LIVESTOCK STUDIES

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Livestock Studies has been monitored: ULAKBIM (Turkish National Academic Network and Information Center), FAO AGRIS, CAP Abstract, CABI Full Text, Animal Breeding Abstracts, Google Scholar.

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RESEARCH PAPER

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Examination of OECD Countries for the Presence of Livestock by Non-Metric Multidimensional Scaling

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Abstract

In this study, by Non-metric multidimensional scaling (NMDS) which is one of the multivariate statistical analysis methods, configuration of 36 OECD countries in two dimensional space was examined and similarities/dissimilarities between these countries were determined for the variables regarding with livestock data. As variables, "number of horses, pigs, donkeys, turkeys, mules, geese, goats, sheep, buffaloes, ducks, cattle and chickens" of OECD countries obtained from the website of FAO was used. Euclidean distance was used as distance measures. According to the results of NMDS; USA, Germany, France, England, Canada and Poland had the highest positive effect on livestock. However, Israel, Iceland, Lithuania and Luxembourg were different from other countries with the lowest effects. Similarly, while buffalo was the lowest effective one; pigs, cattle, chicken and turkey were found the highest effective species on the livestock sector. As a result, it was suggested that NMDS can be used as an effective method in the analysis of multivariate data in agriculture and livestock with simple graphical representation and interpretation of the results.

Introduction

The steady increment in the world population lead to increase importance of animal production. Therefore, properly plans and policies should be developed by obtaining information about animal assets and acting consciously about animal science. According to Food and Agriculture Organization of the United Nations in 2018, There are 1 786 881 743 cattle (buffalo, cattle), 2 557 846 061 ovines (sheep, goat), 123 687 495 single-hoofed (horse, donkey, mule), 1 425 507 453 pig, 32 041 935 000 poultry (chicken, turkey, duck, goose) in 36 OECD countries (FAO, 2020). In the light of these data, it can be stated that OECD countries has approximately 17.17% of world animal wealth. Turkey is placed considerable point among the OECD countries.

Some studies (Ersöz, 2008; Beyhan Acar, 2013; Boz *et al.,* 2016; Akdamar, 2019) have been carried out to identify similarities or dissimilarities between OECD (Organization for Economic Co-operation and Development) countries with Multidimensional scaling (MDS). Akın and Eren (2012) examined the similarities or dissimilarities of OECD countries for education indicators. Furthermore, by using MDS, Çelik (2015) classified Turkish provinces for livestocks.

In the scientific research, as the number of variables increases, the number of dimensions also increases. Thus, there are some difficulties in interpreting of the results obtained. To overcome these difficulties, multivariate statistical methods, especially dimension reduction methods are used and the results are more easily interpreted and understandable by using two or three dimensions instead of multiple dimensions. Therefore, multivariate statistical methods play an important role in statistics and are used in almost all disciplines. These methods examine the relationships between two or

more dimensional variables by considering together. As a one of multivariate statistical methods, MDS examine the data in a smaller scale (Ağgün, 2011; Härdle and Simar, 2015).

MDS is a graphical method that allows determining the relationship between objects by displaying in k-dimensional space as close as possible for their real positions and a less dimensional space by using distance values in cases where the relationships between objects are unknown however computable (Johnson and Wichern, 2007; Özdamar, 2010; Alpar, 2011). The purpose of the graphical representation method is to transform the relationships between objects or variables into a more understandable form (Borg and Groenen, 2005). The application area of MDS is quite common due to using the similarities measures instead of distance or difference measures. MDS can be applied to both metric and non-metric variables in various scientific fields such as Psychology, Sociology, Education, Agriculture, Economics, Marketing, Medicine and Engineering (Tatlıdil, 1992; Ding, 2018).

Multivariate statistical methods such as Principal Component Analysis, Factor Analysis, Correspondence Analysis and Cluster Analysis can be used similar purposes. However, these methods require large sample size as well as some assumptions. MDS can be considered to more advantageous than these multivariate statistical methods due to more flexible for assumptions and small sample size. High-dimensional (multivariate) data sets are quite common in many fields of science, especially, animal science. Thus, MDS can be used effectively to examine the relationships between variables in terms of similarities or dissimilarities.

With this perspective, the aim of this study is to explain MDS briefly and to examine similarities or dissimilarities of OECD countries in terms of livestocks and to present configuration of the countries in two-dimensional space for easy understanding and interpretation.

Materials and Methods

Material of the study consists of number of animals for 36 OECD countries in 2018 (FAO, 2020). Totally 12 variables that horse, pig, donkey, turkey, mule, goose, goat, sheep, buffalo, duck, cattle, and chicken numbers were included into analysis.

In the study, NMDS was used to statistical analysis method. This analysis determines the similarities or dissimilarities between n points and shows the distances between these points in a reduced dimensional space with the help of appropriate distance measures (Johnson and Wichern, 2007; Özdamar, 2010).

MDS can be summarized in 6 steps:

- In case of different scaled variables, these variables are converted into a standard scale for equal effect in determining the distance.
- Using the appropriate distance measurement for the data type, the *nxn* dimensional distance matrix is calculated.
- To display objects in reduced dimensional space, stress value (statistic) that is measure of fit is computed for determination of reduced dimension number.
- According to data type, properly regression method such as linear, polynomial or monotonic regression is performed to calculate original (δ_{ij}) and configuration distances (d_{ij}) of objects.
- The stress value which is a suitable statistic is calculated to determine the fit original and configuration distances.
- To display distances of objects in reduced dimension, coordinates of the objects are determined. Positions of each object are displayed in the determined coordinates or 2 dimensional space (Özdamar, 2010).

In order to reduce the number of dimensions, the configuration distance (d_{ij}) values represented in the new space are used instead of the similarity measures (δ_{ij}) Thus, the configuration distances are accepted to be equal to the original distances and estimated distances of them are shown with $\hat{d}_{ij}$ (Tatlıdil, 1992; Härdle and Simar, 2015).

MDS is based on the distance measures. Therefore, similarities or dissimilarities between the objects are computed by using the distance functions. According to the data structure and variable type, various distance measures are recommended. Therefore, it is very important to use the appropriate distance measure. Euclidean distance is quite common distance function in MDS (Everitt and Dunn, 2001; Özdamar, 2010). Euclidean distance is used to determine the distance between two points. It can also be used to determine the distances of elements on a data matrix. Thus, using the generalized Euclidean distance equation, the configuration distance between i^{th} and j^{th} points in a p-dimensional is follows.

$$d_{ij} = \left[\sum_{a=1}^{p}\left(x_{ia} - x_{ja}\right)^2\right]^{1/2} \quad \textbf{(1)}$$

In the equation (1), x_{ia} and x_{ja} give the i^{th} and j^{th} observation values in a^{th} and a^{th} dimension, respectively. Thus, the distance between the elements of the data matrix can be computed in two-dimensional space (Cox and Cox, 2001; Everitt and Dunn, 2001).

In MDS, number of distances between n objects is computed with [n(n-1)/2]. These distances are symmetrical and known as configuration distances. In order to obtain a low dimensional geometric representation, a coordinate system is tried to be created by obtaining the closest viewing distances to the configuration distances. The distances are calculated with the metric scaling method in case of interval or ratio scaled variables. However non-metric scaling method is used to ordinal scaled variables (Mackay and Zinnes, 1986; Mead, 1992).

Metric MDS uses directly for distance values to

locate a given observation. However, non-metric MDS uses orders of magnitude instead of numerical values of distances, and the only information used in determining the display distances ($\hat{d}_{ij}$) is the sequence numbers of the configuration distance (d_{ij}) values in this analysis (Kruskal and Wish, 1978; Mead, 1992). Here, it is aimed that the distance values $\hat{d}_{ij}$ keep the same monotone increasing order relationship with the corresponding d_{ij} values and scaling accordingly. Iteration method is used to minimize the stress value due to no analytical solution for the method. When the smallest stress value is obtained, iteration process is stopped and the size number is decided (Cox and Cox, 2001; Seber, 2004). NMDS is more preferred than metric MDS due to its more flexible assumptions and providing less dimensional solutions (Özdamar, 2010).

The efficiency of MDS is measured by STRESS (Standardized REsidual Sum of Squares) and the Stress value show the discrepancy between original and configured distances of objects (Kruskal and Wish, 1978). In general, Kruskal stress statistic is used in NMDS. The first reason for this is that this statistic has been widely accepted, and the other is that the statistics can be calculated for good and weak NMDS solutions (Borg *et al.,* 2018). Kruskal stress statistics is calculated by the following equation.

$$Stress = \sqrt{\frac{\sum_{i<j}\left(d_{ij}-\hat{d}_{ij}\right)^2}{\sum_{i<j} d_{ij}^2}} \quad \textbf{(2)}$$

In equation (2), (d_{ij}) shows the configured distance between i^{th} and j^{th} points, and the projection distance estimated by c^{th} iteration between i^{th} and j^{th} points according to $\hat{d}_{ij}$ multidimensional scaling. This value varies depending on the number of dimensions and scale used (Kruskal, 1964; Mead, 1992; Borg and Groenen, 2005; Johnson and Wichern, 2007). There are different versions of this given stress statistic in practice, such as the "Quadratic stress (S-stress) statistic" (Borg *et al.*, 2018). The S-stress statistic is derived from the stress scale to reveal the mismatch between the assumed and original structures. S-stress statistics is calculated by the following equation;

$$S-Stress = \sqrt{\frac{\sum_{i<j}\left(d_{ij}^2-\hat{d}_{ij}^2\right)^2}{\sum_{i<j} d_{ij}^4}} \quad \textbf{(3)}$$

Alternating least squares scaling (ALSCAL) algorithm in SPSS program uses s-stress value (Giguère, 2006; Johnson and Wichern, 2007).

Dimensional solutions that give stress value close to 0 are specified as the most suitable or desired solution (Ersöz, 2008). For easy interpretation, the solution is desired to be smaller than four dimensions and generally, two dimensions are preferred (Mackay and Zinnes, 1986; Özdamar, 2010).

According to Kruskal-Shepherd tolerance rate, stress values can be classified as bad for $\sigma \geq 0.20$, medium for $0.1 \leq \sigma < 0.20$ and good for $0.05 \leq \sigma < 0.10$. If stress value is less than 0.025, this indicated that there is a perfect fit between original and configured distances (Kruskal, 1964; Seber, 2004).

Another criterion is R^2 and this is square of the correlation coefficient (Cox and Cox, 2001; Alpar, 2011). R^2 can be written as follows.

$$R^2 = 1 - \frac{\sum_{i=1}^{n}\left(d_{ij}-\hat{d}_{ij}^2\right)^2}{\sum_{i=1}^{n}\left(d_{ij}-\bar{d}\right)^2} \quad \textbf{(4)}$$

Where $\bar{d}$ is the average of the configured distances. If the value of R^2 is greater than 60%; this is stated that the fit is reasonable and NMDS can be applied to the data (Alpar, 2011; Çelik, 2015). Thus, by using the ordinal numbers of the observed objects, coordinates of the points in space are obtained by NMDS (Cox and Cox, 2001).

Besides, another method used to determine the appropriate number of dimensions for the positions of objects in space is the scree plot. Scree plot shows the relationship between the stress value and the number of dimensions, and the number of dimensions is determined according to the breaking point expressed as elbow (Johnson and Wichern, 2007). In a scree plot, the curve usually decreases monotonous, however gradually there is a decrease in the slope such that it becomes fixed after a point. Elbow point of this curve determines number of dimensions (Borg and Groenen, 2005).

For MDS, ALSCAL algorithm is available in the IBM SPSS v21 statistical package program PROXSCAL algorithm for the scree plot is used (IBM Corp. Released, 2012). In addition, Euclidean distance is taken as a measure of distance.

Developed by Takane *et al.* (1977) to calculate optimal distances between objects in k-dimensional space, ALSCAL is the first applicable algorithm for MDS in non-metric individual differences. This algorithm minimizes the S-stress loss function (Equation (3)) with the least-squares method. ALSCAL represents large differences better than small differences and can be defined as a flexible MDS algorithm that provides models for asymmetric data, propagation and three-way analysis (Borg and Groenen, 2005).

Results and Discussion

The similarities and dissimilarities of 36 OECD countries in terms of the numbers of animals (horse, pig, donkey, turkey, mule, goose, goat, sheep, buffalo, duck, cattle and chicken) were analyzed. The scree plot is given in Figure 1.

Figure 1 shows that the change in stress values after the second dimension is very small and this is less

than 0.025 at this point. Thus, it can be stated that two dimensions are suitable for visualizing the objects. Stress values showing the fit between the positions of these data in multidimensional space and their positions in two-dimensional space are given in Table 1.

