small ruminant breeding is more labor intensive than other types of animal breeding, its financial income is higher. Among the livestock, the fact that goats can better use areas unsuitable for agriculture and rocky areas compared to other animals reveals the importance of small ruminant breeding (Karatekeli, 2020). The goat population is much higher than the sheep population in the Bitlis region (Ertaş, 2019). There is no research on the presence of *M. agalactiae*, one of the infectious agalactia agents seen in sheep and goats in the Bitlis region.

The most frequently used gene regions in the molecular diagnosis of mycoplasmas are *adk*, *gpsA*, *polC*, and *uvrC*. However, it has been reported that these genes can be used as clinical detection markers to distinguish between *M. bovis* and *M. agalactiae*. These genes can be important markers for clinical diagnosis and reveal genetic differences between species. *uvrC* gene region confirms the study results and distinguishes between *M. agalactiae* and *M. bovis* agents. In recent studies, it was reported that point mutations were detected in the *M. bovis uvrC* gene, which may cause false negative PCR results to identify *M. bovis* strains (Sun et al., 2020; Yücetepe et al., 2022). Research has demonstrated the successful utilisation of the *uvrC* gene region for detecting and characterising *M. agalactiae* in small ruminants (Göçmen et al., 2016). In this study, it was aimed to investigate the presence and prevalence of *M. agalactiae*, one of the causative agents of infectious agalactiae in sheep and goats raised in Bitlis and its region, by using the molecular method of PCR and *uvrC* gene region. In addition, the study draws attention to the first study in which the disease agent was investigated using the molecular method in the region.

## MATERIALS and METHODS

### Material

A total of 120 samples (1 joint fluid, two ear swabs, five eye swabs, 19 blood, 93 milk) were collected from 113 different animals (18 sheep and 102 goats) showing clinically infectious agalactia symptoms (milk reduction, milk withdrawal, milk discoloration, keratoconjunctivitis, arthritis) in 46 different enterprises in 29 villages of 6 districts of Bitlis in 2024 (Table 1). Among the 93 milk samples collected, 89 were obtained from goats and 4 from sheep. The collected samples were brought to the laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Van Yuzuncu Yil University, Van Yuzuncu Yil University, as soon as possible under the cold chain.

**Table 1.** Distribution of samples according to species and location of collection

| Sample regions | Samples | | | | | Total |
|---|---|---|---|---|---|---|
| | Milk | Blood | Eye Swab | Ear Swab | Joint Fluid | |
| Centre | 27 | 10 | 1 | 0 | 0 | 38 |
| Mutki | 19 | 5 | 3 | 1 | 0 | 28 |
| Güroymak | 17 | 2 | 1 | 1 | 0 | 21 |
| Tatvan | 12 | 2 | 0 | 0 | 1 | 15 |
| Hizan | 9 | 0 | 0 | 0 | 0 | 9 |
| Ahlat | 9 | 0 | 0 | 0 | 0 | 9 |
| Toplam | 93 | 19 | 5 | 2 | 1 | 120 |

### Reference strains

The quality control analyses performed in the study used the *Mycoplasma agalactiae* AIK, NCTC 10123 strain obtained from Pendik Veterinary Control Institute, Mycoplasma Laboratory.

### Methods

After centrifugation at 16,000 rpm for 20 minutes for 93 milk samples taken in sterile tubes under aseptic conditions from animals with mastitis findings, the fat layer accumulated on the upper part and the clear liquid phase just below it was discarded with the help of an automatic pipette, and 1 ml of the

precipitate was transferred to sterile eppendorf tubes and stored at -20°C until used in the analyses. Swap samples were placed in vortexed tubes containing 2 ml of sterile PBS, and the liquid phase content was transferred to the liquid phase. Then, the tube with PBS was centrifuged at 2,500 rpm for 10 minutes, 1.5 ml of the supernatant was discarded, and the precipitate was transferred to sterile Eppendorf tubes and stored at -20°C until used in the analyses. Blood samples were collected in anticoagulated (EDTA) vacuum tubes (Greiner, Bio-One, Germany) and stored at +4°C until analyses.

**DNA isolation and amplification**

Genomic commercial kits were used for DNA extraction from the study's samples. Two different DNA kits were used for tissue (HY-DDNA-100, Hydra Biotechnology R&D, Van, Türkiye) and milk (Norgen Biotek Corporation, Ontario, Canada). Both DNA extraction procedures were performed according to the manufacturer's specifications.

The *uvrC* gene was selected as the specific primer for *M. agalactiae,* and the amplification length of the product to be obtained was synthesised as 1624 bases. Accordingly, the forward and reverse amino acid sequences of the *uvrC* gene are given in Table 2.

**Tablo 2.** Oligonucleotide sequences of primer pairs used in the PCR step

| Gen | Primers | Oligonucleotide sequences (5′-3′) | Size | Annealing T. | References |
|---|---|---|---|---|---|
| *uvrC* | MAGAUVRC1-L<br>MAGAUVRC1-R | CTCAAAAATACATCAACAAGC<br>CTTCAACTGATGCATCATAA | 1624bp | 60°C | (Subramaniam et al., 1998) |

**Table 3.** PCR mixture and heat cycling protocol were used to detect the *uvrC* gene region of *M. agalactiae* in the analysed samples.

| Component | Amount used | Heat cycling protocol | |
|---|---|---|---|
| 2x Taq PCR Master mix | 12.5 µl | 95°C - 5 min<br>94°C - 30 sec<br>60°C - 30 sec<br>72°C - 90 sec<br>72°C - 7 min | 33 Cycle |
| Primer -F (10 pmol/µl) | 0.5 µl | | |
| Primer -R (10 pmol/µl) | 0.5 µl | | |
| Deionised water | 8.5 µl | | |
| DNA | 3.0 µl | | |
| **Total** | **25 µl** | | |

PCR was performed using a 2x Taq PCR Master mix (K0171, Thermo, Lithuania) according to the procedures specified by the manufacturer. The samples' DNAs were analysed for the PCR step to amplify the *uvrC* gene region using the methods described by Subramani et al. (1998) (Subramaniam et al., 1998). The reaction components, mixtures, and heat cycling protocol for PCR of the synthesised primers were carried out in a Thermal Cycler (Corbett Research, Qiagen GmbH) (Table 3).

DNA obtained from *Mycoplasma agalactiae* AIK (NCTC 10123) reference strain was used as a positive control, and deionised water was used as a negative control. The PCR amplicons obtained were run on an agarose gel electrophoresis setup (Thermo Scientific, OwlR Easy Cast TM B1) using 2% agarose gel and then analysed under UV light in a Gel Imaging device (Genesis®).

**Statistical Analysis**

Descriptive statistical data were given as numbers and percentages. The evaluation of the disease according to districts and animal species was performed by chi-square test. Statistical analyses were performed with the SPSS Ver 26 package programme. The statistical significance level was accepted as $p<0.05$.

**Ethical consideration**

Van Yuzuncu Yil University Animal Research Local Ethics Committee (Decision no: 2023/ 09-06), Van, Türkiye, granted ethical permission for the study.

## RESULTS

In this study, in order to investigate the presence of the *uvrC* gene region of *M. agalactiae*, genomic DNA extraction was performed from a total of 120 specimens collected from the Bitlis region and its

districts and stored at -20˚C in the laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Van Yuzuncu Yil University. After DNA extraction, qPCR was performed with a commercial Master mix kit using appropriate (forward/reverse) primers. As a result of qPCR, the presence of nucleic acid for the *M. agalactiae uvrC* gene was detected in 34 samples out of 120 sheep and goat samples. No positivity was obtained in the study samples except milk. A 36.55% (34/93) positivity rate was detected in milk samples. The positivity rate was determined as 28.30% in all study samples. The agarose gel image of the amplicons of the analysed samples is shown in Figure 1.

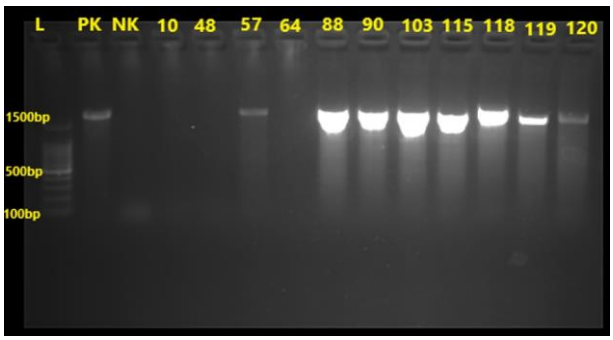


**Figure 1.** Gel Image of *uvrC* gene region (1-L=Ladder, 2-PK= Positive Control, 3-NK= Negative Control, 4-5-7= Negative Study Samples numbered 10, 48, 64, 6-8-9-10-11-12-13-14= Positive Study Samples numbered 57, 88, 90, 103, 115, 118, 119, 120)

**Table 4.** Distribution of *uvrC* gene detected in samples from districts

| Sample regions | Positive (%) | Negative (%) | Total | p |
|---|---|---|---|---|
| Centre | 15 (39.5) | 23 (60.5) | 38 | |
| Mutki | 6 (21.4) | 22 (78.6) | 28 | |
| Güroymak | 5 (23.8) | 16 (76.2) | 21 | |
| Tatvan | 2 (13.3) | 13 (86.7) | 15 | 0.407 |
| Hizan | 3 (33.3) | 6 (66.7) | 9 | |
| Ahlat | 3 (33.3) | 6 (66.7) | 9 | |
| Total | 34 (28.3) | 86 (71.7) | 120 | |

**Table 5**. Distribution of the presence of the *uvrC* gene region according to animal species

| Animal species | Positive (%) | Negative (%) | Total | p |
|---|---|---|---|---|
| Sheep | 1 (5.6) | 17 (94.4) | 18 | |
| Goat | 33 (32.4) | 69 (67.6) | 102 | 0.022* |
| Total | 34 (28.3) | 86 (71.7) | 120 | |

*: $p<0.05$

**Statistical Analysis Results**

The chi-square method analyses showed no statistically significant difference in *uvrC* gene regions between the districts (Table 4).

For the *uvrC* gene region, the highest positivity rate (39.5%) was observed in Bitlis-Centre, while the highest negativity rate (86.7%) was determined in the Tatvan district. The reason statistically significant differences could not be determined in the evaluation of the *uvrC* gene was the small number of samples collected in the Tatvan, Hizan, and Ahlat districts, which limited the study.

In addition, a chi-square test was performed separately for *uvrC* gene regions to determine whether there are differences between species. The results are presented in Table 5.

For the *uvrC* gene region, the positivity rate in goats was 32.4%, while the negativity rate in sheep was 94.4%. Although the low number of sheep in the study may limit the chi-square analyses, no statistically significant difference was detected between sheep and goats (*uvrC*= $p<0.022$).

## DISCUSSION

*M. agalactiae* is an important bacterial pathogen that primarily affects small ruminants, mainly sheep and goats, and causes contagious agalactiae syndrome. *M. agalactiae* is spread primarily through direct contact and contaminated milk, and infected animals often become chronic carriers that can shed the pathogen intermittently (Migliore et al., 2024). It is emphasised that live attenuated vaccination is successfully applied in Türkiye, where the disease

is endemic (Dudek et al., 2022). The disease is particularly prevalent in the East and Southeast of Türkiye, where small ruminant farms are dense and considered an important part of the agricultural economy (Ocak et al., 2023). According to the literature, the disease agent is endemic in many provinces of Türkiye, affecting small ruminant herds, and its seroprevalence varies between 5% and 50%. In addition, studies show that infection rates are higher in traditional farming than in modern farming. This is due to factors such as management practices and biosecurity measures applied in the enterprises (Jaye et al., 2021). This study used molecular methods to investigate the presence of *M. agalactiae* in small ruminants with clinical symptoms in Bitlis and its region. No data on the disease agent in this region existed before. Therefore, the data obtained from this study are considered important epidemiological data about the circulating disease in the region.

There are similar studies in Türkiye. In a study conducted in Mersin to determine *M. agalactiae* from goats with mastitis complaints by molecular and bacteriological methods, the identification of 10 (8%) isolates as molecular methods confirmed *Mycoplasma* spp. and all isolates (100%) were confirmed to be *Mycoplasma* spp. by 16S rRNA PCR. Of the 10 isolates identified based on the genus, 7 (70%) were identified as *M. agalactiae* as a result of *M. agalactiae* 16S rRNA-specific PCR (Uluganligil, 2019). In another study conducted in Elazig, haematological and biochemical parameters were determined in goats with clinical signs of contagious agalactiae caused by *M. agalactiae*. Although the concentrations of biochemical parameters were emphasised in the study, it was reported that *M. agalactiae* was detected as positive by PCR (Kizil & Ozdemir, 2006). In a study investigating the presence of infectious agalactiae in the Isparta and Afyonkarahisar regions in 2018, *Mycoplasma* spp. was bacteriologically isolated from only 3 nasal swabs (1.03%). PCR was performed with the *polC* gene region for *M. agalactiae* for 3 isolates isolated as *Mycoplasma* spp. but the agent could not be detected (Karatekeli, 2020). A study conducted on sheep and goats from Bursa, Balıkesir, Çanakkale, and Edirne provinces aimed to determine the presence of contagious agalactia disease using bacteriological and molecular methods. It was determined that 29 (8,55%) isolates were positive for *Mycoplasma* spp. of these, 25 (7.37%) were identified as *M. agalactiae*. In molecular diagnosis, it was reported that 9.14% were positive for *M. agalactiae* as a result of *polC*-PCR. When PCR findings were compared with bacteriological findings, it was reported that five milk samples and one lung sample were positive for *M. agalactiae* by *polC*-PCR but negative by culture. According to *polC*-PCR results, 14.19% of milk samples, 13.33% of joint fluid samples, 2.72% of eye swab samples, and 50% of lung samples were positive. As a result of the study, it was emphasised that *M. agalactiae* was the primary causative agent of infectious agalactiae (Göçmen et al., 2015). A previous study conducted in the same provinces detected the uvrC gene region in 22 (9.4%) of 234 samples. This research confirmed the presence of both *M. agalactiae* and other *Mycoplasma* species in goats, highlighting the necessity for implementing effective control strategies against infectious agalactia and various *Mycoplasma* species throughout Türkiye (Göçmen et al., 2016). In another study in the Ağrı-Diyadin region, the seroprevalence of ewes during the lambing period was investigated. As the results of the study, it was reported that 1 (0.207%) of the 482 blood samples taken was seropositive and belonged to a 6-year-old ewe showing mastitis symptoms (Yaşar, 2008).