Table 1 shows that the iteration was continued until the proper value of the stress. For two dimensions (p = 2), iteration was stopped when stress value reached 0.00039 in the 4^{th} iteration. In the 4^{th} iteration, stress value was found 0.14790 and this value shows that the fit between the real positions of the data in the multidimensional space. Stress value can also be calculated by the Kruskal equation and the coefficient of determination, which is the square of the correlation coefficient, was given in Table 1. According to Kruskal equation, stress value was 0.13795 and this value indicated that fit is at a "moderate level". Corresponding to this stress value, determination coefficient (R^2) was found 0.91107. This value is

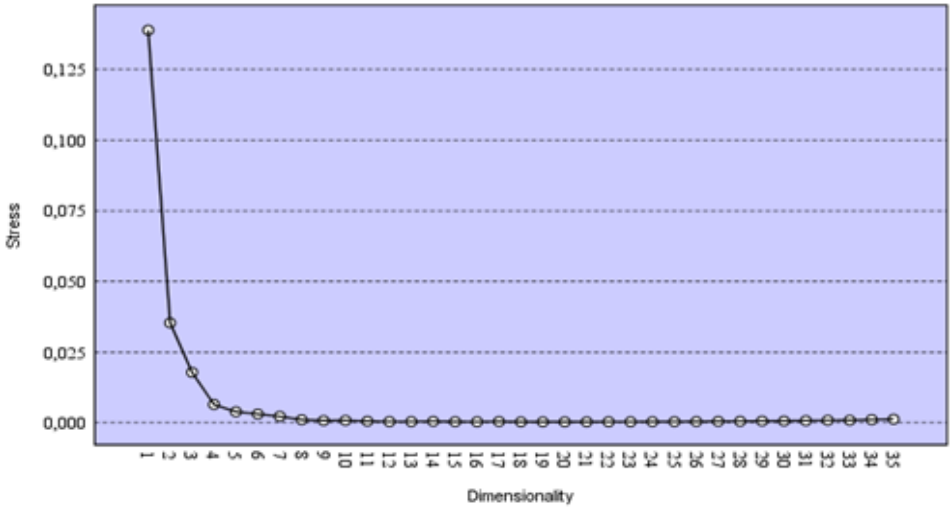

Figure 1. Scree plot showing the relationship between stress and dimension for countries.

Table 1. S-Stress, correction values and Kruskal Stress and R^2 value for two dimensions.

Iteration	S-tress	Improvement	Stress	R^2
1	0.18849	-		
2	0.15100	0.03749	0.13795	0.91107
3	0.14829	0.00270		
4	0.14790	0.00039		

greater than 60% and it indicates that the analysis is reliable.

Coordinates of the countries in two-dimensional space are given in Table 2.

According to the coordinates given in Table 2, locations of the countries in two-dimensional space were visualized in Figure 2. It can be stated that as the distance between objects decreases, the objects begin to look alike. However, as increasing of this distance, the objects begin to differ from each other. In other words, more similar countries are closely located each other on the graph (Çelik, 2015; Ding, 2018).

Table 2. The coordinates of the countries in two-dimensional space.

Variable	1.Dimen	2.Dimen	Variable	1.Dimen	2.Dimen	Variable	1.Dimen	2.Dimen
USA	2.2765	0.3983	Ireland	0.2945	-0.1318	Luxembourg	-2.3159	-0.1365
Germany	1.3632	0.9246	Spain	1.2488	-1.2849	Hungary	0.3612	0.1811
Australia	1.2151	-0.0147	Israel	-0.4317	-1.0245	Mexico	2.3640	-0.0442
Austria	-0.1488	0.1696	Sweden	-1.2294	0.1628	Norway	-0.5519	0.0674
Belgium	-0.2539	0.2743	Switzerland	-0.4628	-0.5426	Poland	0.7227	1.1913
Czech R.	-0.5045	0.3425	Italy	1.3506	-0.9011	Portugal	-0.0999	-0.7540
Denmark	-0.6334	0.7524	Iceland	-1.9565	-0.3866	Chile	0.8694	-0.7811
Estonia	-1.8965	0.0457	Japan	-1.0625	0.9461	Slovakia	-1.1197	0.0117
Finland	-1.1136	0.1997	Canada	1.0248	1.1066	Slovenia	-1.4051	0.0094
France	2.0038	0.0613	S. Korea	-0.1692	1.2065	Turkey	1.8158	-1.3152
Netherlands	0.4110	0.4869	Latvia	-1.2087	0.0762	Greece	0.0476	-1.3980
Britain	1.2929	0.5715	Lithuania	-1.8598	-0.1718	NewZealand	-0.2381	-0.2991

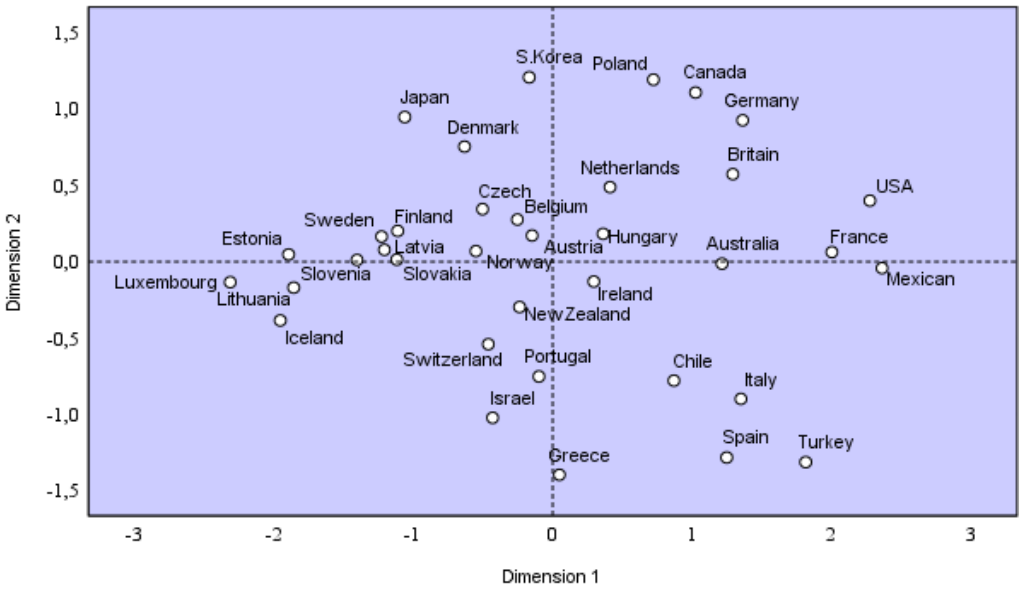

Figure 2. Configuration of 36 OECD countries in two-dimensional space.

As seen from Figure 2; Mexico, USA, France, Turkey, Germany, Italy, UK, Spain, Australia and Canada are the most similar countries in the first dimension. These countries have positive loads and play the most important discriminator role in this dimension. Therefore, it can be expressed that these 10 countries, especially Mexico (2.3640), the USA (2.2765) and France (2.0038), have a great influence among OECD countries. Furthermore, Turkey (1.8158) seems to be high in terms of the effectiveness of the variables. With a higher impact, these countries are differentiating from the other 26 countries.

Luxembourg, Iceland, Estonia, Lithuania, Slovenia, Sweden, Latvia, Slovakia, Finland and Japan were located negative region of the first dimension with greater than 1 (numerically less than -1) loading values in the first dimension. Although these countries are similar to each other, they differ from the general trend since they have negative values greater than 1. Therefore, besides having negative effects, they are not of primary importance. Especially, Luxembourg (-2.3159) and Iceland (-1.9565) have high negative loads and these two countries quite different countries. According to these results, it can be stated that these 10 countries are different from other countries in the first dimension. Mexico and Luxembourg are the most different countries in the first dimension while Belgium and New Zealand are the most similar countries.

According to second dimension, Korea, Poland and Canada are similar to each other and these countries are the most important differentiators in this dimension. These countries have positive loads as well as the effect values of these loads are greater than 1. These 3 countries can be considered to have a great influence among the OECD countries. Thus, it can be noted that these 3 countries are similar to each other and differ from the other 33 countries with higher effects in the second dimension.

In the second dimension with a negative value greater than 1, Greece, Turkey, Spain and Israel were identified as different countries. Although these countries are similar to each other, they are differed from the general trend with have negative values greater than 1. With having a negative effect, they are not of primary importance. According to these results, it can be mentioned that these countries are different from the other 32 countries in the second dimension. In this dimension, Korea and Greece are the most different countries, while Slovenia and Slovakia are the most similar.

When considering of two dimensions together, it can be expressed that the USA, Germany, France, Netherlands, England, Canada, Hungary and Poland have positive loadings. These 8 countries had the highest effect on the livestock sector, especially the USA and France, due to their high values. On the other hand, Israel, Switzerland, Iceland, Lithuania, Luxembourg, Portugal and New Zealand have negative loadings in both dimensions. For this reason, these countries, especially Luxembourg and Iceland, are the countries that have the least effect on the livestock sector.

The stress values showing the fit between the positions of the data in multidimensional space and their positions in two-dimensional space are given in Table 3.

Iteration was continued until the correction value of the stress statistics for two dimensions (p = 2) reached a value less than 0.001 and the iteration was stopped when this value reached 0.00064 in the 6^{th} iteration. In the 6^{th} iteration, the stress value was found 0.13289 and this value indicted that the fit between the real positions of the data in the multidimensional space and the reduced positions in the two-dimensional space was at a moderate level.

Besides, the stress statistics calculated by the Kruskal equation and the coefficient of determination, which is the square of the correlation coefficient, are given. Corresponding to the stress value, determination coefficient (R^2) was found 0.88322. This value is greater than 0.60 and indicates that the analysis is reliable.

Coordinates of the animal species in two-dimensional space are given in Table 4.

Table 3. S-Stress, correction value for two dimensions by animal species; Kruskal Stress and R^2 value.

Iteration	S-Stress	Improvement	Stress	R^2
1	0.18735	-	0.16448	0.88322
2	0.14481	0.04255		
3	0.13747	0.00733		
4	0.13476	0.00271		
5	0.13352	0.00124		
6	0.13289	0.00064		

Table 4. Coordinates of animal species in two-dimensional space.

Variable	1.Dimen	2.Dimen	Variable	1.Dimen	2.Dimen	Variable	1.Dimen	2.Dimen
Buffalo	-0.8287	-0.7671	Duck	1.5181	-0.8634	Mule	-1.3002	0.1362
Cattle	0.5165	0.4262	Goat	-0.9889	0.4640	Pig	1.7400	0.9686
Chicken	0.5908	0.3664	Goose	0.1389	-2.6397	Sheep	-1.1879	0.2796
Donkey	-1.3049	0.4452	Horse	0.4635	0.8823	Turkey	0.6427	0.3017

According to these coordinates, locations of the animal species in two-dimensional space were visualized in Figure 3.

In the first dimension, pig and duck, which have positive and greater than 1 effect values, were determined as the most similar species. Therefore, it can be noted that these two species are similar to each other for 36 variables or countries. donkey, mule and sheep with a negative value greater than 1 in this dimension were defined as different species. Although these species are similar to each other, having negative values greater than 1 shows that they are different from the general trend. Therefore, these are species that have both negative effects and are not of primary importance. Thus, it can be considered that these 3 species are different from the other 9 species in the first dimension for the 36 variables (countries). Two most different species in this dimension are pig and donkey, while the two most similar species are donkey and mule.

In the second dimension, pig and horse are similar to each other and they are the most important discriminant in this dimension. Although these two species have less than 1 loading values, their effects are high among the given species. Goose's loading value is -2.6397 and can be defined as the most different species. Thus, it can be noted that this species is quite different from the other 11 species in the second dimension. In the second dimension, pig and goose are the most different species from each

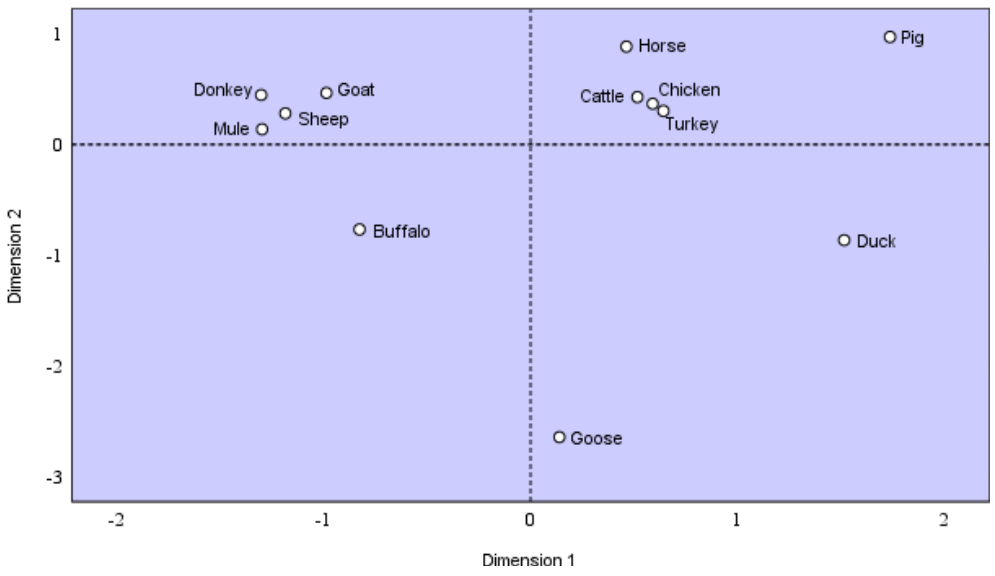

Figure 3. Configuration of animal species in two-dimensional space.

other, while donkey and goat are the most similar species.