Our study is not similar to those of Kizil and Ozdemir (2006) and Yaşar (2008). The reason for this difference seems to be using serological methods in other studies. Because it is known that molecular methods, such as PCR, are more sensitive than serological methods (Balachandra et al., 2021). In addition, classical bacteriological isolation and identification methods were not used in our study. The studies of Göçmen et al. (2015), Uluganlıgil (2019), and Karatekeli (2020) are relatively similar to the method used in our study. However, there are differences in the positive rates detected in these studies. For example, in Uluganlıgil (2019), the fact that only five different herds were collected in Uluganlıgil (2019) explains the low positive rate and suggests that this and the other two studies may be due to the fact that the disease agent circulates at a lower rate in the west (Ocak et al., 2023). It is also challenging to culture *Mycoplasma* bacteria. For example, the use of antibiotics in the diseased animal or the inability to bring the suspicious sample to the laboratory under suitable conditions are seen as disadvantages in isolation. In addition, our study's absence of positive results from swabs and blood samples may be attributed to the limited sample size. The exclusive detection of positive results in milk samples clearly demonstrates that the pathogen is present in milk from clinically

symptomatic animals and is actively shed through this medium.

## CONCLUSION

These findings have important implications for animal health and regional livestock management, as *M. agalactiae* is the primary causative agent of infectious agalactia, which can cause significant economic losses in small ruminant farming. Further studies are needed to determine the prevalence of the agent in the region. The relatively high prevalence rate detected in this initial survey highlights the need for comprehensive surveillance programmes and the development of targeted control strategies. Further epidemiological studies are needed to better understand the distribution patterns, risk factors, and economic impacts of *M. agalactiae* infections in the Bitlis region, which will be crucial for implementing effective disease prevention and control measures. Future studies aim to expand the epidemiological data and provide more detailed information about the pathogen using whole genome sequence (WGS) analysis.

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# The effect of ovarian laterality on various reproductive parameters in Arabian mares

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## ABSTRACT

**Objective:** The present study aimed to investigate the effect of ovarian laterality on pregnancy outcomes and foal sex and to explore the role of age and parity in determining the ovulation side in Arabian mares.

**Materials and Methods:** Data were collected from 358 mares over a five-year period, during which 885 estrus cycles were monitored. The side of ovulation was determined using ultrasonography, and pregnancy was confirmed on days 14, 28, and 45 post ovulations. The mares were categorized into four age groups as 3-8, 9-13, 14-17, and ≥ 18 years.

**Results:** The laterality of ovulation was evenly distributed between the right (49.3%) and left (50.7%) ovaries, with no significant difference in pregnancy outcomes between the two sides. The majority of pregnancies resulted from the first ovulation (51.9%), and pregnancy outcomes did not differ significantly among the ovulatory sequences. Likewise, no significant relationship was found between the ovulation side and foal sex. The distribution of ovulation sides was similar across the age groups; however, a significant relationship was observed between the age group and the ovulation side that resulted in pregnancy ($p<0.05$), with a general decline in pregnancies observed between the younger and older age groups on both sides of ovulation. Younger mares had higher pregnancy rates, with left-side ovulation rates of 50.0% and 41.1% for mares aged 3–8 and 9–13 years, respectively, compared to 13.9% and 5.3% for mares aged 14–17 years and 18 years and older. Similarly, right-side ovulation rates were 43.7% and 33.5% for younger mares, which were higher than the rates of 11.0% and 1.3% for older mares. Additionally, the parous state of the mares did not significantly influence the side of ovulation or pregnancy outcomes.

**Conclusion:** Overall, in Arabian mares, ovarian laterality does not significantly influence pregnancy outcomes or foal sex and is not affected by parity or age, although a decrease in pregnancy rates with increasing mare age was evident. These results provide insights into the dynamics of ovarian laterality in Arabian mares and their impact on reproductive efficiency, which can guide the development of breeding strategies and assisted reproductive techniques.

***Keywords:*** *Age, Equine, Foal sex, Ovulation side, Parity, Pregnancy*

## INTRODUCTION

In many species, the laterality of reproduction holds significant biological and clinical importance in reproductive studies and management, as this phenomenon influences reproductive efficiency, embryo development, and the success of assisted reproductive techniques. While some species exhibit anatomical asymmetry between the right and left sides, others have functional inequality. For instance, in llamas and alpacas, gestation and

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embryo implantation occur solely in the left uterine horn, irrespective of the ovulation side, highlighting a significant asymmetry in which the left horn is larger than the right horn (Sumar and Adams, 2007; Ratto et al., 2020). The dominance of the left ovary is well documented in many avian species, as the right ovary typically remains underdeveloped and nonfunctional (Delehanty and O'Hearn, 2005; Wan et al., 2017; Peng et al., 2023).

In addition to these anatomical asymmetries, ovarian function may differ significantly between left and right ovaries. In golden hamsters, a right-side biased asymmetry in the number of corpora lutea has been noted, regardless of housing conditions (Chow and Chow, 1987; Fritzsche et al., 2000). Similarly, the predominance of the right ovary in producing more corpus luteum and harboring a greater quantity of larger follicles has been observed in several ruminant species, including sheep (Shabankareh et al., 2009) and goats (Grizelj et al., 2013), with similar patterns reported for cattle ovulation frequency (Cushman et al., 2005; Karamishabankareh et al., 2015). Furthermore, in humans, the right ovary demonstrates superior performance compared with the left ovary, exhibiting a higher rate of ovulation, producing oocytes with greater pregnancy potential, and showing better follicle recruitment and oocyte retrieval during in vitro fertilization. This pattern is consistently observed in both fertile and infertile women with healthy ovaries (Fukuda et al., 2000; Lan et al., 2010). However, some studies have suggested an equal distribution of ovulation between the two ovaries (Balasch et al., 1994; Fukuda et al., 1996). Contrasting evidence challenges the categorization of a species into a specific ovulation pattern, as evidenced by research on mares, which reported symmetrical (random) as well as asymmetrical patterns of ovulation favoring the left ovary (Wesson and Ginther, 1981; Sanderson, 1982; Ginther, 1983).

Studies on reproductive laterality have gained increasing interest because of their potential influence on sex-ratio determination. The uneven sex distribution observed in the uterus of cattle, gerbils, rabbits, and mice implies a consistent asymmetry in either the ovaries or uterine horns, which influences offspring sex distribution (Pearson, 1949; Wimsatt, 1975; Younglai et al., 1981; Baird and Birney, 1985; Endo et al., 1987; Hylan et al., 2009). Experimental surgical translocation in Mongolian gerbils has demonstrated that sex ratio inversion is primarily determined by the ovary of origin rather than the uterine environment, suggesting a mechanism involving biased selection of X- or Y-bearing sperm during fertilization (Clark et al., 1994). Comparable mechanisms have been identified in livestock (Grant and Chamley, 2007), with additional contributing factors including testosterone concentrations in follicular fluid (Grant and Irwin, 2005; Grant et al., 2008), insemination timing (Wehner et al., 1997; Martinez et al., 2004), and the maturation state of the oocyte (Dominko and First, 1997; Gutierrez-Adan, 1999). Furthermore, sex distribution appears to be breed-dependent, as studies have reported an asymmetric male-female distribution between uterine horns in beef cattle (Hylan et al., 2009; Giraldo et al., 2010), whereas Holstein dairy cows exhibit a more balanced distribution (Gharagozlou et al., 2013).

In the equine industry, where the ability to predetermine foal sex has significant economic and breeding implications, maternal factors, including age, body condition, and parity, have been closely linked to offspring sex (Cameron et al., 1999; Rezagholizadeh et al., 2015; Santos et al., 2015; Hall et al., 2022). For instance, mares under 15 years of age are more likely to produce male foals, whereas maiden and older mares tend to deliver female offspring, possibly due to mechanisms related to mitochondrial DNA and endometrial function (Kuhl et al., 2015; Flores, 2024). In addition, the Trivers-Willard hypothesis suggests that mares in better body condition at conception are more likely to produce male offspring. Other factors such as natural mating, gestation length, and genetic lineage have also been identified as potential factors influencing foal sex (Gutiérrez-Adán et al., 1999; Martinez et al., 2004; Cameron and Linklater, 2007; Hylan et al., 2009; Giraldo et al., 2010; Santos et al., 2015). Apart from these research, in a recent study conducted on Thoroughbred horses, left-sided ovulations resulted in a significantly higher proportion of male foals than right-sided ovulations (Rezagholizadeh et al., 2015). Collectively, these studies emphasize the relationship between reproductive laterality and sex determination, highlighting the need for further research on the underlying mechanisms of lateral asymmetry in this field.

Understanding the dynamics of asymmetry in ovarian structures and its influence on factors such as ovulation frequency, pregnancy outcomes, and potential offspring sex ratios is crucial for enhancing reproductive outcomes in both natural and assisted reproduction. However, in equines,

conflicting reports have challenged the categorization of mares into specific ovulatory patterns. In addition, to date, there has been a lack of research investigating the relationship between the ovulation side and foal sex distribution in Arabian mares, highlighting a significant gap in our understanding of reproductive laterality in this specific breed. Thus, this study aimed to investigate the effect of ovarian laterality on various reproductive parameters in Arabian mares, including pregnancy outcomes, foal sex, and the influence of age and parity on these parameters. Through the analysis of these aspects, this research sought to expand the knowledge base regarding ovarian functions in mares and provide valuable insights that could potentially inform breeding strategies and assisted reproductive techniques in the equine industry.

## MATERIALS and METHODS

### Animals and data collection

The study was conducted during the breeding season (February-June) over a five-year period, involving data obtained from 358 Arabian mares, which were monitored 546 times across various breeding seasons. The mares, housed individually at a Stud Farm in Eskişehir, Türkiye, were provided with alfalfa hay and commercial concentrate and had unlimited access to water. Only mares with no prior general health or gynecological issues were included. The mares, aged between 3 and 24 years, with previous offspring between 0 (maiden) and 14 years, were mated with a total of 46 stallions. In total, 885 estrus cycles (up to the 6$^{th}$ consecutive ovulation according to the timing within the breeding season) were monitored using ultrasonography with a 5–10 MHz linear endorectal L52x probe (Sonosite II, Fujifilm, Japan. The decision to breed mares was based on the presence of a preovulatory follicle with minimum diameter of 40 mm, the degree of uterine edema, cervical tone, and intrauterine fluid accumulation (Scarlet et al., 2023). The ovulation side was determined based on the location of the emerging corpus luteum following ovulation. Unilateral or bilateral double ovulations were excluded from the study. Pregnancy was determined ultrasonographically on the 14$^{th}$ day following ovulation and confirmed on days 28$^{th}$ and 45$^{th}$ days post-ovulation. Mares who did not maintain their pregnancies after the initial determination were excluded from the study. The sex of foals was recorded after birth. According to their age during the breeding season that they were monitored, mares were divided into four classes (Scoggin, 2015): 3-8 years old (n=243), 9-13 years old (n=206), 14-17 years old (n=74), and ≥18 years old (n=23).

### Statistical analyses

Descriptive statistics were calculated for the data, and considering the distributions of the data, percentages or "Median (Minimum-Maximum)" were provided. Statistical analysis was performed to evaluate differences in the proportions of the side of ovulation based on pregnancy outcome, ovulation sequence, foal sex, age group, and parity variables, as well as to evaluate the significance of differences in the proportions of the side of ovulation leading to pregnancy according to age group and parity variables using the chi-square test. Additionally, the chi-square test was used to evaluate the statistical significance of differences in the proportions of ovulatory sequences according to pregnancy status. As the assumptions for parametric tests were not met, the Mann-Whitney U test was employed to evaluate the statistical significance of differences in the side of ovulation and the side of ovulation resulting in pregnancy according to parity. A significance level of $p<0.05$ was set for all statistical analyses. Data were analyzed using the SPSS 27 software package.

### Ethical approval

This study was conducted in accordance with the decision of the Local Ethics Committee of Animal Experiments at Eskisehir Osmangazi University, dated 20.05.2024, and numbered 108.

## RESULTS

The laterality of all ovulations (n=885) was evenly distributed between right (n= 436, 49.3%) and left (n = 449, 50.7%) ovaries. Likewise, the sides of the ovulations that resulted in pregnancy were similar between the right (n=244, 51.4%) and left ovaries (n=231, 48.6%). There were no significant differences between the right and left ovulations in terms of pregnancy outcomes (Table 1).

**Table 1.** Pregnancy outcome regarding the side of the ovulation.

| Ovulation side | Pregnancy (%) | | P |
|---|---|---|---|
| | Non-pregnant | Pregnant | |
| Left ovary | 219 (48.8) | 231 (51.2) | 0.138 |
| Right ovary | 191 (43.8) | 244 (56.2) | |

The percentage of non-pregnant animals was 12.8%, and the majority of pregnancies resulted from the first ovulation (51.9%), followed by the second (22.9%), third (9.5%), and fourth to sixth ovulations (2.8%) of the breeding seasons. When all ovulations were considered, the pregnancy outcomes of consecutive ovulations did not differ significantly among the ovulatory sequences (Table 2). Similarly, there were no significant differences in the distribution of the ovulation side related to consecutive ovulations within a breeding season. The side of consecutive ovulations within a season was the same in 89 (38.7%) mares.

**Table 2.** Overall pregnancy outcome and the side of the ovulation regarding the ovulatory sequence.

| Ovulatory sequence | Ovulation side (%) | | p | Pregnancy (%) | | p |
|---|---|---|---|---|---|---|
| | Left ovary | Right ovary | | Non-pregnant | Pregnant | |
| 1st Ovulation | 262 (48.0) | 284 (52.0) | 0.117 | 263 (48.2) | 283 (51.8) | 0.253 |
| 2nd Ovulation | 132 (57.4) | 98 (42.6) | | 106 (46.1) | 125 (53.9) | |
| 3rd Ovulation | 42 (49.4) | 43 (50.6) | | 32 (37.6) | 52 (62.4) | |
| 4-5-6th Ovulations | 13 (54.2) | 11 (45.8) | | 9 (37.5) | 15 (62.5) | |

Over the course of five breeding seasons, 230 male and 245 female foals were born. However, no significant relationship was found between the ovulation side and foal sex (Table 3).

**Table 3.** Foal sex regarding the side of the ovulation.

| Ovulation side | Foal sex (%) | | P |
|---|---|---|---|
| | Female | Male | |
| Left ovary | 115 (49.8) | 116 (50.2) | 0.446 |
| Right ovary | 130 (53.3) | 114 (46.7) | |

Mares were divided into four age groups to investigate the effects of mare age. While the majority of the overall ovulations (n=700, 79.1%) were from younger mares aged–3-13 years old, 20.9% of the ovulations were from older mares aged 14–24 years. The distribution of the sides of all ovulations was similar between the age groups (Table 4).

**Table 4.** The side of the ovulation regarding the different age groups of the mares.

| Age groups | Ovulation side (%) | | p |
|---|---|---|---|
| | Left ovary | Right ovary | |
| 3-8 | 180 (48.9) | 188 (51.1) | 0.200 |
| 9-13 | 176 (53.0) | 156 (47.0) | |
| 14-17 | 79 (53.7) | 68 (46.3) | |
| ≥18 | 14 (36.8) | 24 (63.2) | |

The distribution of pregnancies was 46.9%, 37.2%, 12.4%, and 3.4% among age groups, respectively. A general decline in the number of pregnancies was observed between the younger and older age groups on both sides of ovulation. While the younger group aged between 3 and 8 years had a 60.5% (223/368) successful pregnancy rate regarding the number of all ovulations, it was 53.3% (177/332) for the 9-13 years of age group, followed by 40.1% (59/147) and 42.1% (16/38) for the 14-17 years old and ≥18 years old age groups, respectively. There was a significant relationship between the age groups and ovulation side that resulted in pregnancy (Table 5).

**Table 5.** The side of the ovulation resulted in pregnancy regarding the different age groups of the mares.

| Age groups | Ovulation side (%) | | p |
|---|---|---|---|
| | Left ovary | Right ovary | |
| 3-8 | 101[a] (43.7) | 122[a] (50.0) | 0.026 |
| 9-13 | 95[a] (41.1) | 82[a] (33.5) | |
| 14-17 | 32[b] (13.9) | 27[b] (11.0) | |
| ≥18 | 3[b] (1.3) | 13[b] (5.3) | |

a,b: Different letters in the same column indicate statistically significant differences ($p<0.05$)

The rate of ovulations resulting in pregnancy was significantly higher in younger mares aged 3-8 years and 9-13 years (43.7%-41.1%) compared to mares aged 14-17 years and those 18 years and older (13.9%-1.3%) for left side ovulations ($p<0.05$). A similar pattern was observed for right side ovulations as well, with pregnancy rates being higher in mares aged 3-8 years and 9-13 years

(50.0%-33.5%) compared to mares aged 14-17 years and those 18 years and older (11.0%-5.3%) ($p<0.05$).

There was no significant relationship between the side of ovulation and the parous state of the mares. In both maiden and parous animals, the sides of all ovulations, as well as the side of ovulation that resulted in pregnancy, were similar between the right and left ovaries (Table 6).

**Table 6.** The side of the ovulations regarding the parous state of the mares.

| Parous state | Ovulation side (%) | | p | Ovulation side resulted in pregnancy (%) | | p |
|---|---|---|---|---|---|---|
| | Left ovary | Right ovary | | Left ovary | Right ovary | |
| Maiden | 68 (47.6) | 75 (52.4) | 0.406 | 42 (45.7) | 50 (54.3) | 0.524 |
| Parous | 381 (51.3) | 361 (48.7) | | 189 (49.3) | 194 (50.7) | |

The parity of the mares did not differ significantly with respect to the side of all ovulations or the ovulation side that resulted in pregnancy (Table 7). The median parity was 3 for both sides of the ovulations when all ovulations were considered.

**Table 7.** The parity of the mares regarding the ovulation side resulted in pregnancy and the side of all ovulations.

| | | Parity | | p |
|---|---|---|---|---|
| | | N | Median (Min.-Max.) | |
| Ovulation side resulted in pregnancy | Left | 231 | 3 (0-10) | 0.474 |
| | Right | 244 | 2 (0-13) | |
| Ovulation side | Left | 449 | 3 (0-14) | 0.649 |
| | Right | 436 | 3 (0-14) | |

## DISCUSSION

Understanding ovulation laterality is crucial for optimizing breeding strategies, particularly when using techniques such as artificial insemination or embryo transfer, as it can guide veterinarians in determining the most appropriate timing and method for insemination, ensuring that sperm and embryos effectively navigate the oviduct and uterine environment and may also influence the choice of the ovary for follicular aspiration during assisted reproduction. The current study revealed a balanced distribution of ovulation between the right and left ovaries in mares, aligning with earlier equine studies (Bain and Howey, 1975; Sanderson, 1982; Butterfield and Mathews, 1979) as well as with recent research on Thoroughbred mares, in which Rezagholizadeh et al. (2015) reported 51.3% left and 48.7% right ovulations, while Morel and O'Sullivan (2001) observed 50.6% left and 49.4% right ovulations, with no statistically significant differences ($p>0.05$), suggesting that both ovaries in mares have equal functionality, supporting their classification as symmetrical ovulators. Challenging these studies, Najjar et al. (2018) investigated the impact of the ovulation site on pregnancy rates in 62 Pure Arab Breed mares inseminated with frozen semen using the deep intracornual method post-ovulation and showed a significant difference in pregnancy rates based on the ovulation site, with mares ovulating from the right ovary achieving a 59% pregnancy rate compared to only 23% for those ovulating from the left ovary. The contrasting results might be attributed to the limited population size of the study or methodological differences, such as the use of artificial insemination with frozen semen, whereas in the present study, the mares were bred under natural cover.

The complex interplay among mare age, ovulation patterns, and fertility outcomes has significant implications for equine reproductive management and breeding strategies. The current observation that the incidence of left and right ovulations did not differ significantly among age groups is consistent with the results reported by Morel and O'Sullivan (2001), who conducted a comprehensive study on 828 mares across six age groups (3-5, 6-8, 9-11, 12-14, 15-18, and 18-22 years) over three consecutive years. This consistency was further supported by Rezagholizadeh et al. (2015), who also found that age did not influence the incidence of ovulation in the left and right ovaries. However, the current study highlights a general decline in the number of pregnancies between the younger and older age groups, despite a similar distribution of ovulation. This suggests that, while the ovulation pattern remains consistent across age groups, other age-related factors may influence fertility outcomes. An examination of existing research consistently reveals that a mare's reproductive efficiency is

primarily influenced by age, establishing it as one of the most reliable determinants in this field. Studies categorizing mares into age brackets (2-8, 9-13, 14-18, and >18 years) comparable to the current investigation revealed that early stage (days 15-21) pregnancy rates per cycle typically began to decline when mares reached 14 years of age. Notably, the day 40-42 pregnancy rates per cycle in both the United States and the United Kingdom showed signs of a decrease at an even earlier age. These results demonstrate a consistent downward trend in pregnancy rates across successive age groups (Allen et al., 2007; Bosh et al., 2009; Nath, 2010). The relationship between increasing maternal age and declining fertility is complex and requires further investigation, as it is influenced by multiple factors, including reduced viability of oocytes and embryos, deterioration of reproductive structures, and alterations in fetal membrane development, which are not independent of each other and can occur simultaneously, leading to decreased reproductive efficiency across different measures (Scoggin, 2015).

In the current study, no significant differences were observed in pregnancy outcomes among consecutive ovulations or between left- and right-sided ovulations. Notably, 38.7% of the mares consistently ovulated from the same side within a breeding season. There was no literature investigating the relationship among ovulatory sequence within the breeding season, the side of the ovulation, and pregnancy outcome in mares, however, Morel and O'Sullivan 2001 reported a statistically significant ($p<0.05$) higher likelihood that successive ovulatory cycles would ovulate on alternating ovaries rather than on the same ovary. Similarly, research focusing on female patients undergoing infertility treatment has revealed that ovulation patterns across consecutive cycles can significantly affect pregnancy outcomes in both intrauterine insemination and in vitro fertilization treatments. Contralateral ovulation, in which ovulation alternates between left and right ovaries in consecutive cycles, appears to have better outcomes than ipsilateral ovulation (ovulation from the same ovary in consecutive cycles). In particular, the best pregnancy outcomes were observed when ovulation occurred in the right ovary following left ovulation in the previous cycle (contralateral right-sided ovulation) (Fukuda et al., 2006). These findings emphasize the need for further research to explore the relationship between ovulatory sequence and ovulation side in mares.

The relationship between the ovulation side and offspring sex has been a subject of interest in various mammalian species, with conflicting results reported across different breeds and species. In the current study conducted on Arabian mares, 230 male (48.4%) and 245 female foals (51.6%) were born over five breeding seasons, which is comparable to the male foal ratio (46.1%-46.6%) previously reported for the Thoroughbred breed (Gharagozlou et al., 2014; Rezagholizadeh et al., 2014). However, in the present study, there was no significant relationship between the ovulation side and foal sex. This finding contrasts with a recent study on Thoroughbred horses, which reported a significantly higher proportion of male foals resulting from left-sided (57.4%) than from right-sided (35.3%) ovulations. Additionally, right-sided ovulation produced a lower male foal ratio than expected, whereas left-sided ovulation and overall male foal ratios did not differ significantly from the expected 50%. ($p=0.026$). This discrepancy in the results highlights the potential for breed-specific differences in reproductive laterality and its influence on offspring sex ratios. Interestingly, similar species-specific differences have been observed in cattle as well. Research in beef cattle has unveiled an asymmetrical distribution of offspring sex, with right-sided ovulations having a higher likelihood of male offspring, whereas left-sided ovulations were more likely to result in female calves (Hylan et al., 2009; Giraldo et al., 2010), although, in Holstein dairy cows, a parallel distribution of offspring sex was observed between the left and right ovulations (Gharagozlou et al., 2013).

In addition to these patterns, the relationship between ovarian laterality and parity in mares has been a subject of debate in equine reproductive research, with conflicting findings reported in the literature. Ginther (1983) utilized theriogenology reports of a mixed-bred population of mares consisting mainly of Quarter Horses and reported asymmetrical ovulation favoring the left ovary (122 ovulations) over the right ovary (79 ovulations) in a population of maiden mares, hypothesizing that this was due to differences in vascularization and greater blood flow to the left ovary, with the asymmetry reportedly eliminated during or after the first pregnancy. However, Rezagholizadeh et al. (2015) documented no significant effect of parity on ovulation incidence between the left and right ovaries, with nulliparous mares showing 48.2% left and 51.8% right ovulations, and parous mares

showing 52.9% left and 47.1% right ovulations (p>0.05). The present study corroborates these findings, showing no significant difference in the ovulation side with respect to parity. The discrepancy with the results of Ginther (1983) may be attributed to breed differences and the methodology used to determine ovulation, as advancements in diagnostic techniques, particularly ultrasonographic evaluation over rectal palpation, have allowed for a more accurate assessment of ovulation patterns in recent studies.

## CONCLUSION

In conclusion, this study provides valuable insights into the effect of ovarian laterality on reproductive parameters in Arabian mares, highlighting that the side of ovulation does not significantly impact pregnancy outcomes or foal sex, although age-related declines in pregnancy rates were observed. Further research should integrate genetic analyses to provide deeper insights into the mechanisms underlying the observed patterns of ovulation and pregnancy outcomes.

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# Ultrasonographic diagnosis and management of perineal hernia in pregnant Jamunapari doe- a case report

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## ABSTRACT

A two years old Jamunapari cross-breed doe was brought and registered at Shahedul Alam Quaderi Teaching Veterinary Hospital (SAQTVH) with a history of natural mating of five months. The owners have been complaining of a progressively increasing swelling in the perineal region for the past three months. A swelling at the perineal region that extended caudally to the animal's back was seen during a clinical examination of the patient. Ultrasonography scanning was done to confirm the gravid uterus in the gradually swelling perennial area, assess the gestational age and fetal condition. Hematological screening was done to measure the profiles of different blood parameters. Hormonal therapy (prostaglandin) & dexamethasone along with supplements of vitamins and minerals were given to the animal on that day. Three live fetuses were born after 15 hours of treatment. The planned surgical treatment of the hernia's hole could not be carried out in the perineal area because the patient's owner refused to consent to this surgical technique.