When two dimensions are considered together, pig, buffalo, chicken and turkey have a positive loading and the highest effect on the livestock sector. On the other hand, buffalo has a negative loading in both dimensions and has the lowest effect on the livestock sector.

In this study, by NMDS analysis, configuration of 36 OECD countries in two dimensional space was examined and similarities/dissimilarities between these countries were determined for the variables regarding with animal data. In the first dimension, Mexico, the USA, France, Turkey, Germany, Italy, UK, Spain, Australia and Canada were found quite similar to each other. It was observed that these 10 countries, especially Mexico (2.3640), the USA (2.2765) and France (2.0038), have a high level of effectiveness and differ from the other 26 countries. According to results of our study, it can be stated that Scandinavian countries are similar to each other with their positive or negative effects, However, there are quite differences between the results of previous studies for similarities/dissimilarities in the literature.

In NMDS, the relationships between objects are determined by reducing the size and converted the distance matrix into graphing coordinates, thus the method is defined as a graphical method. In this graphical representation, it is expected that the points showing similar objects in the conceptual space are close to each other while the points that are not similar to each other are far from. When determining this distance/proximity, metric or non-metric scaling method is used depending on the data structure (Everitt and Dunn, 2001; Alpar, 2011; Ding, 2018).

In MDS, generally using a two-dimensional space, the results can be better understood and interpreted. However, while applying this dimension reduction method, it is necessary to present a model close to the real structure of the objects of the representation model obtained with as little dimension as possible (Mackay and Zinnes, 1986; Tatlıdil, 1992).

NMDS has fewer assumptions than MDS. This makes it more preferable. In addition, assumptions of NMDS are also more flexible, thus it possible to obtain smaller sized solutions in NMDS (Özdamar, 2004). However, there are some uncertainties regarding its interpretation, reflection and rotation in both methods. All points in the figures can be shifted from one place to another, as well as the entire shape can be rotated or reflected. Based on this information, NMDS uses only the order of magnitude of the distances to solve it, and the ordinal numbers of the distance values constitute the only information used to determine the configuration distance values (Tatlıdil, 2002). In NMDS, analytical solution is not possible in the general algorithm. Therefore, the stress value is tried to be minimized with an iterative approach. Another problem is that the stress value decreases smoothly with increasing dimensions. This lead to difficulties to select the proper number of dimensions. Also, interpreting a spatial map containing more than three dimensions can be difficult. In this case, the stress value is minimized by an iterative method and proper dimension number (mostly as 2) is determined (Basalaj, 2001; Gündüz, 2011). Iterative methods calculated with Monte Carlo also have some disadvantages in NMDS. This method can be time-consuming in large data sets, different estimates are likely to be obtained in each iteration and the graphical representation is shaped accordingly (Alp and Gündoğdu, 2007).

Euclidean distance, which takes into account every variable and does not eliminate the excesses (repeating effects of repeated variables), is an appropriate measure when the variables are continuous and absolute distances are desired to be reflected (Seber, 2004; Giguère, 2006). Therefore, optimum distances between objects in k dimensional space was computed based on Euclidean distance by considering the data structure and scale type in the study. In addition, the data set was analyzed using 4 different distance measurements to show that Euclidean is better than other distance measurements. While Minkowski distance is the same as Euclidean, Manhattan distance was found close to Euclidean with a slightly higher (0.14532) Stress and slightly lower (0.90385) R^2 value, while the Chebyshev measure gives very bad values compared to Euclidean with 0.26555 Stress and 0.63594 R^2 value.

Ersöz (2008) revealed the similarities or differences of OECD countries, taking into account the health level measures and health expenditure indicators. In his analysis, three groups were formed in two-dimensional space. In the first dimension, Turkey, Korea, Mexico, Poland, and Slovakia are similar for 14 variables. In the second dimension, America is differing and according to the difference matrix Turkey, Germany, Austria and Norway were showed to differ from each other. Stress value was found 0.18 as "medium fit". Especially in the first dimension, Mexico and Turkey were similar; Luxembourg and Iceland were similar, however different from the general trend. High negative value of Greece in the second dimension is consistent with our study.

Beyhan Acar (2013) investigated the similarities of OECD countries in terms of labor market key indicators. The results of the research showed that the two countries were most similar to each other. Netherlands and Belgium were differing from each other as well as Slovakia and Iceland. Besides, Turkey was different from all other countries. Spain was the most different country from Iceland.

Boz *et al.* (2016) examined the similarities of OECD countries in terms of health system indicators and stated that Turkey, Mexico, Chile and Korea were quite similar for considered variables. However, Turkey was quite different from Greece, the USA, Switzerland, Portugal, Australia, Spain and Japan. Akdamar (2019) found “good fit” for two dimensions in terms of labor market indicators of OECD countries and Turkey as well as Greece and Spain were found different from other OECD countries.

Akın and Eren (2012) revealed the similarities of OECD countries in terms of education indicators. They found that the fit level was "very good" with 0.03058 stress value. In the first dimension, Mexico, Sweden, Germany and France were found quite similar to each other with negative values. Other countries, except Turkey, were located at the same region with a high positive value. Turkey seemed to be quite different from these countries in this dimension. In the second dimension, Denmark and Norway with a positive value and Korea and Chile with a negative value were quite different from other countries.

Çankaya *et al.* (2003) used MDS to explain the similarities or dissimilarities between 21 morphological characters of 6 honeybee genotypes collected from different regions of Turkey. The results showed that the selected genotypes fit each other perfectly from the examined morphological characters.

Çelik (2015) examined the similarities of 81 provinces in Turkey in terms of livestock data using the data of 2014 with MDS analysis. According to the results of the analysis, it has been determined that Şırnak, Antalya, Siirt and Bitlis are different from other provinces, and that Tunceli, Hakkari, Van, Şanlıurfa, Siirt, Bitlis and Şırnak provinces are the provinces that have the most positive impact on animal production in Turkey. Similarly, Kandemir *et al*. (2019) were examined the similarities of 12 statistical regions in Turkey in terms of sheep and mutton prices by MDS. As a result, the differences of the regions from each other have been revealed and it has been emphasized that the carcass meat price is high in the regions where the number of sheep is high.

On the other hand, Güler (2021) examined the similarities of 24 regions in terms of silkworm breeding with MDS analysis by using 2019 data about number of enterprises (households), silkworm breeding, the number of opened boxes and the amount of fresh cocoon production. As a result, the author noted that the region that contributed the most to silkworm breeding was the TRC2 (Şanlıurfa and Diyarbakır) region, and the region that contributed the least was the TRA1 (Erzurum, Erzincan and Bayburt) region. The author also indicated that TR51 (Ankara), TR32 (Aydın, Denizli and Muğla), TR41 (Bursa, Eskişehir, Bilecik) and TR42 (Kocaeli, Sakarya, Düzce, Bolu and Yalova) regions are similar to each other in terms of silkworm breeding.

Conclusion

In the study, the coefficient of determination was found 91% and it can be stated that MDS can be used as an effective method for visualization of the similarities between OECD countries in terms of animal species in two-dimensional space. When two dimensions are considered together, it can be stated that Turkey shows similarities with Spain, Italy, Chile, Ireland, and Greece among OECD countries in terms of animal species.

On the other hand, Turkey is differing from 13 countries that Korea, Japan, Denmark and Estonia. According to the two dimensions, animal's species can be clustered into 3 groups: duck and goose in group 1; horse, pig, cattle, chicken and turkey in group 2; and donkey, mule, sheep and goat in group 3.

As a result, it is important to determine the animal species raised in the countries with similar culture and nature for planning of short, medium and long-term livestock programs and investments of the countries. In this context, the study may contribute to the literature.

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RESEARCH PAPER

LIVESTOCK STUDIES

Examination of Blood Cortisol and Some Parameters at Parturition and On 30th Day Postpartum In Single and Twin - Pregnant Ewes

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Abstract

Lambs gain about 70 % of their birth weight in the last 6 weeks of pregnancy. In this period, many physiological events occur in ewe metabolism. The increase in the number of fetuses also affects these events. The aim of this study was to determine the effect of single and twin pregnancy on serum cortisol, glucose, triglyceride, HDL-cholesterol, total protein, total cholesterol, calcium and phosphorus levels at birth and postpartum days. This study was conducted on 2 - 3 aged single (n = 11) and twin (n = 8) pregnant Anatolian Merino ewes which housed the Department of Animal Breeding and Animal Production of Konya Bahri Dağdaş International Agricultural Research Institute in Turkey. Blood samples were collected from the jugular vein immediately after parturition and on 30th day postpartum in single and twin pregnant ewes. According to the data obtained, the differences between single and twin pregnant ewes of serum cortisol and phosphorus values at parturition were found statistically significant. The differences between parturition and 30th day postpartum were found statistically significant for the cortisol and glucose values in single pregnant ewes. The differences between the parturition and 30th day postpartum for glucose, triglyceride and phosphorus values were found statistically significant in twin pregnant ewes. In conclusion, it can be said that cortisol and some blood parameters in ewes are affected by pregnancy and the number of fetuses.

Introduction

Pregnancy is an event that causes changes in cardiovascular, respiratory, gastrointestinal, urogenital central nervous system, blood parameters and immunological functions in animals (Özyurtlu *et al*., 2007). Pregnant ewes are exposed to severe distress on the metabolism, especially on ewes with more than one fetus. It is known that the fetus number affects some blood parameters (Siggurdson, 1988). Lambs gain approximately 70 % of their birth weight in the last 6 weeks of pregnancy (Siggurdson, 1988). Pregnancy can cause physiological stress that increases maternal plasma cortisol (Keller-Wood, 1996; Medan *et al*., 2015). Cortisol is a glucocorticoid hormone that produced by the adrenal cortex in response to stress (Medan *et al*., 2015). In sheep, lambing causes a sharp release of cortisol from the fetal adrenal glands. Cortisol increases estrogen concentration and decreases progesterone concentration. Thus, by stimulating PGF2α release, the level of oxytocin which provides myometrial contractions increases (Nagel *et al*., 2019). Thus, it is

thought that the cortisol level at parturition may be affected in the number of fetus.

Fetal development in pregnant animals causes some metabolic changes due to increased requirements of nutrition (Ismaeel *et al*. 2019). The metabolism of mineral substances plays an important role in the regulation of physiological functions of the periparturient and puerperal period (Atakişi *et al*., 2009). Metabolic problems in ewes generally occur in the periparturient period and these problems affect lipid metabolism (Nazifi *et al*., 2002). In ewes, pregnancy toxemia is the most common metabolic disease in late gestational period. In this period, the increase in the energy needs of the fetus can cause negative energy balance and trigger lipid mobilization (Xue *et al*. 2019). Some studies have reported that the reproductive status in ewes affects blood parameters. (Antunović et al., 2002; Karapehlivan *et al*., 2007; Balıkçı *et al*., 2007). In addition to the reproductive status, the number of fetuses may also affect to blood parameters, especially cortisol. In addition, blood mineral levels are affected in pregnant ewes due to the mineral needs of the fetus (Yıldız *et al*. 2005). In this study, we aimed to determine the effect of fetus number on serum cortisol, glucose, triglyceride, HDL-cholesterol, total protein, total cholesterol, calcium and phosphorus levels in Anatolian Merino ewes at parturition and on the 30th day of postpartum.