***Keywords:*** *Perineal hernia, Pregnant doe, Ultrasonography, Live fetus, Hormone*

## INTRODUCTION

Ultrasonography has become as an important diagnostic tool in the modern veterinary industry, and its uses are becoming more varied and highly advantageous. Ultrasonography has made it possible for researchers and veterinarians to evaluate and monitor enormous events by investigating, diagnosing, and tracking various aspects of physiological and pathological issues in veterinary practices (reproduction, internal medicine, surgery, and cardiology) in farm, companion, and wild animals (Samir et al., 2021).

A herniation is the protrusion of an organ or tissue through a congenital hole or traumatic wall defect, which causes bodily components to shift to an inappropriate place (Das et al., 2012). A hernia develops when the abdominal wall becomes weaker and produces a hole called a hernial ring, part of the abdominal cavity's viscera then flows through the hole and bulges out, making the hernia visible on the skin (Singh et al., 2014). Majority of hernias in young animals happened around natural openings like the umbilical and inguinal canal (Misk et al., 2008). The prevalence of hernia can be varied from breed, sex, species etc. where found that 81% of the cases involved ovine species and 72.4% are in females (Amare and Haben, 2020).

Among types of hernia, the perineal hernia, differs from others in that its contents aren't covered by the peritoneum. Additionally, the perineal muscle is weak, which makes it easier for gravid uterus or some viscera of the abdominal and pelvic cavity to droop, which can result in unilateral or bilateral abdominal swelling (Shridhar, 2021), with 59% of

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perineal hernias being unilateral and 41% being bilateral (Bellenger and Canfield, 2003). The swelling frequently causes a perineal hernia, which manifests as an abdominal shape ventrolateral to the anus (Kealy, 2011). The history and examination of the hernial area like swelling, discomfort etc. are used as the main diagnostic tools for perineal hernia. Radiography, ultrasound or exploratory puncture can also be used to diagnose & distinguish hernias from others like fibrino-cystic, abscess, and inflammatory swellings (Al-sobayil and Ahmed, 2007). Depending on the size of the hernial ring present at the location, there are many surgical methods for treating hernias. Herniorrhaphy and hernioplasty or both are preferable for treating simple and big or complex hernias respectively (Kingsnorth and LeBlanc, 2003; Whitfield-Cargile, 2011). Medicinal treatment can also be used in case of perineal hernia containing gravid uterus for delivery of fetus and surgical approach can be done in further to remove the herniated part. In the current investigation, a goat perineal hernia containing gravid uterus was successfully managed through medicinal treatment. This is a case that is written for the first time on the diagnosis of perineal hernia by ultrasonography and the treatment and management of it in pregnant goat.

## MATERIALS and METHODS

### Case history

A two-years-old Jamunapari cross-breed doe weighing 35 kg was brought to the Shahedul Alam Quaderi Teaching Veterinary Hospital (SAQTVH), CVASU, Chattogram, Bangladesh with the complaint of gradual swelling in the perineal region from last three months. The doe was also unable to deliver the fetus despite having reached the end of the gestation period. In addition, the owner stated that the swelling had started loosely and grown gradually in the perineal area without any trauma as the pregnancy advanced. The doe's appetite had decreased recently, but her urination and defecation were both regular.

### Clinical examination

A general examination revealed mild dehydration and evidenced rectal temperature of about 103.9 F, pulsation: 104/min, and pink, moist mucous membrane indicating the vital signs were normal. However, the doe developed perineal swelling and abdominal distension brought on by pregnancy (Figure 1). Cervix was closed and there was no vaginal discharge during the vaginal examination.

**Figure 1.** Herniated gravid uterus

### Ultrasonographic examination

A real-time ultrasound examination was done to detect the content of herniated region. The ultrasonography scanning revealed that the gravid uterus containing amniotic fluid with fetus was herniated from its location to perineal region. Three fetuses were observed and their viability were confirmed by observing fetal heart rate (Figure 2a). The gestational age was calculated by measuring the Trunk Diameter (TD) (85.2 mm) (Figure 2b) revealed 148 days of pregnancy.

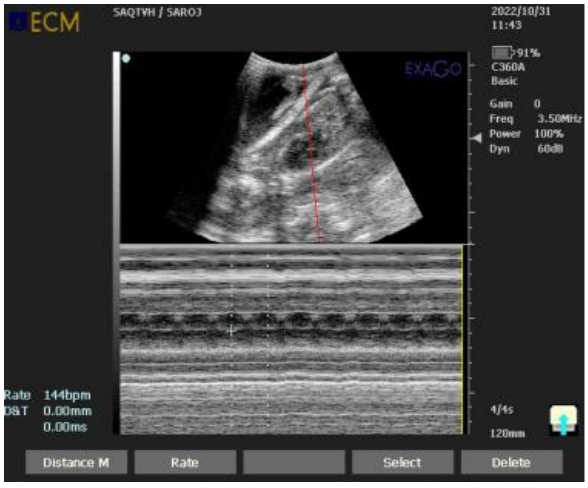


**Figure 2a.** Heart rate of the fetus

### Blood sampling and hematological screening

Blood collection was done to perform a hematological and biological examination of the Jamunapari doe. Hemoglobin and PCV, calcium, phosphorus, magnesium, glucose and total protein level in blood was measured where every parameter was in normal range except calcium and magnesium level, was in below the range (Table 1)

The case was determined to be a perineal hernia containing gravid uterus on the basis of the

anamnesis and the findings of clinical and ultrasonic examination.

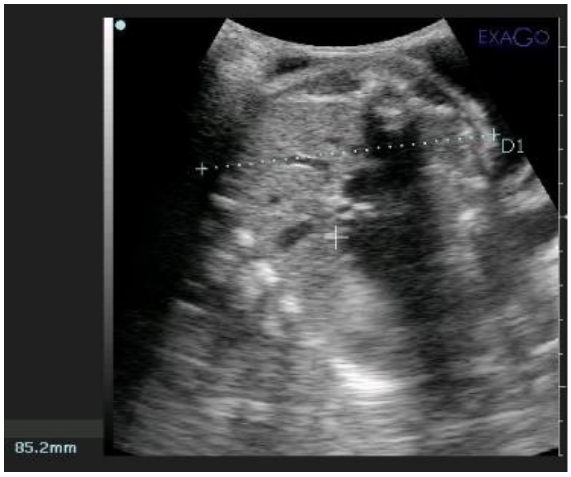


**Figure 2b.** TD measurements of fetus

**Table 1.** The value of different blood parameters-

| Parameters | Calculated value |
|---|---|
| Hemoglobin (Hb) | 8.2% |
| Packed Cell Volume (PCV) | 27% |
| Calcium | 6.08 mg/dl |
| Phosphorus | 7.14 mg/dl |
| Magnesium | 1.73 mg/dl |
| Glucose | 97.84 mg/dl |
| Total protein | 5.91 mg/dl |

**Treatment**

The doe was treated initially with Prostaglandin 250 µg (Inj Prostenol®, ACI Ltd, 1 ml equivalent to 250µg IM), Dexamethasone 4 mg (Inj Dexa vet®, Techno drugs Ltd, 4 ml IM), Calcium boro-gluconate (Inj. Cal-D-Mag Vet®, Renata Ltd. 35 ml IV) with normal saline (Inj. NS, ACME Ltd, 500 ml IV), vitamin ADE (Inj. Renasol ADE, Renata Ltd. 3.5 ml IM) for inducing delivery of fetus. After 15 hours of treatment, the animal was delivered to three live fetuses (Figure 3). Calcium boro-gluconate with normal saline was also suggested for 5 days of post parturition. Physical condition of the doe was improving after delivery but the herniated part remained slightly swollen. We offered the owner to remove the herniated part surgically as there is a chance to occur again and for improving the animal's condition but the owner refused surgery. The patient was in follow up and continue the post parturient treatment for 5 days. After the treatment, animal was goodly well and the feeding condition, posture and gait was normal.

**Figure 3.** Herniated part and fetus after delivery

**DISCUSSION**

Hernia can damage an animal's production and ability to reproduce. The uterus can herniate through the perineal hernia ring in pregnant animals, causing the organ to dislocate and may cause dystocia (Jettennavar et al., 2010). Congenital susceptibility, structural pelvic diaphragm weakness, hormonal imbalance, or ripping of weak abdominal muscles owing to abdominal expansion associated with pregnancy are some suggested reasons for perineal hernia (Das et al., 2012). In the present case, it was observed that the perineal swelling had increased as the pregnancy progressed. Although the reason could not be pinpointed with certainty, it was presumably caused by the rupture of weak abdominal muscles.

The hernial swelling was discovered during the clinical examination in the perineal area of the studied pregnant doe. The fetal heads and extremities were discernible during palpation. The ultrasonographic examination was performed in the current investigation and found live fetal heart rate and movements, can be used to diagnose the fetal components (Salci and Guner, 2016).

Although surgical repair is mostly required in cases with perineal hernia including the uterus (Salci and Guner, 2016) but medical treatment was applied in the current situation. Since prostaglandin F2$\alpha$ (PGF2$\alpha$) or its analogs and dexamethasone have been used successfully to induce parturition in goats, they were utilized in the current investigation to induce luteolysis (Pollock et al., 2021). Additionally, it is advised against using PGF2$\alpha$ injection to induce parturition until at least 144 days have passed in the pregnancy since it might decrease the survivability of triplet or quadruplet offspring who might be born with

small-sized heads (Ott, 1986). Calcium was suggested during and after delivery in the studied doe as it is essential for the contraction of all smooth, cardiac, and skeletal muscles which helps for inducting parturition and for reduction of peri-parturient diseases like retained placenta, dystocia etc. Peri-parturient hypocalcemia causes metabolic and oxidative stress in does as well as the retardation of uterine involution in postpartum periods (Bayoumi et al., 2021).

## CONCLUSION

Pregnancy can cause an increase in abdominal size with heavy weight, which can cause muscles to rupture and herniate. Perineal hernia, which can occur in ruminants and is different from abdominal, inguinal, and umbilical hernias, was clearly identified in the pregnant doe in the current investigation. It was simple to identify uterus with perineal hernia using ultrasonic testing. Goat dairy enterprises can effectively utilize prostaglandin and dexamethasone to induce parturition, even in the perineal hernia.

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# Diagnosis and treatment of retrobulbar abscess in the White New Zealand Rabbit *(Oryctolagus cuniculus L.)*

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## ABSTRACT

Although retrobulbar abscesses are known as commonly encountered in domestic rabbits, there is insufficient information regarding the diagnosis, treatment, and outcomes of the disease. It is also known that retrobulbar and skull abscesses are among the most difficult to treat. A 4-yr-old, 3 kg, male, White New Zealand rabbit *(Oryctolagus cuniculus L.)* was presented for exophthalmos in the left eye which started a while ago and progressed rapidly. On ophthalmologic examination deviation of the globe, third eyelid prolapse, chemosis, inability to blink and subsequent severe keratitis was noted. Diagnostic CT and ocular ultrasound were used and a mass compatible with abscess was detected in the retrobulbar area. Following unresponsive medical treatment, the abscess was surgically removed and the eye globe with irreversible damage was enucleated. Postoperative anti-inflammatory and antibiotic were prescribed, a drain was placed into socket. Although the treatment process was prolonged due to the relapse of the abscess 2 weeks after the operation, the patient fully recovered following local and systemic medical treatment for another 2 weeks. This study was to provide detailed information about the clinical findings, diagnosis, treatment and outcomes of retrobulbar abscess in a domestic rabbit.

***Keywords:*** *Exophthalmos, Rabbit, Retrobulbar abscess, Tomography, Ultrasound*

## INTRODUCTION

Retrobulbar abscess is characterized by the collection of purulent material in the retrobulbar space. Unilateral exophthalmos is the most prominent clinical sign of a retrobulbar abscess and may be accompanied by other symptoms such as stagnation, facial swelling, weight loss, gastrointestinal disease, ocular or nasal discharge, chewing or swallowing problems (Wagner and Fehr, 2007; Levy and Mans, 2023).

Diagnostic imaging modalities can be used to diagnose retrobulbar abscesses. Ultrasonography reveals a round pathologic structure filled with dense material. CT and MRI play significant role for diagnosis and surgical planning. (Capello, 2011; Levy et al., 2023).

Retrobulbar and skull abscesses are the most difficult to treat, of all the abscesses occurs in rabbits. The treatment usually involves either administration of wide-spectrum antibiotics alone or in combination with surgical removal of the abscess. In small animals, drainage of the abscess through the pterygopalatine fossa is the most used approach (Bennett, 2004; Van Den Top et al., 2007).

This report was aimed to provide elaborative information about the clinical findings, diagnosis, treatment and outcomes of retrobulbar abscess in a domestic rabbit based on lack of the detailed information on this topic.

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## CASE HISTORY

In this case report, a 4-year-old, 3 kg, male, New Zealand rabbit (Oryctolagus cuniculus L.) was complained of exophthalmos in the left eye which started 4 weeks ago and progressed rapidly.

The patient had a previous history of local treatment with moxifloxacin (Demoxif Eye Drops, 5mg/ml, Deva Holding Istanbul, Turkey) which was administered for one week without response. It was stated that the general condition of the patient was slightly affected with mild loss of appetite.

Body condition score was poor (thin) and no other abnormal findings were found in physical examination. In the complete blood analysis (H 60 Vet- Edan Veterinary Hematology Analyzer), all data were within the reference values. Blepharoconjunctivitis, xerophthalmia, and exophthalmos were noted, and pupillary light and threat reflex was negative in the left eye. Tear volume with the Schirmer Tear Test-I and intraocular pressure with a tonometer were measured as 6 mm and 13 mmHg, respectively, in the healthy right eye, but no measurements could be taken in the left eye. (Figure 1). Ultrasonographic examination (Esaote Mylab 25 Vision Portable Ultrasound Scanner, micro convex probe was used) revealed a mass consisted of hypoechoic material filled with multiple small echogenic foci looks like inflammatory cells and small fibrous strands the caudal to the eye (Figure 2).

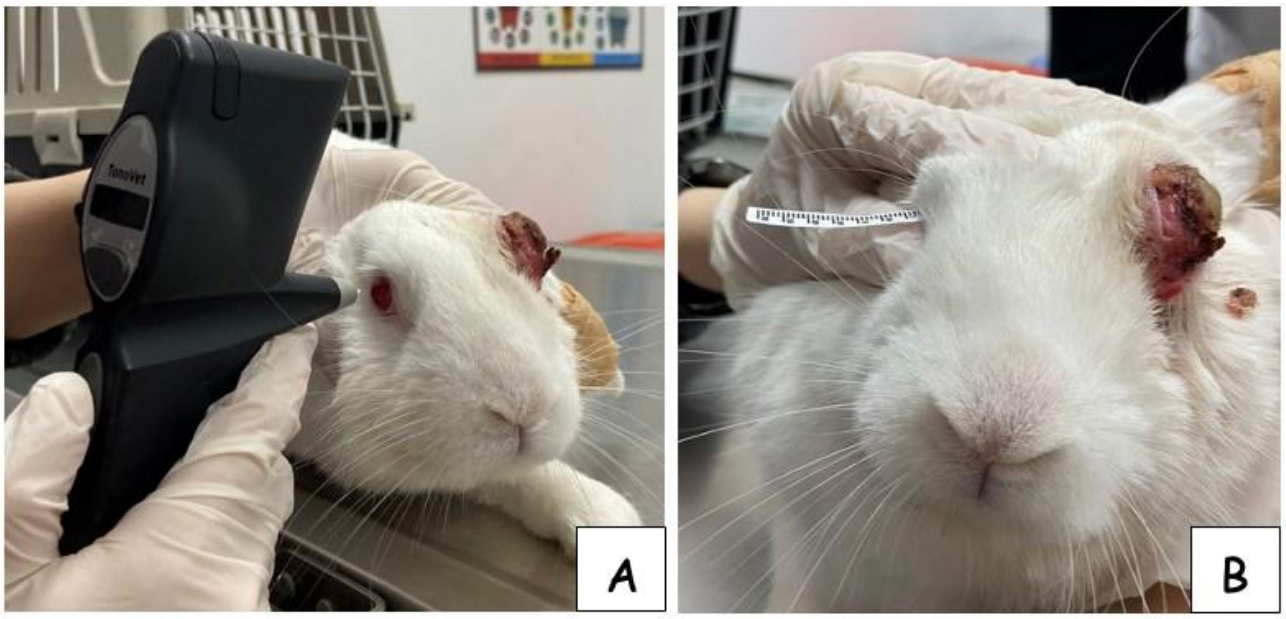


**Figure 1.** Intraocular pressure measurement with tonometer (A), Schirmer Tear Test-I application (B).

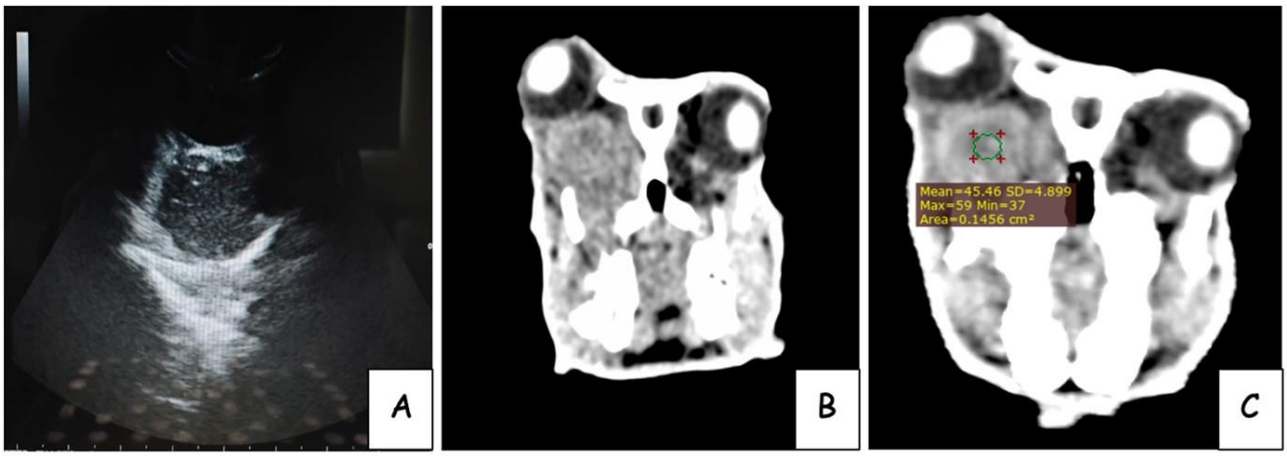


**Figure 2.** USG image of a swelling consisting of hypoechoic material with a small echogenic foci caudal to the left eye (A). Tomography image taken in the soft tissue window in the transversal section showing an abscess structure surrounded by a capsule caudal to the eyeball (B). HU value of the abscess content (C).

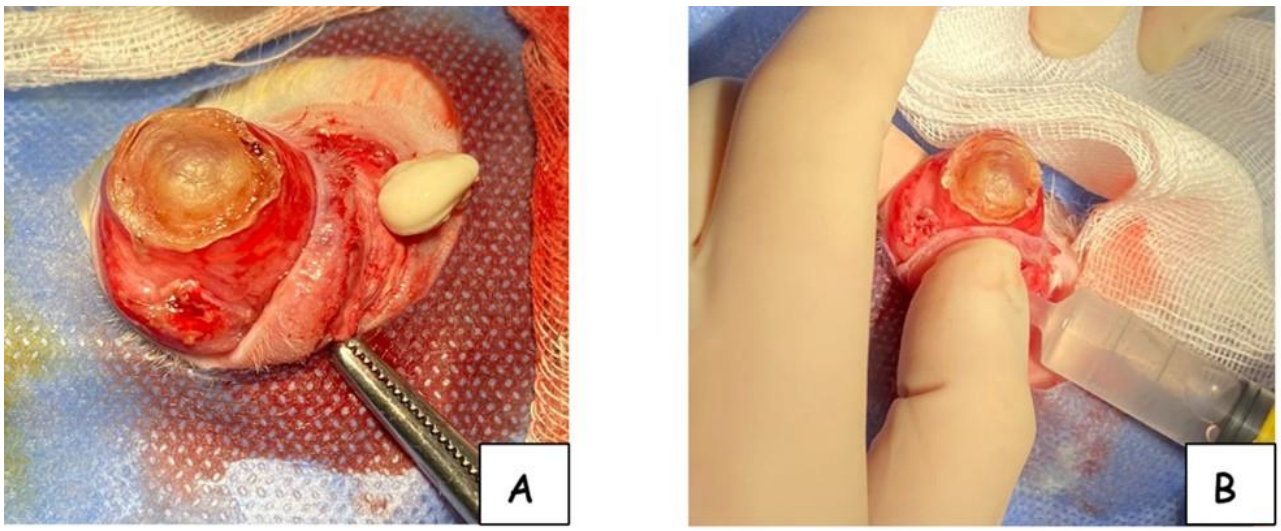


**Figure 3.** Drainage of retrobulbar abscess. The abscess sac was reached through an incision made just below the eye, over the zygomatic arch and the toothpaste-like pus was completely cleaned (A) by irrigating with 3% boric acid solution (B).

**Figure 4.** The eye cavity filled with pus again 2 weeks after the operation (A). The eye after cleaning and treatment (B). The eye 2 months after the operation (C).

Systemic enrofloxacin, which is a safe, easy-to-dose and easy-to-administer first-line antibiotic for rabbits if there is no antibiogram, (5 mg/kg, PO, 2x1, Baytril® 2.5%, 25 mg/ml, Bayer Türk Kimya San. Ltd. Şti.) and, 3 times a day local Carbomer (Viscotears®, 2 mg/g eye gel, Bausch & Lomb, İstanbul, Türkiye), Diclofenac Sodium (İnflased Eye Drops, 1mg/1ml, Bilim İlaç, İstanbul, Türkiye) and moxifloxacin (Demoxif® Eye Drops, 5mg/1ml, Deva Holding, İstanbul, Türkiye) was prescribed as medical treatment for a few days to support the general condition of the patient and to protect the integrity of the eyeball until the operative intervention.

When the patient was brought back to the clinic aggravated corneal lesions and ulceration was seen despite the treatment. Tomography images showed a mass in the caudal part of the eyeball with a relatively low attenuation density of 45.46 HU, surrounded by a ring-shaped capsule with clear borders, which was clearly pushing it, supporting the suspicion of an abscess. No lysis of adjacent bone structures was observed and no dental infection finding was seen. Since the eye damage was irreversible, remove the abscess concurrently transpalpebral enucleation was performed.

Anesthesia maintained with in the intubated patient be inhaling Isoflurane (Isoflurane USP) in 100% oxygen after administering xylazine hydrochloride (5 mg/kg, IM, Xylazinbio® 2%, 20 mg/ml, Intermed, Yenişehir/Ankara, Türkiye) and ketamine hydrochloride (30 mg/kg, IM, Ketasol® 10%, 100 mg/ml, Richter Pharma, Ankara, Türkiye) for induction. The patient was intubated with the

help of an otoscope. The surgical area was prepared following the principles of asepsis.

First, abscess was removed to ease enucleation of the eye and to prevent hematogenous infection (Figure 3). Then, transpalpebral enucleation was performed. Incisions were closed with simple separate sutures using 3-0 multiflament absorbable material (GMD, İstanbul, Türkiye). An opening was left in the last part of the lateral suture line and a drain was applied from this area. A sample was taken for microbiologic examination, but no results were obtained.

Meloxicam (0.2 mg/kg, SC SID, Bavet® Meloxicam, 5 mg/ml, Arion İlaç, Tuzla/İstanbul, Türkiye) for 2 days and Enrofloxacin (5 mg/kg, PO, BID, Baytril® 2.5%, 25 mg/ml, Bayer, Ümraniye/İstanbul, Türkiye) for 7 days were prescribed postoperatively. Also, a collar was recommended, and the drain was changed every 2 days and povidone iodine was consistently applied to the suture line and drain. No other applications such as ointment etc. was made into the cavity.

The drain was completely removed 7 days after the operation and in two days pus accumulation relapsed (Figure 4A). The abscess sac was irrigated daily with 3% boric acid and batikon was applied (Figure 4B) and a different antibiotic, doxycycline (Levadoks® 20%, 200 mg/ml, Netfarma, Istanbul, Türkiye), which is another safe option that does not have any negative effects on the intestinal flora of rabbits, was prescribed. No discharge was encountered in subsequent follow-up visits. One month after the operation, the patient recovered completely, and 2 months later, we received information from the owner that there was no problem or recurrence in our patient (Figure 4C).

## DISCUSSION

Retrobulbar abscesses may be idiopathic, result from endodontic or hematogenous infection, or local injury (Martínez-Jiménez et al., 2007; Tremolada et al., 2015). In presented case, no dental or gingival problem was found. It was learned that he had only a mild trauma to his eye. Therefore, the cause of retrobulbar abscess formation in this case was thought to be posttraumatic inflammation.

Orbital abscesses are usually a chronic condition and can affect other organs through vascular or lymphatic bacterial dissemination. Patients present with normal to decreased appetite and physical activity depending on the degree of pain. Since the pain is not very pronounced in rabbits, owners may not recognize the problem in the initial stages of the disease (Thomas et al., 2020). Our case was mildly lethargic and anorexic and, there was severe exophthalmos, keratoconjunctivitis without pupillary light and threat reflex.

Ocular ultrasound is useful for obtaining samples or draining abscess sites. However, it is difficult to assess the extent of orbital involvement with ocular ultrasound alone. (Van Den Top et al., 2007; Herrmann et al., 2021). CT, the most common diagnostic modalities, can help determine the size of a mass lesion and has the advantage of detecting bone changes such as osteolytic or osteoproliferative lesions, including dental structures. However, CT is not reliable when differentiating between inflammatory and neoplastic formations in soft tissue. In our case, both ultrasound and CT were performed without the need for anesthesia. In both procedures, the patient was kept wrapped in a heavy towel. The echogenicity of the pathological structure detected behind the eye on ocular ultrasound was not compatible with fluid, it had multiple echogenic foci and was thought to be inflammation cells and fibrous fragments, so an abscess was considered. On the CT image, the pathologic structure located behind the eye and clearly pushing it was limited by an annular capsule and contained a mass with a density of 45 HU, which was consistent with a retrobulbar abscess. The diagnosis of retrobulbar abscess was confirmed with the toothpaste-like pus coming from the retrobulbar space during the operation.

Treatment of a retrobulbar abscess in the rabbit can be particularly challenging. Because of the thick nature of the abscess material and the anatomy of the alveolar bulla. In some cases, aggressive surgical debridement may be necessary. Enucleation of the eye for access to the retrobulbar space may be considered (Van der Woerdt, 2012; Pumphrey and Wayne, 2022). In our case, because of irreversible corneal damage and severe proptosis, it was decided to enucleate the eye with retrobulbar abscess. Because pus accumulation relapsed in the following periods antibiotic was changed, and no discharge was seen after 1 week of treatment.