Materials and Methods

Animal

This study was conducted on 2 - 3 aged single (n = 11) and twin (n = 8) pregnant Anatolian Merino ewes which housed the Department of Animal Breeding and Animal Production of Konya Bahri Dağdaş International Agricultural Research Institute in Turkey. Except for enterotoxemia, vaccinations and antiparasitic applications are mostly completed before breeding season. Enterotoxemia vaccine administered in the third month of pregnancy. Fetus numbers were determined by ultrasonographic examination at 60^{th} day of pregnancy (DP 50 VET, Mindray Ltd. China). Live weight after shearing of ewes were about 60-65 kg. During the last 45 days of pregnancy, concentrated feed (750 gr) containing 16 % crude protein and 2500 kcal metabolizable energy were used in addition to 500 gr dry alfalfa grass, 250 gr wheat stem, 500 gr corn silage daily for ewes. After parturition, approximately 1000 gr concentrated feed were given in addition to 500 gr dry alfalfa grass, 250 gr wheat stem, 500 gr corn silage. The births occurred in a normal course and there was no dystocia. There was no negativity (trauma, illness, death, unfavorable environmental conditions) until the 30th day postpartum. The lambs stayed in separate compartments with their mothers for 30 days on the postpartum and during this period they were fed only colostrum-breast milk. After this period, in addition to breast milk, ad libitum concentrate feed and roughage were given for lambs and ewes was never milked. This study was approved by the Konya Bahri Dağdaş International Agricultural Research Institute Animal Experiments Local Ethics Committee (30.04.2020-106)

Blood Samples

Blood samples were collected from the jugular vein immediately after parturition (the sheep usually gave birth between 22:00 and 05:00) and on 30th day postpartum (at 14:00) in single and twin pregnant ewes. Blood samples (5 ml) were centrifuged at 5000 g for 5 minutes to obtain serum and stored at - 20 for analysis. Glucose, triglyceride, HDL, total protein, cholesterol, calcium and phosphorus analyzes were performed using Abbott Architect C 8000 brand autoanalyzer (ABD) using colorimetric method. Cortisol analysis was performed with immunoassay analyzer (Abbott Architect i2000, ABD) using the CLIA method (Darwish, 2019). CLIA kits (Abbott Architect-8D15, ABD) were used for analysis. The intra- and interassay CV were less than 10 %.

Statistical Analysis

SPSS 23 statistics program was used to evaluate the obtained data. Paired T-Test was used to compare serum parameters levels between the same ewes data at parturition and postpartum 30th day. Independent-Sample T-Test was used to compare serum parameters of single and twin pregnancies at parturition and postpartum 30th day.

Results

Serum cortisol, glucose, triglyceride, HDL-cholesterol, total protein, total cholesterol, calcium, and phosphorus values of the single and twin pregnant Anatolian merino ewes at parturition and on 30th day postpartum are presented in Table 1. According to these data, the differences of serum cortisol and phosphorus values between single pregnant and twin pregnant obtained at parturition were found statistically significant ($P < 0.05$).

In single pregnant ewes, differences between parturition and 30th day postpartum of cortisol ($P < 0.01$) and glucose ($P < 0.01$) were found statistically significant. In twin pregnant ewes, differences between parturition and 30th day postpartum of glucose ($P < 0.05$), triglyceride ($P < 0.01$) and phosphorus ($P < 0.01$) were found statistically significant.

Discussion

There are important differences in biochemical parameters of animals before and after parturition due to changing physiology (Gürgöze *et al*., 2009). Pregnancy

Table 1. According to parturition and 30th day postpartum serum values in single and twin pregnant ewes

Parameters	Parturition		30th day Postpartum		P values			
					Part.	Post.	Single	Twin
	Single (n = 11)	Twin (n = 8)	Single (n =11)	Twin (n = 8)	S/T	S/T	Part./Post.	Part./Post.
Cortisol (µg / dl)	2.45 ± 1.23	1.05 ± 0.72	0.62 ± 0.82	0.65 ± 0.46	***0.011****	0.920	***0.004****	0.184
Glucose (mg / dl)	129.90 ± 49.1	106.25 ± 57.5	52.81 ± 14.64	58.50 ± 13.16	0.348	0.397	***0.001****	***0.045****
Triglycerid (mg / dl)	17.72 ± 6.00	16.50 ± 4.95	13.00 ± 4.31	12.12 ± 2.23	0.643	0.608	0.074	***0.009****
Cholesterol (mg / dl)	38.73 ± 15.37	32.25 ± 6.67	39.27 ± 8.17	38.00 ± 14.90	0.233	0.814	0.925	0.372
HDL (mg / dl)	21.90 ± 7.63	17.37 ± 3.24	20.63 ± 4.15	21.62 ± 8.65	0.099	0.744	0.648	0.245
Total Protein (g / dl)	4.50 ± 1.78	3.80 ± 1.18	4.75 ± 0.91	4.86 ± 1.21	0.345	0.827	0.730	0.075
Phosphorus (mg / dl)	2.86 ± 0.99	2.06 ± 0.32	3.85 ± 0.72	3.77 ± 0.73	***0.027****	0.818	0.055	***0.001****
Calcium (mg / dl)	6.51 ± 2.18	5.71 ± 1.52	6.35 ± 1.11	7.03 ± 1.35	0.385	0.244	0.849	0.080

*P < 0.05 **P < 0.01 Part.= Parturition; Post.= 30th day Postpartum; S/T= Single/Twin

can increase the level of maternal cortisol by causing physiological stress. In sheep, lambing causes a sharp release of cortisol from the fetal adrenal glands. Cortisol enhances estrogen concentration and decreases progesterone concentration. Thus, by stimulating PGF2α release, the level of oxytocin which provides myometrial contractions increases (Nagel *et al*., 2019). Some studies are reported that there were no significant differences in serum cortisol levels in the prepartum period between single and twin pregnant ewes (Medan *et al*., 2015) before and after pregnancy (Brunet and Sebastian, 1991) in ewes. Drost *et al*. (1973) found that serum cortisol levels of parturition higher than the levels of prepartum period in ewes. In this study, serum cortisol levels obtained from blood taken immediately after parturition were found as 2.45 µg / dl in single-bearing ewes and 1.05 µg / dl in twin-bearing ewes. On the 30th day postpartum serum cortisol levels were found as 0.62 µg / dl for single-bearing ewes and 0.65 µg / dl for twin-bearing ewes. According to these results, serum cortisol levels obtained immediately after parturition were found to be statistically significant ($P < 0.05$) in single-bearing ewes compared to twin-bearing ewes. Besides, the difference between serum cortisol levels at parturition and on 30th day postpartum in single-bearing ewes was found to be statistically significant ($P < 0.01$). The birth weights of single and twin lambs were found as 5.14 kg and 4.25 kg, respectively in this study. In this case, it can be said that the birth weights of single-born lambs are higher than those of twin-born (Roubles *et al*., 2003) and the single-bearing ewes have been exposed to more stress. Thus, the effect of fetal number on maternal cortisol levels can be evaluated as important at parturition.

Blood glucose levels are affected by increasing energy needs during pregnancy (Atakişi *et al*., 2009). Fetal growth and energy consumption of the fetus are mostly related to glucose (Clapp, 2006; Chlumbohm and Harmeyer, 2008). Chlumbohm and Harmeyer (2008) found that serum glucose levels of single-pregnant ewes were higher than twin-pregnant ewes. Fırat (1994) found that glucose levels at parturition were higher statistically than at pregnancy. Kaya (2004) reported that there was no significant fluctuation in serum glucose levels in the late pregnancy, but increased by 109 % vertically at parturition. In our study, serum glucose levels were found in single and twin - pregnant ewes at parturition as 129.90 mg / dl and 106.25 mg / dl, respectively. On the 30th day postpartum, serum glucose levels were found in single and twin - bearing ewes as 52.81 mg / dl and 58.50 mg / dl, respectively. These results are similar to those of Fırat (1994) and Kaya (2004). The increase in plasma glucose level at parturition can be explained by the sudden release of ewes from the hexose requirement for the fetus and the increase in the glucose level or it may reflect an urgent requirement of the ewe for readily available energy (Fırat, 1994).

In pregnant ewes, triglyceride levels are reported as lower in postpartum measurements than in the last three days of pregnancy (Kaya, 2004; Karadaş, 2008). Toker (2004) reported that ewes suffered a significant loss in terms of cholesterol during the last week of pregnancy. Kaya (2004) found that plasma triglyceride levels followed a linear course until parturition and showed a significant decrease postpartum period in their study in ewes. In the other study, it was found that total cholesterol levels decreased to the lowest level on

the 1st day postpartum while the highest was on the 3rd day prepartum (Karadaş, 2008). Nazifi *et al*. (2002) reported that triglyceride, total cholesterol, HDL-cholesterol and VLDL-cholesterol levels were higher in the last week of pregnancy than in other periods of pregnancy in ewes. In our study, serum total cholesterol levels were found in single and twin - bearing ewes at parturition as 38.73 mg / dl and 32.25 mg / dl and in 30th day of postpartum, 39.27 mg / dl and 38.00 mg / dl respectively. Also, serum triglyceride levels were found in single and twin - bearing ewes at parturition as 17.72 mg / dl and 16.50 mg / dl and in 30th postpartum day, 13.00 mg / dl and 12.12 mg / dl respectively. At parturition and in 30th day of postpartum, the difference of serum total cholesterol levels between single and twin - bearing ewes was nonsignificant. There were no significant differences between single and twin - bearing ewes at parturition, whereas differences between serum triglyceride levels between parturition and 30th day postpartum were found significant in twin births. ($P < 0.01$). Karadaş (2008) reported that serum HDL-cholesterol levels fluctuated throughout pregnancy, tended to fall towards the end of pregnancy, and increased again in the postpartum period. In our study, serum HDL-cholesterol levels were found in single and twin - bearing ewes as 21.90 mg / dl, 17.37 mg / dl respectively at parturition, and as 17.37 mg / dl and 21.62 mg/dl respectively in 30th day postpartum. In this study, lower serum HDL-cholesterol levels were obtained than other studies (Nazifi *et al*., 2002; Karadaş, 2008) and there was no significant difference between the groups.

The fetus synthesizes its proteins from the amino acids derived from the mother; proteins are used mainly for synthesis rather than oxidation or gluconeogenesis (Jainudee and Hafez 2000). Balıkçı *et al*. (2007) found a decrease in serum total protein levels on the 150th day of pregnancy, compared to another period of pregnancy in both single and twin pregnant ewes. In the same study, differences in serum total protein values between single and twin - bearing ewes on 45th day postpartum were found statistically insignificant. Kaya (2004) reported that total protein levels in single and twin pregnant ewes were 5.9 g / dl and 5.6 g / dl respectively at parturition. Altiner (2006) found that total protein level as 6.3 g/dl at parturition, while he found 7.1 g / dl in 15th day of postpartum. In our study, at parturition serum total protein levels were found to be 4.50 g/dl for single pregnant ewes and 3.80 g / dl for twin pregnant ewes, besides in 30th day of postpartum were found as 4.75 g / dl and 4.86 g / dl respectively. These values are lower than the other studies (Kaya, 2004; Altiner, 2006).

Retention of calcium, phosphorus and iron increases according to fetal body weight during pregnancy. The fetus has a unique ability to consume the maternal skeleton (Jainudee and Hafez 2000). Roubles *et al*. (2003) reported that serum calcium (Ca) values decreased markedly in the late period of pregnancy, reached the lowest level at parturition and continued to decrease until the 3rd week postpartum in the single and twin - bearing goat. Gürgöze *et al*. (2009) stated that serum Ca values were significantly higher in the postpartum period compared to the peripartum period. On the 150th day of pregnancy, Yıldız *et al*. (2005) found that Ca and P values of single and twin pregnant ewes lower than values of 45th day postpartum. In our study, Ca levels of single and twin pregnant ewes were found to be 6.51 mg / dl and 5.71 mg / dl at parturition, while Ca levels of 30th day postpartum was found to be 6.35 mg / dl and 7.03 mg / dl respectively. As in our study, Özyurtlu *et al*., (2007) found that there was a small increase in serum Ca levels of postpartum compared to the prepartum, but statistically nonsignificant. Phosphorus levels of single and twin pregnant ewes were found to be 3.85 mg / dl and 3.77 mg / dl at parturition, P values of 30th day postpartum were found to be 2.86 mg / dl and 2.06 mg / dl respectively. While the Ca and P levels of parturition were found to be lower than levels of the 30th day postpartum similar to some studies (Roubles *et al*., 2003; Özyurtlu *et al*., 2007), differences of P values between the parturition and 30th days postpartum was found statistically significant ($P < 0.01$) only in twin pregnant ewes. This situation may be an indication that twin pregnant sheep need more calcium and phosphorus during pregnancy.

Conclusion

In conclusion, it can be said that cortisol and some blood parameters in ewes are affected by pregnancy and the number of fetuses. This situation depends on the various mineral needs of the fetus, the energy needs and the stress which the mother is exposed to at parturition. Cortisol levels are higher in ewes carrying single fetus compared to twins. In addition, glucose levels are higher in ewes at parturition compared to the 30th day of postpartum due to the energy need. Phosphorus levels of sheep are affected by the number of fetuses.