Retrobulbar abscess is an important disease in rabbits because it may cause visual impairment and may be life threatening. This report summarizes the clinical findings, diagnosis, treatment and outcomes of retrobulbar abscess in a domestic rabbit.

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**Author contributions:** ZBÜ and ESA performed surgery. ZBÜ designed the study. ZBÜ and ESA collected data. ZBÜ and ESA made analysis/or interpretation. ZBÜ and ESA made literatüre review. ZBÜ and ESA wrote the article. ZBÜ criticized the article. ZBÜ: Zeynep Bozkan Ünal, ESA: Ezgi Sude Aybak

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# Integrative treatment method in Veterinary Medicine: Hirudotherapy

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## ABSTRACT

Traditional and complementary medicine methods have a long history in veterinary medicine. Medicinal leech therapy (hirudotherapy) is one of these methods. Medicinal leeches are sanguivorous invertebrates. While sucking blood to feed, they secrete saliva into the host's body. They contain over 100 bioactive molecules with analgesic, anti-inflammatory, anti-platelet, anticoagulant, and antimicrobial extracellular matrix degeneration effects. Consequently, the leeches treat the host by exhibiting a phlebotomic effect, a range of pharmacological effects due to bioactive molecules, and a reflex stimulation effect at the bite site. Recently, leeches have been used successfully in veterinary medicine to treat many diseases of animals, especially dogs, cats, and horses. The most common indications for the use of leeches are venous congestion, acute and chronic arthritis, laminitis in horses, diseases associated with inflammation of tendons, ligaments and fascia, vertebral diseases, and treatment of scars. Despite its frequent application in different countries, there needs to be more scientific literature on the veterinary use of leeches. This article aims to encourage further scientific investigation to expand the veterinary use of leech therapy, which is a cost-effective and relatively safe alternative to other methods, and to elucidate the potential effects of leech secretions.

***Keywords:*** *Veterinary leech therapy, Hirudotherapy, Medicinal leech, Leech saliva*

## INTRODUCTION

The domestication of animals has enabled humans to use them as a sustainable source of food and livelihood and companions in various forms of transportation, freight transport, everyday life, and warfare. This situation has resulted in prioritizing animal health on a par with humans (Aslan, 2023). The fact that over 60% of infectious diseases are zoonotic and that epidemics can rapidly globalise and become pandemics due to the rapidity of contemporary communication is further evidence that veterinary health is of great importance (Maden, 2020).

Both medicine and veterinary medicine aim to achieve and maintain equilibrium within the body. The role of a physician is to maintain homeostasis, to rebalance it if it is disturbed, and to ensure the maintenance of mental and cognitive well-being. The practices carried out for the prevention of physical diseases, diagnosis, treatment and cure of these diseases have collectively developed a body of knowledge that forms the basis of today's veterinary and medical science (Aslan, 2023). Veterinary medicine has been supported by traditional medical practices for thousands of years, with preparations, drugs, and applications have been made against hundreds of medical conditions. These include parasite control; vertebrae, neck, and

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head diseases; joint, foot, and bone diseases; lameness; eye diseases; mouth, jaw, and facial muscle disorders; skin and subcutaneous connective tissue disorders; diseases of the chest and abdomen; dental diseases, stomach, and intestinal diseases (Brennan, 2001; Sinmez, 2011). One such traditional method is leech treatment, also known as hirudotherapy (Ben-Yakir, 2008a).

Hirudotherapy involves the use of blood-sucking leeches. The use of medicinal leeches has a vast historical background, extending from Ancient Egypt to the Ottoman Empire and Europe. The earliest written records date back to 200-130 BCE in *Alexipharmaca*, a medical poem by Nicander of Colophon, who lived in what is now Menderes, Izmir. During the Roman period, Galen extensively employed leech therapy for bloodletting (Wells et al., 1993; Whitaker et al., 2004). In Islamic medicine, Ibn Sina (Avicenna) in *Al-Qanun fi't-Tibb* described leeches as an effective treatment for deep vascular diseases and skin disorders, emphasizing their ability to draw blood from vessels that could not be accessed by cupping therapy. Similarly, Ottoman physicians, including Sabuncuoğlu Şerefeddin and Sanizade Mehmet Ataullah Efendi, provided detailed records on leech applications for a wide range of conditions, from fevers in children to post-surgical wound healing (Uzel, 1992; Kahya, 2009; Acıduman, 2009). In Europe, hirudotherapy was extensively practiced during the Napoleonic era, particularly in France and England, where it was used for brain, kidney, and liver diseases, ophthalmic disorders, rheumatism, epilepsy, and inflammatory conditions (Rolleston, 1959; Adams, 1988). However, with the rise of modern medicine in the late 19th century, leech therapy declined in favor of synthetic pharmaceuticals and surgical advancements (Whitaker et al., 2004). Historically, medicinal leeches have also been used in veterinary medicine for the treatment of various conditions, including acute equine laminitis, canine aural hematoma, laryngitis, and prolapsed uterus in cattle (Ben-Yakir, 2008a).

Hirudotherapy encompasses a range of biological properties inherent in medicinal leeches. Until the end of the nineteenth century, the only known property was that leeches feed on the blood of their hosts (phlebotomic property). However, with the discovery of anticoagulant substances in the secretion extract by Dr. John Haycraft in 1883 and subsequent studies, it became evident that leeches release a range of bioactive substances, particularly anticoagulants, anti-inflammatory agents, and anesthetics, into the host's body while sucking blood. The main aim of therapy is now to use the potential benefits of leech saliva secreted during leech feeding (Weinfeld et al., 2000; Alaama et al., 2024).

In veterinary medicine, medicinal leeches are applied to obstructed or inflamed areas of the animal body, particularly in cases where conventional treatment methods are ineffective or have insufficient efficacy (Buote, 2014; Sobzcak and Kantyka, 2014). In recent times, leeches have been used with considerable success in veterinary medicine for the treatment of a wide range of diseases affecting animals, particularly dogs, cats, and horses. The popularity of its use in microsurgery and reconstructive surgery in human medicine has led to its adoption in veterinary medicine for reconstructive surgery on swollen faces, limbs, and digits following successful arterial revascularization but limited venous repair (Ben-Yakir, 2008a; Fernee, 2011). The efficacy of leeches in the healing of inflammatory conditions, peripheral venous and arterial diseases, non-granulating wounds or ulcers in aged and immunocompromised animals, tendon injuries and muscle fatigue, and many other diseases has been demonstrated (Ben-Yakir, 2008a; Sobzcak and Kantyka, 2014; Kermanian et al., 2022).

Currently, leech therapy, which has become a prominent practice in both human and animal medicine and a valuable raw material in the cosmetic and pharmaceutical industries, is endorsed by international organizations and demonstrates a growing prestige within mainstream health services (Rados, 2004; Anonym, 2023). However, the scientific literature on the veterinary use of leeches remains limited, and standardized treatment protocols have yet to be established.

This review systematically examines the literature on veterinary hirudotherapy using databases such as PubMed, Scopus, Web of Science, and Google Scholar. Search terms included 'hirudotherapy', 'veterinary hirudotherapy', 'medicinal leeches in veterinary medicine', 'leech therapy in animals', and 'hirudotherapy and veterinary application'. Inclusion criteria were peer-reviewed studies published between 2000-2024 focusing on the veterinary use of leech therapy. Studies not related to veterinary applications or lacking sufficient data were excluded.

The objective of this review is threefold: firstly, to contribute to the existing literature on the veterinary use of leeches by providing a comprehensive analysis of their therapeutic applications; secondly, to increase awareness of this treatment method among veterinary professionals and researchers; and thirdly, to highlight potential research gaps and future directions for studies in this field. By critically evaluating the available scientific data, this review aims to establish a foundation for further clinical research and the integration of hirudotherapy into veterinary practice.

**1. Medicinal Leeches**

In 1758, Linnaeus classified medicinal leeches in his monograph *Systema Naturae*, assigning the name *Hirudo medicinalis* to emphasize their therapeutic applications. Today, the classification of *H. medicinalis* and other medicinal leech species places them within Kingdom Animalia, Phylum Annelida, Class Clitellata, Order Arhynchobdella, Family Hirudinidae, and Genus Hirudo (Sawyer, 1986; Gileva and Mumcuoğlu, 2013). The subclass *Hirudinea* comprises over 800 species, though only about 15 are used in medical treatments (Kvist et al., 2013; Ünal et al., 2023). While *Hirudo medicinalis* is the most widely recognized species, several other members of the *Hirudo* genus are also used for medicinal purposes, including *Hirudo verbana*, *Hirudo orientalis*, *Hirudo troctina*, *Hirudo nipponia*, and the newly identified *Hirudo sulukii*, endemic to Turkey. Additionally, medicinal leeches outside the *Hirudo* genus, such as *Hirudinaria manillensis* and *Macrobdella decora*, are also utilized in therapeutic applications (Sağlam et al., 2016; Trontelj and Utevsky, 2005; Utevsky et al., 2010).

- *Hirudo medicinalis*: Found across Central and Western Europe, including the United Kingdom, Lithuania, and Ukraine (Elliott and Kutschera, 2011).
- *Hirudo verbana*: Distributed in the Balkans, Eastern Mediterranean, Moldova, Ukraine, Russia, Turkey, and Armenia (Elliott and Kutschera, 2011).
- *Hirudo orientalis*: Native to Transcaucasia, Iran, and Central Asia (Trontelj and Utevsky, 2005; Utevsky et al., 2010).
- *Hirudo troctina*: Found in North Africa and parts of Spain (Hechtel and Sawyer, 2002).
- *Hirudo nipponia*: Found in Asia (Whitman, 1886)
- *Hirudo sulukii*: Recently discovered in southeastern Turkey, specifically in Kara Lake (Adıyaman), Sülüklü Lake (Gaziantep), and Segirkan Wetland (Batman) (Sağlam et al., 2016).

Furthermore, within the Turkish context, both state institutions and private farms are engaged in the production of medicinal leeches. The purpose of this production is to satisfy both domestic and foreign demand.

Medicinal leeches feature a cylindrical, dorsoventrally flattened body comprising 33-34 segments and are hermaphroditic. They feed by ingesting the blood of mammals or, on occasion, that of frogs, tadpoles, and small fish (Blair, 1927; Sawyer, 1986). These animals have photoreceptors, comprising five pairs of eyes, as well as sensilla that function as chemoreceptors (for chemicals in the air and water) and sensilla that function as mechanoreceptors (for vibrations or sounds). These allow them to locate potential prey and hosts (Nesemann and Neubert, 1999; Phillips et al., 2020). The body segments are differentiated into anterior suckers at the beginning and posterior suckers at the end. The posterior sucker is usually larger than the anterior sucker and only helps with surface attachment and movement (Mann, 1962; Lukin,1976). The anterior sucker, consisting of a buccal cavity and jaws, is used for attachment and feeding. The leech has three rigid jaws arranged in a tri-radiated configuration. About 100 pyramidal, sharp teeth in each jaw are used to cut the skin, leaving a characteristic Y-shaped bite. On the side of each tooth, close to the piercing tip, is an opening through which saliva is injected into the skin (Orevi et al., 2000; Ayhan et al., 2021). The row of teeth in each jaw works like a saw to cut open the host's body surface. After piercing the skin and injecting its saliva, the leech begins to feed on the blood flowing through the destroyed vessels and lymph from the interstitial space, which is then sucked into the crop by the pumping action of the pharyngeal muscles while gradually releasing the salivary chemicals responsible for the therapeutic benefits of hirudotherapy following the bite (Hildebrandt and Lemke, 2011). Adult leeches typically feed for approximately 30 to 40 minutes, ingesting approximately 10 to 15 milliliters of blood (Sawyer, 1986). After feeding, the blood is stored in the crop, which contains ten pairs of lateral pockets, providing additional storage space (Wenning et al., 1980). The blood is combined with anticoagulation secretions during ingestion. The blood in the digestive tract is preserved by endoenzymes, and

blood cells are concentrated by excreting plasma from the ingested blood. After feeding, leeches have been observed to survive for approximately a year without sucking blood (Wenning and Cahill, 1989; Hildebrandt and Lemke, 2011).

Furthermore, the digestive process is facilitated by endosymbiotic bacterias, such as *Aeromonas veronii* and *Aeromonas hydrophila*, which are present within the leech's digestive tract (Graf, 1999; Özbey et al., 2020). In medicinal leeches, the body surface is adapted in such a way as to facilitate gas exchange. The leeches respire by taking in oxygen from the water during locomotion, and they also respire by attaching the posterior sucker to something and undulating their body (Mann, 1962; Kaestner, 1967). The nervous system of medicinal leeches comprises a pair of cerebroid ganglia, 21 body ganglia, and seven caudal ganglia. These ganglia contain thousands of microglial cells (Le Marrec-Croq et al., 2013). The body of leeches typically possess both male and female reproductive systems, with the former consisting of tube-like male reproductive organs (including the duct for sperm transport) and the female reproductive organs located caudally. Following mating, full embryonic development occurs within a cocoon. In the natural environment, eggs in cocoons are laid in moist soil on the banks of inhabited ponds. After hatching from the cocoon, juvenile leeches enter the aquatic environment, where they grow by feeding on the blood of aquatic animals (Kutschera and Elliott, 2014).

## 2. Mechanism of Action

The therapeutic effects of medicinal leeches on their hosts while feeding can be broadly categorised as follows: a bloodletting effect during active blood absorption and passive wound oozing; the injection of biologically active substances into the host with saliva; and neuro-reflex (cuto-visceral) effects (Isakhanjan, 2003). To date, a substantial body of scientific literature has been produced on the secretions and mechanisms of action of leeches. In particular, more than 100 specific bioactive molecules with different molecular masses and their mechanisms of action have been identified in their secretions (Baskova et al., 2004). The mechanisms of action have been categorised to facilitate comprehension; however, these mechanisms are closely interrelated and should be considered as a whole. Following a leech bite, the leech must create a suction pathway by degrading the extracellular matrix. It must then inhibit platelet function and anticoagulant action to prevent adhesion, aggregation, and coagulation. The leech must also increase blood flow, protect itself by showing antimicrobial activity, and avoid detection in the host's body by exhibiting analgesic and anti-inflammatory action. Furthermore, the bioactive molecules present in leech saliva have been demonstrated to exhibit anti-cancer properties (Sig et al., 2017; Shakouri and Wollina, 2021).

### 2.1 Extracellular matrix degradation, increasing blood flow, and antimicrobial effect

The enzymes collagenase and hyaluronidase are produced by leeches immediately following a bite, facilitating the absorption and diffusion of their bioactive compounds (Sig et al., 2017). Hyaluronidase reduces the viscosity of tissues and renders them more permeable to injected fluids, thereby increasing the rate of fluid absorption. This effect allows the resorption of excess fluids and extravasated blood in the tissues, thereby increasing the efficacy of local anesthetics. Hyaluronidase thus increases the diffusion of all salivary secretions (Hovingh and Linker, 1999). Furthermore, it has antibiotic properties (Hallaji et al., 2019). Hyaluronidase can be used to treat various complications associated with hyaluronic acid. Collagenase has been demonstrated to diminish scar tissue density and inhibit fibroblast formation in keloids and hypertrophic scars (Rigbi et al., 1996).

Leech saliva contains the vasodilator acetylcholine and histamine-like molecules, as well as a carboxypeptidase A inhibitor that increases blood flow and reduces local edema. Furthermore, acetylcholine induces relaxation of the endothelial muscles. These molecules facilitate the continuity of microcirculation by transporting oxygenated blood to areas of compromised circulation, thereby restoring normal circulation (Segal, 2005; Herlin et al., 2016).

Destabilase has been observed to possess β-glycosidase activity, which enables it to break down β1-4 bonds in the bacterial cell walls' peptidoglycan layer directly (Zavalova et al., 2003). Lysozyme, frequently present in lacrimal fluid and both human and animal saliva, has exhibited the same action as described above (Franken, 1989). In addition to the enzymatic action of glycosidases, non-enzymatic components also contribute to the antimicrobial activity (Zavalova et al., 2006). The bacteriostatic action of the substance is effective against several bacterial species, including *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Indergand and Graf, 2000). Leech secretions

include the powerful antibiotic chloromycetin, yet there is a paucity of knowledge regarding this compound (Wilmer et al., 2013).

**2.2 Analgesic and anti-inflammatory effects**

The kallikrein system plays a significant role in the inflammatory response and is associated with the coagulation cascade. Bradykinin, a pro-inflammatory protein, kallikreins, and kininogen (a precursor to kinin) are components of the complex system known as the kallikrein-kinin system, which is produced in different organs. Bradykinin functions by increasing vascular permeability, which induces vasodilatation and intensifies pain (Sharma and Al-Sherif, 2011; Kashuba et al., 2013). Antistasin and hirustasin found in leech saliva act as potent factor Xa inhibitors and have an inhibitory effect on the kinin-kallikrein system (Nutt et al., 1991). Factor Xa is a prothrombin activator and plays a critical role in the common pathway of the coagulation cascade (Schenone et al., 2004). Some studies have shown that some kininases and antistasins may inhibit the kinin kallikrein mechanism, an important nociceptive pathway (Nutt et al., 1991). In addition, it has been observed in animal experiments that antistasin prevents vascular graft thrombosis, accelerates reperfusion, reduces restenosis after balloon angioplasty of atherosclerotic femoral artery, and affects mitosis of aortic smooth muscle cells in cell culture as a result of selective factor Xa inhibition (Zaidi et al., 2011).

Mast cell tryptases are serine proteases found in cell granules, and their release causes inflammatory reactions in animals. Strong correlations exist between these effects and leukocyte activation, chemotaxis, the kinin-kallikrein system, vasoactive activities, and the ensuing pain-producing interactions. Allergy and inflammatory conditions like arthritis, asthma, and anaphylaxis can be caused by chemicals that are secreted by mast cells (Vitte, 2015; Caughey, 2016). Three isoforms of leech-derived tryptase inhibitor (LDTI), known as a, b, and c, function by preventing mast cell proteolytic enzymes from acting. In addition to specifically inhibiting mast cell tryptase, the serine protease inhibitor LDTI also inhibits trypsin and chymotrypsin (Campos et al., 2004).

Eglins (elastase-cathepsin G leech inhibitors) are small proteins found in leeches that exhibit potent inhibitory activity against chymotrypsin and subtilisin-like serine proteinases acting on non-cationic substrates. A single leech contains approximately 20 μg of eglin. Eglin C has been identified as a potential therapeutic agent for treating inflammatory diseases. Its efficacy has been demonstrated in the management of shock and emphysema in experimental models (Seemuller et al., 1986; Zaidi et al., 2011). Eglins also improve diabetic neuropathy due to their anti-inflammatory effect on the nerve and improve microangiopathy by increasing blood circulation through the effect of many other bioactive substances in saliva, which are the main mechanisms of the positive impact on diabetic wounds (Dwivedi, 2012).

Additionally, the saliva of leeches contains bdellins. Bdellins are a class of substances that act as inhibitors of trypsin, plasmin, and sperm acrosine. They have anti-inflammatory effects. Furthermore, they act as proteinase inhibitors. In particular, bdellin A can be utilised as a plasmin inhibitor to regulate bleeding (Fink et al., 1986; Seemuller et al., 1986). These substances, which are secreted by leeches, are efficacious in the treatment of inflammation-induced diseases, anaphylaxis, allergy, and pain management in animals.

**2.3 Anticoagulation effects**

To maintain blood flow into their host's body while extracting blood, leeches inject anticoagulant chemicals. Leech secretions contain bioactive compounds that act at distinct sites in the coagulation cascade, a series of reactions that ultimately lead to the formation of a blood clot. Factor Xa plays a role in breaking the chain reaction, destabilase has a fibrinolytic effect, and hirudin primarily functions as a thrombin inhibitor (Sig et al., 2017).

Hirudin irreversibly binds to thrombin, thereby inhibiting blood coagulation by blocking all biological functions caused by thrombin (Markwardt, 1955; Bichler and Fritz, 1991). The administration of hirudin or its derivatives may be indicated for the prophylaxis and treatment of postoperative venous thrombosis, particularly in the context of cardiac surgery; the augmentation of fibrinolytic therapy and/or angioplasty to prevent re-occlusion; and flap surgery. Hirudin and its analogs may be utilized in the treatment of vascular diseases, including strokes and myocardial infarction (Zaidi et al., 2011; Alaama et al., 2024). Factor Xa inhibitor is an anticoagulant, and it also plays a critical role in the treatment of osteoarthritis and rheumatoid arthritis (Das et al., 2014).

The monomerising activity of destabilase has the potential to degrade stable fibrin by isopeptidolising $\alpha$ and $\gamma$ fibrin chains linked by $\varepsilon$ ($\gamma$

Glu) Lys isopeptide bonds (Baskova et al., 2001). It can be concluded that the anticoagulant effect of bioactive substances is of great importance in the therapeutic management of diseases associated with anticoagulant deficiency in humans and animals.

**2.4 Antiplatetelet effect**

Normally, in animals and humans, when the wall of a blood vessel is damaged, it causes the spread and release of collagen particles targeting the von Willebrand factor (vWF). After the collagen particles are released, a plug is formed in the vessel following a series of events, and the bleeding is stopped. This reaction also initiates another chain in which substances such as thromboxane A2, platelet activation, and the coagulation cascade are released (Kumar et al., 2015). In leech secretions, various molecules (saratin, calin, and apyrase) react against different parts of this chain (Das, 2014). Saratin affects only the first stage of platelet adhesion and inhibits the collagen-vWF reaction. Some animal studies have shown promising results with recombinant saratin molecule as a potential local therapeutic agent for antithrombotic treatments and atherosclerosis (Gronwald et al., 2008).

Calin exhibits rapid action (within 10 minutes) on collagen, effectively suppressing platelet adhesion to collagen-coated microcarrier particles and collagen-induced platelet aggregation (Munro et al., 1991). In addition to preventing direct platelet-collagen contact, Calin also obstructs the binding of von Willebrand factor to collagen. This is believed to be one of the initial stages of thrombus formation in injured endothelial areas (Harsfalvi et al., 1995). A disruption to this mechanism may have antithrombotic effects. Research conducted on hamsters has demonstrated that calin selectively and in a dose-dependent manner prevents platelet aggregation brought on by collagen (Deckmyn et al., 1995). The chemicals released by leeches have the potential to suppress platelet aggregation by blocking the platelet receptor vWf and degrading collagen particles. This approach may prove effective in the management of micro thrombus formation following surgical procedures in animals.

A summary of the bioactive molecules mentioned above can be found in Table 1.

**Table 1**. Potential bioactive substances in leech secretions.

| Mode of action | Bioactive Substance | Function |
|---|---|---|
| ***Analgesic and anti-inflammatory effect*** | Bdellins | Used as a plasmin inhibitor to control bleeding |
| | Hirustasin/ Antistasin | Prevents vascular graft thrombosis, accelerates reperfusion |
| | LDTI | Inhibits mast cells that occur in conditions such as arthritis and asthma |
| | Eglins | Improves diabetic neuropathy and promotes healing of diabetic wounds |
| | Carboxypeptidase A inhibitor | Inhibits carboxypeptidase A, accelerates blood flow |
| ***Anticoagulant effect*** | Hirudin | Hirudin inhibits thrombin. It is a powerful anticoagulant alternative to heparin |
| | Destabilase | Dissolves stabilised fibrin |
| | Factor Xa inhibitor | Inhibits Factor Xa |
| ***Extracellular matrix degradation*** | Hyaluronidase | Inhibits hyaluronic acid. Makes vessels more permeable to fluids |
| | Collagenase | Dissolves collagen particles, accelerates wound healing |
| ***Increasing blood flow*** | Histamine-like molecules | Increase blood flow |
| | Acetylcholine | Helps relaxation of endothelial muscles. |
| ***Anti-platelet function*** | Calin | Inhibits collagen-induced platelet aggregation |
| | Saratin | Potential agent for atherosclerosis |
| ***Antimicrobial effect*** | Destabilase | Destroys the peptidoglycan layer in the bacterial cell wall. |

## 3. The application of Veterinary Hirudotherapy

### 3.1. Procedure

It is recommended that leeches for therapeutic use are obtained from hygienic leech farms that are certified to cultivate leeches for medicinal purposes. This is more effective than acquiring them from natural habitats as it protects against various pathogens, including viruses, bacteria, fungi, and parasites. Leeches taken from farms should be kept in groups of no more than 10-15 in glass jars of approximately 3 liters, covered with a layer of dense linen fabric, and held in place by an elastic band or secure plastic lid with small perforations to facilitate ventilation (Figure 1). The water in the container should be free of chlorine. The water in the container should be changed once or twice a week, and the container should be thoroughly cleaned once a month without using chemicals. The leech container should be kept in a dark and cool place, away from strong odors and vibrations. These conditions have been shown to support the survival of leeches for up to one and a half years (Gileva and Mumcuoğlu, 2013).

**Figure 1.** Glass container suitable for storage of leeches.

The number of leeches to be used in a therapeutic application is contingent upon the size and disease state of the animal. It is recommended that one leech be used for animals weighing less than 10 kg, 1- 2 leeches for animals weighing 10-15 kg, 3-4 leeches for animals weighing 30-40 kg, and 5-15 leeches for a horse (Sobczak and Kantyka, 2014). Before the application of leeches, it is imperative that the area be cleansed with sterile water, as the utilization of disinfectant or antiseptic solutions within the application area will impede the leech's ability to attach. In order to facilitate the attachment of the leech, it may be necessary to shave the treatment area, depending on the area to be applied. However, shaving is not a prerequisite for applications in auricular hematomas (Anonym, 2024a). Leeches are typically willing to feed due to their starvation before use. However, if the leech is reluctant to feed, pricking the skin with a small needle to induce bleeding facilitates its attachment, or applying a sugar and water solution can facilitate the attachment of the leech. Once the leech has attached itself, it is likely to remain in place until it has ingested sufficient blood, at which point it will fall off of its own accord. Nevertheless, monitoring the process to prevent the leech from relocating to an unintended area is essential. The feeding period for leeches in dogs and cats is typically 30 to 60 minutes, while in horses, it is 40 to 120 minutes. During this period, the leech expands to approximately five times its original size (Sobczak and Kantyka, 2014). It is of the utmost importance to allow the leech to feed until it detaches naturally; the leech should never be forcibly removed during this process. The application of force may result in the leech vomiting its stomach contents into the wound, which could potentially lead to infection. The optimal method for leech removal is to shake the leech's entire body several times gently or to gently loosen the posterior sucker with a fingernail, allowing the leech to descend due to its body weight (Michalsen et al., 2007). Following the application of the leech, it is important to maintain the bleeding of the tissue at the bite sites. The wound should be kept open in a clean environment.

Following application, leeches should not be reused on another patient. To mitigate the potential for complications, it is advised that physicians applying leeches disinfect them prior to use in accordance with established protocols, apply the leech on one occasion only, and then dispose of it. The most humane method of leech disposal is to initially place the leech in an 8% alcohol solution and then apply a stronger 70% solution (Ben-Yakir, 2008a).