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RESEARCH PAPER

LIVESTOCK STUDIES

First Commercial Semen Cryopreservation and Main Spermatological Features of Anatolian Buffalo

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Abstract

Conventional buffalo semen freezing studies are limited in Anatolian buffaloes, which are overly sensitive to exogenous stimulation. The present study's object was to determine the main features of Anatolian Buffalo semen obtained by artificial vagina method for the first time. A total number of 150 ejaculates were collected from three Anatolian Buffalo bulls (app. 4 years of age). The mean pH, volume and concentration of semen were found 6.63±0.15, 1.61±0.5 ml, 1629±222.67 x10^6 spermatozoa/ml, respectively. The sperm motion characteristics were determined by using a computer-assisted sperm analysis system (CASA); the total and progressively motile sperm values were 57.12±5.63%, 23.22±4.47% and other kinetic parameters such VAP, VSL, VCL, ALH, BCF, STR, LIN were found 94.71±8.48 µm/s, 72.6±7.08 µm/s, 160.9±15.66 µm/s, 7.8±3.75 µm, 29.15±1.56 Hz, 76.91±3.87%, 46.21±2.61%, respectively after thawing. Among buffalo bulls, differences in semen pH values were statistically significant ($P<0.05$), while differences in ejaculate volume, semen concentration, total motility, progressive motility, VAP, VSL, VCL, ALH, BCF, STR, and LIN were not ($P>0.05$). As a result, frozen Anatolian buffalo semen can be obtained economically and can be used for animal breeding in assisted reproductive biotechnology such as artificial insemination or in vitro embryo production commercially.

Introduction

Domestic water buffaloes are studied under two classes as river (Bubalus bubalis) and swamp buffaloes (Bubalus carabenensis). Despite of, one breed comprises swamp buffaloes, there are many breeds such as Murrah, Nilli-Ravi, Kündi, Surti, Jafarabadi, Nagpuri, Pandharpuri and Mediterranean buffalo in river buffaloes (Kelgokmen, 2015). Anatolian water buffalo (as shown in Figure 1) located in Turkey originated from the Mediterranean subgroup of river buffaloes (Soysal *et al*., 2007). Although Turkey had a high population of Anatolia buffalo earlier in this century, the buffalo population had fallen below 100 thousand head in 2010 (Atasever *et al*., 2008; Aköz *et al*., 2017; Çolak *et al*., 2017). Thus, genetic materials (DNA, somatic cell) of Anatolian buffalo against the danger of extinction; have been stored in the national gene bank of Turkey (Arat, 2011).

Sperm cryopreservation forms the basis of gene banks with its ease of method, success in its transfer to the field, economical storage and alternative fertilization application options. Besides, the use of frozen semen by artificial insemination is the most common biotechnological practice that enables rapid and inexpensive genetic progress in many animal species for increasing yield and product quality (Morrell, 2011).

The first buffalo sperm cryopreservation in the world was carried out in the 1950s (Roy *et al*., 1956). In this sense, conventional cryopreservation of sperm with different buffalo species is a method that has been used for a long time (Andrabi, 2009). Until recently, artificial inseminations in Anatolian buffaloes were generally performed with Mediterranean buffalo semen that

imported from Italy. Because of there was no frozen Anatolian buffalo semen for commercial purposes (Aköz *et al*., 2017). Due to the high prices of imported frozen buffalo semen, breeders in Turkey prefer natural breeding rather than artificial insemination (Isık, 2015; Yılmaz, 2013; Okuyucu *et al*., 2018). For these reasons, the National Anatolian Buffalo Breeding Project, coordinated and supported by the General Directorate of Agricultural Research and Policies (TAGEM) was initiated in 2010. As an outcome of this leap sperm production from Anatolian buffalo bulls, with its superior genomic characterization and acceleration of breeding activities by artificial insemination, is one of TAGEM's priority strategic plans (Soysal *et al*., 2020). For these reasons, the first commercial Anatolian buffalo sperm production was started at the Lalahan International Livestock Research and Training Center, Ankara.

Cryopreservation of epididymal Anatolian buffalo sperm has been studied, and valuable data have obtained (Selcuk *et al*., 2015; Yeni *et al*., 2017). However, collection process was not performed with artificial vagina. It is revealed that it is not suitable for commercial production and it is necessary to conduct new studies with common methods due to its effects on ejaculate's quality.

In the present study, unlike the studies mentioned above, it was the first time to use a controlled semen collection system (Lalahan Model) in terms of occupational safety and animal welfare for semen collection from Anatolian buffaloes which are overly sensitive to exogenous stimulation (Korkmaz *et al*., 2019; Baştan, 2020). For this purpose, a novel collection system was used and semen characteristics of frozen Anatolian buffalo semen collected by artificial vagina were investigated after freezing.

Materials and Methods

Animals

In this study, three Anatolian Buffalo bulls (apr. 4 years of age) were used at the Lalahan International Center for Livestock Research and Training (Ankara, Turkey), and maintained under uniform feeding and housing conditions that individually in pens.

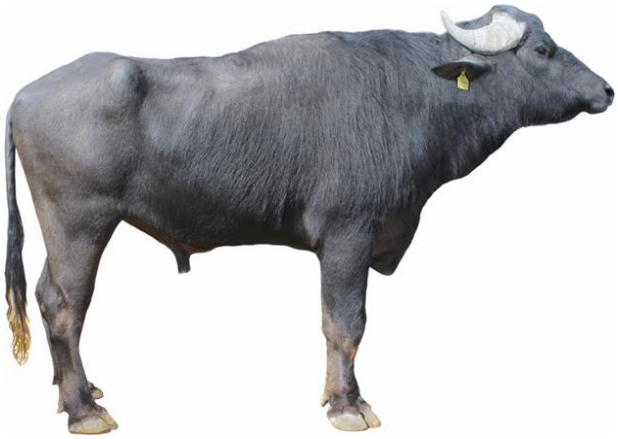

Figure 1. Anatolian buffalo bull.

Preparation of Artificial Vagina

Semen was collected using a bovine artificial vagina with soft neoprene liner (AV; 30 cm long; 5 cm internal diameter; IMV, France). The internal temperature of the artificial vagina was maintained 40 oC. In order to lubricant, the contact of bull penis with the soft neoprene liner of the AV, the inner surface of the liner was covered with a thin layer of sterile petroleum jelly (Vaseline®). Sterile glass conical cylindrical 15-ml collection tube, which used to collect semen and transporting it to the laboratory was placed at the end of artificial vagina. The protective felt cover was used to keep the artificial vagina and collection tube at the correct temperature during the collection process and prevent semen thermal shock after the bull ejaculation (Ansari *et al*., 2017; Baştan 2018).

Semen Collection

In terms of occupational safety and animal welfare, a controlled semen collection model (Lalahan model) was used to collect semen from Anatolian buffalo bulls (as shown in Figure 2). In this way, each bull came to the semen collection arena from its individual pen without a bull handling person. A female Anatolian buffalo was used as a teaser animal to steer male buffaloes. A total number of 150 ejaculates were collected by using the Lalahan Model with the aid of an artificial vagina twice a week during 30 weeks (Korkmaz *et al*., 2019; Baştan, 2020).

Determination of Semen Volume, Concentration and pH

The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals, and sperm concentration was determined by using the Accucell photometer (IMV, L'Aigle, France). The pH of semen samples was determined by indicator paper strips and digital pH meter simultaneously (Hanna-Hi 221, Smithfield, RI) (Khawaskar *et al*., 2012).

Semen Freezing Processing

A soybean-based semen extender (Andromed, Minitüb, Germany) was used for diluting ejaculates with >80 total motile sperm to a final concentration of 100 x 10^6 spermatozoa/ml. Samples were cooled at +4°C for 3 hours. Afterwards, they were packaged in 0.25 ml French straws (IMV, L'Aigle, France) by using an automatic straw filling and sealing machine (MX4, IMV, L'Aigle, France). The straws were frozen to -140°C (- 3°C/min from +4 to -10 °C; -40 °C/min from 10 to 100 °C; -20 °C/min from -100 to -140 °C) by using an automatic freezing machine (Digital cool 5300ZB 250, IMV, L'Aigle, France), plunged into liquid nitrogen and

stored at -196 °C (Tuncer *et al*., 2010; Ansari *et al*., 2017).

Thawing and Post-Thaw Evaluation

After one month of storage period at -196 °C, the straws were thawed in a water bath (37 °C, 30 s) for post-thaw kinetics analysis. Afterwards, a 3µl sample of semen was put onto a prewarmed four chamber slide (20 µm, Leja slides, IMV, L'Aigle, France) and sperm kinetics parameters were determined by using computer-assisted sperm analysis system, as shown in Figure 3 (CASA; IVOS I, Hamilton Thorne Inc., Beverly, USA). CASA was set up as follows: frame rate 60 Hz; minimum contrast 80; low and high intensity gates 0.30–1.70; low and high static size gates 0.10–3.40; low and high elongation gates 8–97; default cell size 5 pixels; default cell intensity 70. In the analysis settings, spermatozoa with VSL 70% and VAP 50 µm/s were evaluated as progressively motile. The motility parameters were expressed in percentage units. Other kinematics average path velocity (VAP, µm s–1), straight line velocity (VSL, µm s–1), curvilinear velocity (VCL, µm s–1), straightness (STR = [VSL/VAP] × 100), linearity (LIN = [VSL/ VCL] × 100), beat-cross frequency (BCF, Hz), amplitude of lateral head displacement (ALH, µm) were also evaluated and expressed with their own units (Tuncer *et al*., 2010; Sahin *et al*., 2020).

Statistical Analysis

For statistical analyses, data were examined with Shapiro-Wilk test for normality and with Levene test for homogenity of variances as parametric test assumptions. The statistical control of the difference between the variables was done with ANOVA. The Tukey test used to evaluation of differences between the buffalo bulls. Descriptive statistics for each variable were calculated and presented as mean ± standard error (Mean ± SE). All statistical analyzes were examined using the SPSS® 22.0 package program and P<0.05 level was considered significant.

Figure 2. Semen collection by Lalahan Model in Anatolian buffalo bull.

Results

The present study aimed to describe some sperm characteristics and spermatological parameters of Anatolian Buffalo sperm. In particular, the data important for AI stations and parameters that routinely used by andrology laboratories were prioritized. The data pH, volume, concentration of semen, total motility and progressive motility were given Table 1. The mean pH, volume, concentration of semen, total motility and progressive motility were found 6.63±0.15, 1.61±0.5 ml, 1629±222.67 x 10^6 spermatozoa/ml, 57.12±5.63%, 23.22±4.47% respectively after thawing.

The sperm kinetic values (VAP, VSL, VCL, ALH, BCF, STR, LIN) were given Table 2. The mean VAP, VSL, VCL, ALH, BCF, STR and LIN were found 94.71±8.48 µm/s, 72.6±7.08 µm/s, 160.9±15.66 µm/s, 7.8±3.75 µm, 29.15±1.56 Hz, 76.91±3.87%, 46.21±2.61%, respectively after thawing. While the pH values are statistically significant among the bulls (P<0.05), differences in terms of other spermatological and CASA parameters (ejaculate volume, sperm concentration, total motility, progressive motility, VAP, VSL, VCL, ALH, BCF, STR, LIN) were not significant (P>0.05).

Discussion

According to this study, the mean semen volume of the Anatolian buffalo was determined as 1.61±0.5 ml. When compared to domestic buffalo breeds such as Murrah, Surti, Jafarabadi, Nili Ravi, Kundihi and swamp buffaloes (2.58 ml, 3.16±0.76 ml, 4.72 0.24 ml, 2.80±1.62 ml, 2.25±0.01 ml, 2.9 ml, respectively), it can be evaluated that the volume of semen obtained in Anatolian buffalo ejaculation is lower than other breeds (Malik *et al*., 1974; Jainudeen *et al*., 1982; Dhami *et al*., 2005; Bhakat *et al*., 2011; Kaka *et al*., 2012; Khawaskar *et al*., 2012).

The sperm concentration of the Anatolian buffalo, another parameter obtained in the study, was

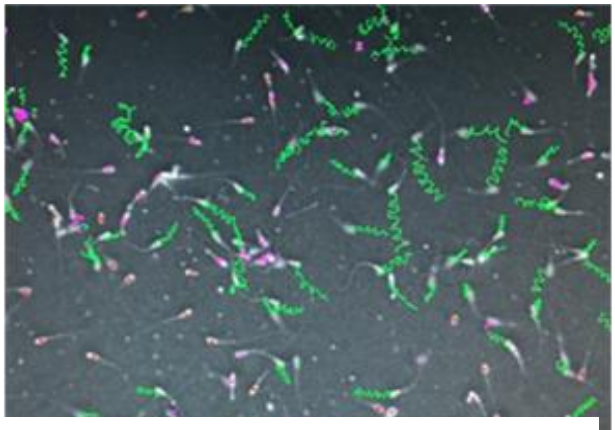

Figure 3. Assessment of motion and kinematic characteristics of semen using computer-assisted semen analysis (CASA).
Yellow color: Progressive motile sperm, Pink color: Motile sperm, Red color: Immotil sperm.

determined as 1629±222.67 x 10^6 sperm/ml. This value is in other domestic buffalo breeds; 990.65±143.9 x 10^6 sperm/ml in Mediterranean buffaloes, 998.91 x 10^6 sperm/ml in Murrah breed, 885.42±24.9 x 10^6 sperm/ml in Surti breed, 838.30±25.74 x 10^6 sperm/ml in Jafarabadi buffaloes. It has been reported that 1000±50.0 x 10^6 sperm/ml in Nili ravi, 1542±9.20 x 10^6 sperm/ml in Kundhi, and for Swamp buffalo 1006 x 10^6 sperm/ml. As a matter of fact, although Anatolian buffalo semen volume was found low compared to other domestic buffalo breeds, it is observed that the concentration of spermatozoa is higher (Malik *et al*., 1974; Jainudeen *et al*., 1982; Galli *et al*., 1993; Bhakat *et al*., 2011; Ghodasara *et al*., 2018; Kaka *et al*., 2012; Khawaskar *et al*., 2012).

In the study, the semen pH value was determined as 6.63±0.15. This value varies between 6.10 and 6.92 (Murrah; 6.92, Surti; 6.89±0.16, Nili-Ravi 6.55±0.50, Kundhi; 6.10±0.007) in some domestic buffalo breeds. The semen characteristics of a bull in frozen semen production are important for extender optimization before freezing. In this process, post-thaw sperm concentration and production efficiency are evaluated according to the variables specified. Nevertheless, sperm characteristics such as semen volume, concentration, and pH may in a large variation specific to the breed, as well as they may vary depending on factors such as age, climate, number of false mounting and ejaculation frequency (Khawaskar *et al*., 2012; Murphy *et al*., 2018; Sankhi *et al*., 2019; Şahin *et al*., 2020).

Computer aided sperm analysis systems (CASA), which reveal in vitro spermatozoon morphology and kinetic parameters in a detailed and systematic manner, are an analysis technique widely used in andrology laboratories. For validating the commercial license of frozen buffalo semen in Turkey, it must have at least 40% total motility and 15 million motile spermatozoa after thawing basically, according to Republic of Turkey Ministry of Agriculture and Forestry. Hence, the percentages of total motile and progressive motile spermatozoa are important among the sperm kinematic parameters in the evaluation of sperm quality. There are many studies indicating the correlation of these parameters with conception rates (Mahmoud *et al*., 2013; İnanc *et al*., 2018; Vincent *et al*., 2018). In this context, kinematic parameters determined by CASA analysis are presented in Table 1 and 2. Considering the parameters obtained from samples that were frozen with a soy lecithin-based plant-derived extender (AndroMed®), it would be more accurate to compare the results with studies using similar extenders. Ansari *et al*. (2017) and Singh *et al*. (2018) determined the total motility values in the sperm cryopreservation studies performed with the same extender in Nili Ravi and Murrah buffaloes as 49.2±1.7% and 38.3±2.3% at post-thaw evaluation. The progressive motility value in Murrah buffaloes with the same extender was determined as 22.3±1.8% at post-thaw. Compared with the total motility and progressive

Table 1. The Anatolian Buffalo semen characteristic and post-thaw motility parameters.

Parameters	Buffalo Bull No: 1 (n=40)	Buffalo Bull No: 2 (n=50)	Buffalo Bull No:3 (n=60)	Mean ± SE	Range	P-Value
Ejaculate Volume (ml)	1.75±0.52	1.60±0.55	1.55±0.44	1.61±0.5	0.2 – 4.1	P=0.545
Concentration (x10^6 sperm/ml)	1589±254.65	1622±227.84	1683±189.86	1629±222.67	625 - 2678	P=0.352
pH	6.65±0.17^a	6.62±0.13^{ab}	6.61±0.11^b	6.63±0.15	6.45 – 6.72	P<0.05
Total Motility (%)	56.74±6.45	56.66±5.11	57.81±5.29	57.12±5.63	48 - 76	P=0.808
Progressive Motility (%)	23.25±4.24	23.49±4.8	22.92±4.25	23.22±4.47	16 - 35	P=0.931

a,b: Different letters on the same line for each parameter represent a statistically significant difference (P < 0.05).

Table 2. The Anatolian Buffalo sperm kinetics parameters.

Parameters	Buffalo Bull No: 1 (n=40)	Buffalo Bull No: 2 (n=50)	Buffalo Bull No: No: 3 (n=60)	Mean ± SE	Range	P-Value
VAP (µm/sec)	94.94±9	94.25±8.5	94.97±7.97	94.71±8.48	79.9 – 137.8	P=0.963
VSL (µm/sec)	73.58±6.44	71.49±8.35	72.91±6.03	72.6±7.08	60.5 – 103.6	P=0.706
VCL (µm/sec)	161.74±15.21	158.44±17.66	162.57±13.59	160.9±15.66	125.8 – 207.8	P=0.723
ALH (µm)	7.24±0.44	8.81±6.22	7.24±0.38	7.8±3.75	5.9 – 8.6	P=0.397
BCF (Hz)	29.36±1.3	29.45±1.93	28.71±1.26	29.15±1.56	25.7 – 32.6	P=0.330
STR (%)	77.87±2.4	76.16±5.61	76.9±2.36	76.91±3.87	68 - 84	P=0.489
LIN (%)	46.82±2.15	45.82±3.35	46.13±2	46.21±2.61	43 - 55	P=0.578

motility (57.12±5.63% and 23.22±4.47%) values obtained in this study, it can be expressed that the first Anatolian buffalo sperm cryopreservation trial was successful and its cryotolerance was higher when compared with other breeds. To clarify, in some domestic buffalo breeds were frozen with different extenders, the total motility values were between 37.92% and 67.84% (37.92±1.12%, 49.3±12.8%, 43.25±3.40%, 57.41±0.92%), and the progressive motility values vary between 20.4% and 30.64% (Kaka *et al*., 2012; Gaviraghi *et al*., 2013; Kumar *et al*., 2016; Singh *et al*., 2017; Ahmed *et al*., 2020; Pathak *et al*., 2020).

Singh *et al*. (2017), studied on Murrah buffaloes, and stated that VAP, VSL, VCL, kinematic values and ALH values were higher in buffaloes showing high fertility characteristics than buffaloes showing low fertility characteristics. He also stated that BCF, STR and LIN values were lower in buffaloes with high fertility characteristics. It has been reported that high motility does not only play a role in determining fertility, but also other sperm kinematics such as swimming pattern and sperm head movements play crucial roles also (Singh *et al*., 2017). The data obtained from this study are similar to the values stated in previous studies conducted on different domestic river buffalo breeds (Gaviraghi *et al*., 2013; Kumar *et al*., 20

16; Singh *et al*., 2017; Singh *et al*., 2018). Preliminary field studies (not statistically sufficient at the moment) were also carried out which pregnancy was obtained with Anatolian buffalo semen.

In conclusion, Anatolian buffalo semen collected by artificial vagina and frozen commercially with the evaluation of some spermatologically parameters (pre-freeze and after thawing), non-return rates for the first time with this study. However, further studies are needed in order to determine Anatolian Buffalo sperm characteristics in a large sample count and field studies.

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RESEARCH PAPER

LIVESTOCK STUDIES

The Effect of the Combination of Various Training Methodologies in Horse Training on the Learning Performances of Arabian Horses

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Abstract

This research aims at examining the learning performance of Arabian horses with the use of Join-up, Parelli's Seven Games, and Clicker methods in combination and separately. In the research, thirty-six Arabian mares were examined and combinations of training methods were applied. Before and after the application of each training method, horses were directed to pass through a narrow-spaces and to walk on a tarp. While applying training methods, stress parameters, behavioral responses, and learning responses of horses were evaluated. The highest heart rates of the training groups were being during the application of the Join-up method. When the Parelli method was performed last, the training duration was 13.3% shorter. In walk on tarp task, the highest success score was in Clicker Method (75%). In the triple combination of training, when the Join-up method was performed last, task success rates decreased (33.3%). However, when the Join-up method was performed first, the success rate was 100%. When Parelli's methods were applied last, conflict behaviors were prevented, all horses learned vocal cues, and trusted their trainers. During the application of the Clicker method, all horses learned vocal cues and trusted their trainers. It was concluded that the order of methods is so crucial.

Introduction

Horses, being herd animals in their nature, are isolated from other members of their species during the training, get into contact with humans, and be exposed to unfamiliar objects and stimuli (Parkin et al. 2018). This situation causes fear and anxiety in horses, and as a result, undesired behavior may occur. If obedience is due to oppression applied by the human, and not based on a reciprocal trust, the horse may feel insecure, and develop some instinctual behaviors such as escaping, resisting and fighting (Blanchard, 2005). Therefore, simple veterinary and animal husbandry interventions such as routine examinations and grooming can pose risks for human safety (Lansade et al. 2019). On the other hand, in all riding disciplines, appropriate methods improve the learning skills of horses, and decrease their undesired behaviors (McGreevy and McLean 2007). Although ensuring a high level of animal welfare is an important issue for contemporary animal husbandry, the development of optimal programs in the training of racehorses who started their sports career at a very young age cannot be achieved due to the lack of scientific knowledge on stress (Witkowska-Piłaszewicz et al. 2021). Though the physical exercise, if well organized, determines forms of adaptation that improve performance and "correct or optimal stress level" may have a positive impact on welfare (McEwen, 2019). Studies show that acute state-related behavioural patterns and horse's behaviour are used in the evaluation of horse welfare (Czycholl et al. 2018, Dalla Costa et al. 2016). The way to ensure the welfare of the horse and the safety of the people working with the horse is through understanding the factors that affect

the horse's learning. Learning has been important for survival of equids during their evolutionary history changing their behaviors through experience and adapting to new environments and threats (Beaver, 2019). To minimize the risks associated with horse training, training methodologies must apply scientific knowledge on equine ethology, cognition, and learning (Fenner et al. 2019). Most of horse training methods are based on conventional practices, however these methods ignore the natural behaviors of horses. Therefore, in horse training, underlying processes beneath horse behaviors have to be comprehended (Waran et al. 2007). Waran and Randle (2017) stated that while scientific debates continue about the nature of consciousness, cognitive abilities and emotions that horses have compared to humans, it is important for horses to express their natural behavior in order to ensure the best quality of life. They also pointed out that keeping them away from poor training and management-related stresses is important for welfare.

Conventional, Behavioral and Conspecific Horse Training

The conspecific models typically explain the human-to-horse attachment through the application of the herd-leader premise (McGreevy et al. 2009). Conspecific models for instance Parelli's Seven Games and Join-up method focus on motivating the horse by the pressure-release (negative reinforcement) principle, using the horse's natural instincts or ethogram (Parelli 1993, Roberts 2000). This model implies that horses would innately respond to human interventions in the same way as they would when receiving analogous signals from conspecifics (Hartmann et al. 2017). The Behavioral training model maintains that horses are not culpable participants in training and that they learn through the correct timing application of positive and negative reinforcement schedules (McGreevy and McLean 2010). It is proposed that human attachment to horses is reliant on learning principles, such as the correct timing of positive and negative reinforcement, tactile rewards and praise as secondary reinforcers, and operant and classical conditioning processes. Kydd et al. (2017) drew attention to the importance of excellent timing when using negative reinforcement in horse training. The Conventional training model rests on a model of the benevolent/malevolent horse otherwise known as the "cooperative model" (McGreevy et al. 2009). There has been a recent tendency in horse training to not only incorporating positive reinforcement, but to completely eliminate the use of aversive stimuli in horse training (McLean and Christensen, 2017).

The aim of this study is to examine the learning performance of Arabian horses by applying Join-up, Parelli's Seven Games (Friendly Game, Porcupine Game) and Clicker methods together and separately.

Materials and Methods

Ethical Statement

This study was approved by the Ankara University Animal Ethics Committee (Approval no: 2015-05-91)

Horses

In the research, 36 Arabian mares in the General Directorate of Agricultural Enterprises (TİGEM) were used. Twelve one-year-old Arabian mares, twelve two-year-old Arabian mares, and twelve Arabian mares over three years old were examined. Horses were routinely reared in a similar way. The horses studied did not show clinical symptoms of any illness nor show external symptoms of estrus. The experiment was conducted on the same stud farm where the horses had been raised.

Equipment Used

During each training test, horses were equipped with a Polar Equine M400 Heath Rate monitor. The monitors continuously recorded Heath Rate (HR). During each training tests, each horse was under constant video surveillance.