### 3.2 Indications

Hirudotherapy is used in traditional and complementary medicine to treat a wide range of diseases and support existing therapies. The anticoagulant, vasodilator, thrombolytic, analgesic and anti-inflammatory properties of leeches can be used to treat various conditions (Ayhan and Mollahaliloğlu, 2018). Using leeches in veterinary medicine has the same indications as in human medicine. The most common applications of hirudotherapy in animals include flap surgery, orthopedic trauma, limb amputation, post-castration, venous congestion, presence of blood clots, poorly healing wounds, and inflammatory diseases (Sobczak and Kantyka, 2014; Abdisa, 2018; Kermanian et al., 2022).

The diseases in which the use of hirudotherapy is indicated in various animal species are as follows.

#### 3.2.1. Cats

The therapeutic efficacy of medicinal leeches as an adjunct treatment for polycythemia vera has been demonstrated in veterinary medicine. In a cat diagnosed with polycythemia vera, leeches were applied as an alternative to more invasive procedures, such as phlebotomy, which was initially not feasible. Hirudotherapy aids circulation by reducing blood viscosity, thereby improving hemodynamics. However, the optimal dosage and duration of application vary depending on the patient and disease severity. Studies indicate that hirudotherapy contributes to the stabilization of blood cell levels and may play a supportive role in long-term disease management (Net et al., 2001).

Conventionally, polycythemia vera is treated with therapeutic phlebotomy and chemotherapeutic agents, such as hydroxyurea. While phlebotomy is effective in acutely reducing hematocrit levels, it necessitates frequent and repetitive procedures, which may be challenging in some clinical scenarios. Additionally, chemotherapeutic agents pose risks of immunosuppression and myelosuppression, requiring careful patient monitoring (Plumb, 2008). In contrast, hirudotherapy presents a minimally invasive alternative with fewer systemic side effects, making it a potentially beneficial complementary treatment in polycythemia vera management. However, further research is warranted to establish standardized protocols and confirm its long-term efficacy.

In another case, a cat was presented with acute hind limb paralysis and pain in these limbs. Furthermore, no femoral pulses could be discerned in either limb. The distal limbs were observed to be cold and bluish, and no bleeding was observed from the cut nail beds. Two medicinal leeches were applied to both sides of the inner thigh, close to the abdomen. Twenty-four hours after the leeches were applied, the cat exhibited a return of femoral pulses to normalcy, a reduction in pain, and the ability to walk without assistance. It was documented that other similar cases were presented to the clinic within two years, and each was successfully treated with medical leeches. (Ben-Yakir, 2008b).

Following a constrictive injury from a bandage, a cat with considerable swelling in its paw and impaired limb use was treated with leech therapy for four days, during which time a notable reduction in swelling was observed. The cat was able to use its limbs, and the constrictive wound healed further without any complications. In this case, it was reported that the cat's severe venous congestion had been resolved and that there was no further skin loss due to leech therapy (Figure 2) (Buote, 2014).

**Figure 2.** Leech treatment of cat paw with venous congestion (Buote, 2014).

Additionally, medicinal leeches are used in the treatment of conditions such as inflammation and diseases of the peripheral veins and arteries, including feline aortic thromboembolism. They are

also used in FIV-positive cats with salivary gland diseases, including severe stomatitis, gingivitis, sialadenitis, and sialoadenosis, acute and chronic inflammatory responses of the oral mucosa and periodontal lesions. Furthermore, they are applied in the management of postoperative wound complications, discopathies, eczema, abscesses, strained ligaments, dysplasia of the knee, knee inflammation, neuritis, and mastitis (Sobczak and Kantyka, 2014; Anonym, 2024b).

**3.2.2. Dogs**

An aged dog presenting with fractures of all four metatarsal bones in its hindlimb underwent surgical intervention to stabilize the affected bones via internal fixation. Following the surgical procedure, leeches were applied to various regions of the dog's foot. As a consequence of the therapeutic intervention, the foot exhibited a notable degree of recovery (Fernee, 2011).

A 10-year-old spayed female Mastiff dog with an upper respiratory tract obstruction was intubated and received a propofol and fentanyl infusion, as well as a series of antibiotics, during the initial 18 hours of treatment. Subsequently, a total of 11 leeches were applied to the sublingual and cervical regions of the dog, resulting in a reduction in sublingual swelling observed 6-8 hours after the treatment. The patient was extubated at the 44th hour and subsequently discharged from the hospital (Trenholme et al.,2021).

A study conducted by Canpolat and Sağlam (2004) explored the use of medicinal leeches as a non-invasive alternative for treating aural hematomas. In their experiment, ear hematomas were induced in five adult dogs by traumatizing the arteries and veins of the outer ear surface. On the following day, three medicinal leeches were applied to each hematoma without anesthesia. The leeches were allowed to feed until they were fully engorged and detached naturally, with the procedure repeated daily for three days. The study reported that all cases exhibited complete resorption of the hematoma without any complications, demonstrating the potential of hirudotherapy as an effective alternative to conventional methods (Canpolat & Sağlam, 2004).

Aural hematomas are commonly managed through medical or surgical interventions, depending on their size and severity. Smaller hematomas may be treated conservatively with corticosteroids and drainage, whereas larger hematomas typically require surgical intervention as they are less responsive to therapeutic management. Surgical treatment involves waiting for the hematoma to coagulate over 8–10 days before surgical evacuation, but this approach carries risks such as fibrosis, chronic inflammation, and auricular deformation (Dubielzig et al., 1984; Cowley, 1976).

Compared to surgical intervention, hirudotherapy offers a less invasive and low-risk treatment, reducing the likelihood of fibrosis and structural deformities associated with surgical procedures. Additionally, the anticoagulant and anti-inflammatory properties of leech saliva aid in blood resorption and tissue healing, further supporting its use as a viable alternative or adjunct therapy in aural hematoma management. However, more clinical studies are needed to establish standardized treatment protocols and validate its long-term efficacy.

Furthermore, the treatment is indicated mainly in instances where the vessels are particularly thin, as well as in cases of mastitis and gastritis, the reduction of scrotal edema in adult dogs following a castration procedure, postoperative wounds, spinal osteoarthritis, discopathies, and cauda equina syndrome, hip and elbow dysplasia, neuritis, ear eczema, poor wound healing, postoperative scars, tendinitis, tenosynovitis (Sobczak and Kantyka, 2014; Ben-Yakir, 2008a).

**3.2.3. Horses**

Laminitis is a painful and debilitating inflammatory condition affecting the laminae of the horse’s hoof, often leading to severe lameness and structural hoof damage. Conventional treatment typically includes NSAIDs (phenylbutazone), cryotherapy, and corrective farriery (Glöckner, 2002). While these methods aim to control pain and inflammation, many cases become chronic, and NSAID toxicity presents a concern (Rasch, 2010). Surgical interventions, such as deep digital flexor tenotomy, are reserved for refractory cases but have variable success rates (Obel, 1948).

An alternative approach using medicinal leeches has been investigated for its anticoagulant, anti-inflammatory, and vasodilatory properties. A study conducted in Germany between May 2008 and August 2009 examined the effects of hirudotherapy in 57 laminitic horses, with a total of 112 leech applications (Rasch, 2010). Results indicated that 84% of the horses exhibited clinical improvement, with some cases showing symptom relief within 48 hours. Unlike NSAIDs, which can contribute to renal and gastrointestinal complications, leech

therapy resulted in only mild to moderate itching at the application site in 6 cases out of 112 (Rasch, 2010).

Compared to conventional therapy, hirudotherapy offers a less invasive, systemic toxicity-free alternative. By improving local circulation and reducing venous congestion in the hoof, medicinal leeches facilitate the resolution of inflammation without the adverse effects associated with NSAIDs. However, further research is required to determine long-term efficacy, develop standardized application protocols, and establish its role as a routine adjunctive therapy in laminitis management.

The team led by Dr Sagiv Ben-Yakir used leech therapy on four horses with acute laminitis. Four leeches were implanted into each affected leg. Following a 12-hour course of treatment, the horses were reported to have returned to their normal behavioral patterns (Ben-Yakir, 2006).

A haematoma was observed on the penis of a stallion whose penis had been kicked during mating. As the initial treatment was unsuccessful and exhibited delayed efficacy, a second course of treatment was initiated, comprising two applications of leeches with a five-day interval between each application. Following the administration of anti-inflammatory and antibiotic agents, the horse made a full recovery within 45 days. The stallion's erectile function was restored, and the stallion was able to resume reproductive activities (Nowicki et al., 2021)

In an interview with a leech practitioner, it was reported that the application of leeches was observed to be 80-90% effective in the treatment of mud fever in horses (Figure 3) (Bergsma, 2023).

**Figure 3.** Leech application in horse (Bergsma, 2023)

In addition to the previously mentioned conditions, leeches are also used in the treatment of ophthalmia, lymphangitis, congestion, tendinitis, tenosynovitis, ataxias, myositis, and spinal osteoarthritis in horses, as well as arthritis in the shoulder joint (Sobczak and Kantyka, 2014; Anonym, 2024b).

#### 3.2.4. Ruminants

In a study examining the effects of hirudotherapy on hemostasis-plasma link in cows with clinical mastitis, two leeches were attached to the tissue of the diseased quarter of the udder for a period of three days, with each application occurring at 24-hour intervals for 40-50 minutes per therapy session. The results indicated that leech therapy had a corrective effect on the plasma-coagulation coupling of hemostasis (Soboleva, 2018).

The use of medicinal leeches for the treatment of mastitis, uterine prolapse, and inflamed vulva in other ruminants has also been reported (Ben-Yakir, 2008a; Haq et al., 2021).

### 3.3. Contraindications and complications

The following conditions are contraindications to the use of leeches in medical therapy: coagulopathy or animal on anticoagulant therapy, anemia and/or low blood pressure, hemophilia, the last stages of malignant tumour, pregnancy in the animal, lactation, bleeding in estrus time, immunosuppressive diseases and malignant skin abnormalities, fungal diseases or in animals with known allergic reactions to the active components

of leech saliva (hirudin, hyluronidase, destabilase) (Sobczak and Kantyka, 2014).

It is important to note that there is a risk of transmission of *Aeromonas spp*. bacteria when leeches suck blood. These bacteria are carried in the leech's body and may cause infection if transmitted to a host. The presence of *Aeromonas spp*. and other gram-negative bacteria within the leech body can result in various bacterial infections, including intestinal disorders, pneumonia, septicemia, necrosis, and flap failure (Abdualkader et al., 2013). Another potential complication is the reuse of the leech, which carries the risk of contamination with various blood-borne diseases. Additionally, allergic reactions, unstoppable bleeding, and anaphylaxis may occur following leech application (Sobczak and Kantyka, 2014; Abdisa, 2018).

Some authors have proposed that prophylactic parenteral antibiotics may be administered to the animal prior to leech application or that leeches may be immersed in 0.02% chlorhexidine hydrochloride for 10 to 15 seconds before application to the treatment area to reduce the risk of infection (Haycox et al., 1995).

## DISCUSSION and CONCLUSION

The bioactive compounds found in medicinal leeches have been utilized for centuries in the treatment of various diseases. Modern scientific research has confirmed the anti-inflammatory, anticoagulant, and circulatory-regulating effects of key bioactive molecules such as hirudin, eglins, bdellins, and destabilase (Sig et al., 2017; Shakouri and Wollina, 2021).

Laboratory studies on leech secretions have provided promising evidence regarding the therapeutic potential of hirudotherapy. However, the pharmacokinetic and pharmacodynamic properties of these bioactive compounds remain incompletely elucidated (Bayıroğlu, 2023; Erol, 2023).

Due to its minimally invasive nature, cost-effectiveness, and biological compatibility, hirudotherapy has been suggested as a potential alternative to conventional treatments, particularly in the management of joint diseases, vascular obstructions, and postoperative recovery (Abdualkader et al., 2013). Recent studies have highlighted its critical role in reducing venous congestion, accelerating soft tissue healing, and preventing postoperative complications following microsurgical procedures (Gileva and Mumcuoğlu, 2013).

In veterinary medicine, hirudotherapy presents a promising complementary treatment option. Nevertheless, the absence of standardized treatment protocols, interspecies variability in biological responses, and concerns regarding sterilization procedures pose significant limitations to its widespread clinical application. Therefore, further scientific investigations are warranted to establish the safety and efficacy of hirudotherapy as a viable therapeutic approach in veterinary practice. However, future research should focus on evaluating the long-term outcomes and safety profiles of leech therapy in veterinary patients.

In conclusion, hirudotherapy holds significant potential as a complementary and alternative treatment in veterinary medicine. However, further empirical evidence is required to substantiate its clinical efficacy and safety, and the development of standardized treatment protocols remains a priority. Consequently, future studies should prioritize controlled clinical trials and veterinary-specific applications to establish a robust scientific foundation for the widespread implementation of hirudotherapy.

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***Review Article***: A Review Article is an in-depth analysis of the current status of a particular topic. It is not an original article with new data but represents a well-balanced summary of a timely subject with reference to the literature.

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This first page should contain the complete title of the article, manuscript category, names and credentials of all authors, institutional affiliations of all authors, orcid (all authors of the manuscript must have a orcid number) of all authors and a short running title (≤60 characters). The corresponding author should be indicated, including a complete e-mail address and telephone number.

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**Whole Book**

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**Edited Book**

Underwood LE, Van Wyk JJ. Normal and aberrant growth. In: Wilson JD, Foster DW, eds. Wiliams' Textbook of Endocrinology. 1st ed. Philadelphia: WB Saunders; 1992. p.1079-1138.

**Website**

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