Study Protocol

Thirty-six Arabian mares are divided into six groups. All groups have six horses, two mares of each age group (Figure 1). All training applied to horses within the scope of the research was carried out by the researcher/trainer. The trainer, who was the first author

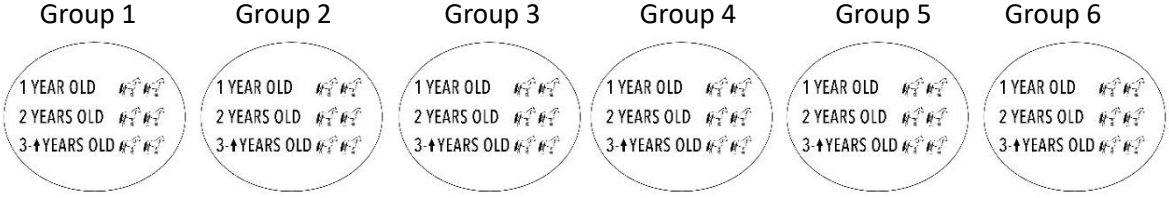

Figure 1. Distribution of horses by groups

Table 1. The training methods and combinations

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
J*	J	P**	P	C***	C
J-C	J-P	P-C	P-J	C-P	C-J
J-C-P	J-P-C	P-C-J	P-J-C	C-P-J	C-J-P

*Join-up Method, **Parelli's Games (Friendly Game, Porcupine Game), ***Clicker Method

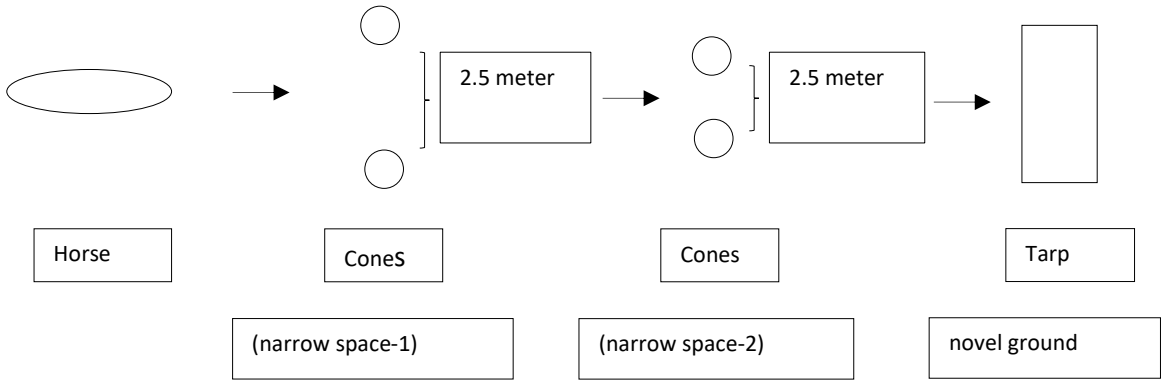

Figure 2. Study design

of the study, is a licensed trainer unfamiliar with the respective horses. For each horse, a training session lasting a maximum of 75 minutes, using three training methods, was carried out in one day. While a horse was training, the other two horses that would be trained that day were kept in the training area to reduce the stress of leaving the herd. All training has its own learning goals. However, for the standardization of the research, regardless of whether the learning goals were reached or not, fixed times were applied. The respective times were measured with the help of a stopwatch. The training methods and combinations applied in each group can be seen in Table 1.

Before and after the application of each training method, horses were directed to pass through a narrow-spaces and walk over a tarp (novel/typically frightening tasks) with trainer. The first narrow space was 2.5 meters between two red and white cones of 55 cm in height. The second was 1.5 meters between cones again. In the task of walking on the tarp, horses were directed to walk on an orange tarp of 70 x 150 cm size (Figure 2. Study design). During these tasks, their heart rates (as an indicator of stress), behavioral responses (fright, curiosity, etc.), and their learning responses (the duration of the completion of the task) were examined. In Trainings, the behavior of each horse was assessed by the trainer with the use of video recordings, trainer's notes, and Equid ethograms.

In the study, all three horse training methods were applied to the same horse with all their combinations to test whether these three methods have the potential to complement each other or to increase their effectiveness. Within the scope of the research, Parelli's Seven Games and Join-up methods from conspecific horse training methods and Clicker method within the scope of behavioral horse training were examined. Join-up method used to impose the dominancy of the trainer to the horse and to manipulate its behaviors in a controlled environment (the round pen) (Roberts, 2000; Roberts, 1997). In our study, in Join-up method, the terms of obedience and trust are used instead of "respect," "dominance," and "leadership" because of can jeopardize the welfare of the horse (ISES, 2017). During the research, it was observed and recorded whether the horses were performing one or more of the signs (lowered head, licking-chewing, eye contact, smaller circle) as well as "Join-up" and "Follow-up", which Monty Roberts revealed in the Join-up method. Seven Games developed by Pat Parelli (Friendly Game, Porcupine Game, Driving Game, Yo-yo Game, Circling Game, Sideways Game, Squeeze Game) focuses on enhancing friendship and dominancy relationships between the trainer and the horse (Parelli, 1993). In Parelli method, negative reinforcements are used to make the horse behave in the desired way, and after the desired response is achieved, positive reinforcements are applied. In this research, Friendly and Porcupine Games were applied to horses within the scope of Parelli's Seven Games. Friendly game is designed to persuade the horse that the trainer is a reliable friend. Porcupine game aims at teaching the horse to avoid any pressures applied with fingers. Clicker training is a method in which positive reinforcement is efficiently used in learning theory (Turner, 2013). The unfamiliar sound of the clicker conditions the horse to a reinforcement. The principal reinforcement is food reward (Mills and McDonnell, 2005). In scope of Clicker method, Carrot Stick and Clicker were used. Carrot Stick

Table 2. Assessment of variables

Variable	Definition
Obedience	The horse walking alongside the trainer without using any pressure on the rope.
Disobedience	The horse dragging the trainer, rearing up, walking too close to the trainer, pushing or pulling the trainer.
Trust	The horse staying calm next to the trainer and does not display frightening behavior towards any stimulus.
Fright	The horse widening of the eyes (the eyes widen, thereby exposing the white around the pupil), widening of the nostrils (the nostrils widen and exhalation becomes obvious and louder), and avoidance (walking backward, trying to escape from the trainer, startling from the trainer's movements) (McLean, 2003, Waring 2003).
Curiosity	The horse touching the whip during the Games of Parelli and the target stick in the Clicker method with their nose, sniffing and looking carefully.
Precision	The horses do not allow the body parts to be touched.
Positioning the Horse	The horse is positioned in four directions in round pen in the Join-up method.
Conflict Behaviors	The horse ears laid back, nipping, balking, pushing, head-threat, bite threat, head bumping, chasing, pawing, kicking, kick-threat.
Success Rate	The horse passing through the narrow spaces created within the scope of the research and being able to walk on a tarp.
Lack of Attention	The horse does not respond to the stimuli given by the trainer within the scope of training. The reaction to the applied effects is reduced or eliminated.

Table 3. Heart rates in training groups

Groups	N	Minimum	Maximum	Mean ($X\pm S_x$)
1	6	40.6	201.1	79.1± 3.9
2	6	43.5	186.5	78.0± 4.0
1+2	12	42.0	193.8	78.5± 2.7
3	6	42.0	191.5	67.0± 3.5
4	6	41.3	188.8	73.6± 3.5
3+4	12	41.6	190.1	70.3± 2.7
5	6	45.3	154.5	70.0± 5.3
6	6	42.1	191.1	73.3± 6.5
5+6	12	43.7	172.8	71.6± 4.0
P				-

X±Sx: Arithmetic mean and standard error; 1 (J-C-P); 2 (J-P-C); 3 (P-C-J); 4 (P-J-C); 5 (C-P-J); 6 (C-J-P); -: not significant

was approached to the nose of the horse and the word "Target" was used. When the clicker was touched with the nose of the horse, the click was done and the reinforcement (food reward-small pieces of carrot) given to the horse by the trainer within 3 seconds. Then the carrot stick was moved half a meter away from the horse and the word "target" was used. In this research, the aim of the Clicker training is that the horse follows the carrot stick, therefore the trainer willingly.

Statistical Analysis

Significance tests between the groups in terms of heart rates and training durations were conducted as variance analysis test. Their success in completing the required tasks were checked by chi square test in order to see the impacts of training methods. SPSS 14.0 software (SPSS 2005) was used for statistical analysis.

Assessment of Variables

The explanations of the variables used in the research are presented in the Table 2. “Assessment of variables” below.

Results

Heart rate data of training groups can be found in Table 3. Mean heart rates varied from 67.0 to 79.1. As each method was used as the initial method, the number of groups was reduced to three (1+2, 3+4, 5+6), and no significant statistical difference was found among the group in variance analysis (78.5) ($P>0.05$). The highest mean heart rate was recorded in the groups which started the training with Join up method, and the lowest (70.3) in those who started with the Parelli method.

The total training duration in each group is demonstrated in Table 4. It can be seen that the longest training duration was in the groups in which Join up method was practiced as the last method (Groups 3 and 5), and the shortest training duration was in those which practiced the Parelli method as the last one (Groups 1 and 6). The differences in training durations among groups are not significant (P>0.05). Examined individually, Join-up and Clicker methods take 15-20 minutes, while the Parelli method takes 30-35 minutes to apply.

Table 4. Total training duration (minutes)

Groups	Minimum	Maximum	Total ($X\pm S_x$)
1	42.3	57.8	51.9± 2.8
2	39.5	71.1	57.0± 5.0
3	51.0	63.8	57.4± 1.7
4	43.6	71.5	56.2± 4.6
5	50.5	67.8	61.0± 2.4
6	47.6	57.1	52.0± 1.4
General	39.5	71.5	56.0± 1.3
P			-

X±Sx: Arithmetic mean and standard error; 1 (J-C-P); 2 (J-P-C); 3 (P-C-J); 4 (P-J-C); 5 (C-P-J); 6 (C-J-P); -: not significant

Task completion success rates, obedience, and conflict behaviors of the horses were examined as each method was applied as the first method separately, and according to the sequence of each method in the order or the combination. Therefore, the impacts of Join-up, Parelli, and Clicker methods were examined in terms of learning and behavioral characteristics.

Table 5. demonstrates the task completion success rates of the horses in reference to each training method. Each group of 12 horses starts the training with one of the methods. When these methods are applied by themselves, the success rates in the task of passing through a narrow space are similar, however, those in the task of walking on a tarp vary dramatically (J, 50%; P, 41%; C, 75%). Moreover, single-use of every method resulted in lower success rates than the application of double and triple combinations except the combination in which the Join-up method was applied as the last method (J-C, 83.3%; J-P, 100%; P-C, 83.3%; P-J, 50%; C-P, 100%; C-J, 100%; J-C-P, 100%; J-P-C, 100%; P-J-C, 66.6%; C-J-P, 100%). In the triple combination groups in which the Join-up method was applied as the last method, the success rate in the task of walking on a tarp was the lowest (P-C-J 33.3%; C-P-J 33.3%), and the differences among the groups were highly significant (P<0.01). When the Join-up method was applied as the first method in the training, all the horses were more successful in completing the tasks. Combinatory application of methods also increased the success rates, except for the use of the Join-up method as the last part of the training. The highest success rate was the result of triple combinations starting with Join-up methods (100%), and double combinations starting with the Clicker method (100%).

Obedience and conflict behaviors with reference to training methods are demonstrated in Table 6. Obedience rates were low and conflict behavior rates

Table 5. Success rates of the horses in completing the tasks with reference to training methods

		Narrow Space 1		Narrow Space 2		Walking on Tarp	
Training Method	N	Number	Rate (%)	Number	Rate (%)	Number	Rate (%)
J	12	11	91.6	11	91.6	6	50.0
P	12	12	100	12	100	5	41.6
C	12	12	100	12	100	9	75.0
x^2			-		-		-
J-C	6	6	100	6	100	5	83.3
J-P	6	6	100	6	100	6	100
x^2			-		-		-
P-C	6	6	100	6	100	5	83.3
P-J	6	6	100	5	83,3	3	50.0
x^2			-		-		-
C-P	6	6	100	6	100	6	100
C-J	6	6	100	6	100	6	100
x^2			-		-		-
J-C-P	6	6	100	6	100	6	100[a]
J-P-C	6	6	100	6	100	6	100[a]
P-C-J	6	6	100	5	83,3	2	33.3[b]
P-J-C	6	6	100	6	100	4	66.6
C-P-J	6	3	50,0	3	50,0	2	33.3[b]
C-J-P	6	6	100	6	100	6	100[a]
x^2			-		-		**

-: not significant; **: *P*<0,01 a,b,c: Rates with different letters in the same column differ significantly.

were high in the solo use of Join-up method. The use of Parelli method only decreased the conflict behavior rates to minimum, and the use of Clicker method only resulted in the highest rates of obedience. The use of Clicker method the last method in the combination resulted in the obedience of almost all horses. The use of Join-up method as the last method in the combination decreased obedience to minimum and increased conflict behavior rates to maximum. In double combinations, there were statistically significant differences between P-C and P-J applications (P<0.01). The differences among triple combinations were also significant (P<0.01). These differences are due to the use of the Join-up method as the last method.

Table 7. includes data on the learning and behavioral characteristics of the horses in the Join-up method. As can be seen in the table, solo and combinatory uses of the Join-up method resulted in high rates of conflict behaviors. Lack of attention was around 50% in J and C-P-J applications, and 16.6% in other groups. As the lack of attention decreased, the rate of the positioning of the horses increased. While Follow-up rates were generally low in all groups (16.6%-58.3%), they were highest in the J group.

Two Parelli games were used in this research: The Friendly Game and the Porcupine Game. Success

Table 6. Obedience and conflict behavior rates with reference to training methods

		Obedience		Conflict Behavior	
Training method	N	Number	Rate (%)	Number	Rate (%)
J	12	2	16.6	8	66.7
P	12	6	50.0	3	25.0
C	12	7	58.3	4	33.3
x^2			-		-
J-C	6	5	83.3	1	16.6
J-P	6	4	66.6	2	33.3
x^2			-		-
P-C	6	6	100	0	0.0
P-J	6	1	16.6	5	83.3
x^2			**		**
C-P	6	4	66.6	3	50.0
C-J	6	1	16.6	5	83.3
x^2			-		-
J-C-P	6	5	83.3ª	0	0.0ª
J-P-C	6	6	100ª	2	33.3^b
P-C-J	6	1	16.6^b	4	66.6
P-J-C	6	6	100ª	0	0.0ª
C-P-J	6	2	33.3	4	66.6
C-J-P	6	5	83.3ª	0	0.0ª
x^2			**		**

-: non significant; ** : *P*<0,01 a,b,c: Rates with different letters in the same column differ significantly.

Table 7. Learning and behavioral characteristics in Join-up method (%)

Characteristics	J (n=12)	P-J (n=6)	C-J (n=6)	P-C-J (n=6)	C-P-J (n=6)	x^2
The stress of separation from the herd	91.7	83.3	83.3	83.3	66.7	-
Communication with the herd (neigh)	91.7	83.3	83.3	66.7	66.7	-
Disobedience	83.3	83.3	83.3	83.3	66.7	-
Conflict behaviors	66.7	83.3	83.3	66.7	66.7	-
Lack of attention	58.3	16.6	16.6	16.6	50.0	-
Positioning the horse	33.3	83.3	50.0	66.6	33.3	-
Obedience at the end of training	100	66.7	50.0	100	50.0	-
Trust to trainer at the end of the training	75.0	66.7	100	100	33.3	-
Follow-up	58.3	50.0	16.6	50.0	16.6	-

-: non significant

rates of the horses with the use of this method can be seen in Table 8. The application of the Parelli method in double and triple combinations increased the obedience rates of the horses. In solo application, the success rate was 50%, whereas it was 66.7% in double and 83.3% in triple combinations. In the groups Parelli method was applied as the last method of three, all the horses learned vocal cues, trusted their trainers and all the conflict behaviors disappeared. Except for the C-P group, the rates of fright behavior were high and the rates of Precision-2 (to touch its leg) were low. Parelli method results in an increase in obedience rates in double and triple method combinations.

The impact of the Clicker method on the learning and behavioral characteristics of the horses can be seen in Table 9. Almost all the horses demonstrated attention to Carrot Stick. When the Clicker method was applied as the only method and as the last method in combination with others, trust rate was 100%, and all the horses learned the vocal cues. The use of the Clicker method in double and triple combination with other methods resulted in higher obedience rates. With the use of this method, all the horses demonstrated fright, and conflict behaviors decreased dramatically.

Discussion

Mean heart rates of horses during trainings were high in the groups which started with Join-up method, and low in those which started with Parelli method. Accordingly in our study, when comparing the HR of horses during training, no significant statistical difference was found among the training groups in variance analysis. The increase in heart rate of horses during Join-up training in our study is thought to be caused by physical activity (active beginning of the training), and the stress of separation from the herd due to the fact that the horses have not been separated from the herd before. Lesimple (2020), states that heart rate is an indicator of the welfare of horses. In a study of Loftus et al. (2016), when comparing the HR of horses during Join-up to overall training, there were no significant differences. Similar to our research results, Physick-Sheard et al. (2000) pointed out that the increase in HR may be due to physical exertion and/or an increase in psychological stress. Loftus et al. (2016) stated that the Join-up method, which includes short episodes of canter, may cause an increase in HR due to more physical activity in horses. The findings of the present study are convenient with those of the research conducted by Fureix et al. (2009), which focused on conventional and natural horse breeding methods, and which demonstrated that horses under stress communicate with the herd by neighing. In our study, the use of Join-up method resulted in conflict behaviors more than the use of other methods; and obedience decrease with this method. McGreevy et al. (2005) claim that conflict behaviors are indicators of physical and mental disturbance as a response to treatments during training. At the same time, similar to our research results, they provided that the horse is directed to leave the herd, and to get used to the training field, its stress might be minimized, the success of the method might be increased. Boivin et al. (2003) shows that manipulating the behavior of horses by training them and making them familiar with new conditions will reduce stress-related behaviors such as neighing, galloping, rearing and defecation, which is consistent with our research results.

Clicker method includes positive reinforcements only. The reason why mean heart rate in Clicker method is higher than in Parelli method is the excitement of the horses to food reward used as the positive reinforcement in Clicker method (Williams et al. 2004). In the groups Parelli method is applied as the last method in combination with others, the application takes less time. As the other methods result in trust and obedience with the trainer, the Parelli method takes the advantage of the application of other methods. Fureix (2009) et al. claim that natural horse breeding practices improve the interaction between the horse and humans. Lansade and Bouissou (2008) state that the amount of contact between the horse and human is in direct correlation with the balancing rate of the responses of the horse.

The success rates in completing the task of passing through narrow space are similar in all methods. However, the rates of success in the task of walking on

Table 8. Data on the rates of learning and behavioral characteristics in the use of Parelli method (%)

Characteristics	P (n=12)	J-P (n=6)	C-P (n=6)	J-C-P (n=6)	C-J-P (n=6)	x^2
Obedience	50.0	66.7	66.7	83.3	83.3	-
Curiosity in whip	100	83.3	100	83.3	100	-
Fright	66.7	66.7	33.3	100	83.3	-
Conflict behaviors	25.0	33.3	50.0	0.0	0.0	-
Precision-1 (to touch to its head)	50.0	66.7	66.7	83.3	50.0	-
Precision-2 (to touch to its leg)	8.3	16.7	66.7	16.6	16.6	-
Learning vocal cues	100	83.3	83.3	100	100	-
Trust to the trainer at the end of the training	91.7	83.3	83.3	100	100	-

-: non significant

Table 9. The impact of Clicker method on the learning and behavioral characteristics of the horses (%)

Characteristics	C (n=12)	P-C (n=6)	J-C (n=6)	J-P-C (n=6)	P-J-C (n=6)	x^2
Obedience	53.3	100	83.3	100	100	-
Curiosity to the stick	100	83.3	100	100	100	-
Fright (*walking backwards and escaping)*	25.0	16.6	33.3	16.6	16.6	-
Conflict behaviors	25.0	0.0	16.6	33.3	0.0	-
Learning vocal cues	100	100	100	100	100	-
Trust to the trainer at the end of the training	100	100	100	100	100	-

-: non significant

a tarp vary dramatically. Christensen et al. (2012) made a research on the learning performances of the horses, and applied Clicker training. Similar to these findings, the present study demonstrated that varying reinforcements increase motivation. Researches by Visser et al. (2003) and Lansade and Simon (2010) resulted in similar findings on the learning performances of horses. Comparing the success rates of the methods, it was found out that C>J>P and Clicker method is 34-55% more successful than the other methods. The clicker method is recommended if only one method is to be applied. Similarly, Pryor (2002) claims that the Clicker method improves the learning skills of horses in new tasks. Lethbridge (2009) claims that the Clicker method can be used to manipulate horse behaviors during horseshoeing applications. Combinations of two methods generally increase success, and except for the groups in which Join-up is applied as the last method, three methods combined also increase the success. The success of completing the task of walking on a tarp was about 33.3% in Group 3 and 5, in which Join-up was applied as the last method. The highest success rate in the present study was of the applications of three methods starting either with Join-up or Clicker methods and double combinations starting with Clicker methods. Therefore, it can be concluded that the contents and the sequences of the methods have to be compatible. This finding is compatible with those of the research by Janczarek et al. (2013), in which the researchers claim that horses' responses vary in each method and that this should be taken into consideration in order to guarantee horse welfare.

In terms of obedience, the scores in the solo application of the Clicker method are the highest scores, and those of the Join-up method is the lowest. It should not be forgotten that the most important purpose of round-pen training should be to establish stimulus control in the horse (Fenner et al. 2019). In the combinations of three methods, when the Join-up method was applied as the last one, obedience decreased, and conflict behavior increased dramatically. Similar to our study results, Dai et al. (2019), comparing the loading into a truck time of the horses that using positive reinforcement-based training and without training, it was shown that the training horses had shorter loading time and the clicker training reduced loading stress. Hall and Heleski (2017), define fear as an innate behavioral response that motivates the horse to escape from potential danger. This causes a conflict response to the desired behavior. This finding is similar to the findings of the present study. The Join-up method establishes the hierarchy between the horse and the human, however, when other methods are applied before Join-up, conflict behaviors occur. The application of the Join-up method after other methods in training decreases the stress of separation from the herd to a degree. This is possible with the familiarity and trust other methods may enhance. Communication with the herd and disobedience can be decreased with the combination of methods. Many other types of research also show that the application of learning theory in training gets successful results. Repeated applications of methods decrease stress and increase the success (McGreevy, 2007; McGreevy and McLean, 2007; McGreevy and McLean, 2010).

When Join-up is applied as the only method, Follow-up rates increase. Other methods establish a friendly relationship between the horse and human, however, the Join-up method separates the horse from the herd again. This makes the horse confused and results in difficulties in learning. The application of the Join-up method decreases the rate of positioning, but increase obedience and Follow-up. For successful training, the Join-up method should be applied by itself, or as the first method in combination with others.

The use of Parelli just after Clicker (C-P) decreases fright. Positive and negative reinforcements without pressure keep the horse calmer. Similarly, Dougherty and Lewis (1992), showed that horses' responses depend on positive reinforcement a great deal. Many other studies emphasize the correlation between positive reinforcement and training horses in new responses (Feng et al. 2016; Flannery, 1997; Sappington and Goldman, 1994; Williams et al. 2004). The findings of the present study are similar to many other experimental types of research (Heird et al. 1986; Lansade et al. 2004; Visser et al. 2002) in that horse training decrease the stress horses are exposed to and improve their emotional responses in facing unfamiliar conditions. The researchers could not explain why the J-C-P combination resulted in anxiety and fright, but it might be suggested that it is due to the individual temperaments of the horses. In their research, Lansade and Simon (2010) showed that the influence of tem-

perament on learning performance was task-dependent. They found out that temperament did not directly influence learning, but horses develop some dispositions with their responses to stimuli in training. The present study shares the emphasis on the necessity of developing individual programs for each horse and choose an appropriate method for appropriate temperament.

The present study showed that almost all the horses were curious about the target stick. This made the application of the method easier and increased its efficiency due to the horses' willingness to training.

Moreover, fright and conflict behaviors were minimum in this method. Easy application, success in the short term, and minimum danger for the horse and the trainer make the Clicker method an advantageous one. Training systems based on positive reinforcements such as the Clicker method make training processes much easier.

Conclusion

In the natural behaviors of horses, the hierarchy in the herd is determined initially, and social communication in the limits of this hierarchy occurs then. This process should be taken into consideration while applying methods based on ethology in horse training. This is why the application of the Join-up method before others is successful: It enables the determination of hierarchy between the horse and human. When the Join-up method is followed by the Parelli and Clicker methods, this situation positively affects the success of the training since the communication between horse and human is formed similar to the natural herd dynamics of the horses. In the Join-up method, horse follows the trainer and feels secure with him. After determination of hierarchy, conflict behaviors decrease and horses interact easily. Afterwards, horses can establish friendships, and develop social behaviors such as protecting and grooming each other. Parelli's Games establish friendship, and the trainer can touch the horse's body trustfully. Clicker method uses a very strong positive reinforcement, i.e. feed, as reward. The horse, having developed obedience and become friend with the human, gets ready for cooperation.

In conclusion, as it takes short time and can be applied easily, in solo applications, the Clicker method is the most advantageous one. In triple combinations, Join-up should not be used as the last method, since it takes longer times and decreases success. Join-up is more successful as the first method in combination with others. With this research, it is seen that ordering is important when applying behavioral and conspecific training methods to horses in combinations. It has been revealed that planning the training of horses by considering the dynamics of the herd in their natural life increases the success in training.

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RESEARCH PAPER

LIVESTOCK STUDIES

The Influence of Corpus Luteum Size on the Conception in Embryo Transfer Recipient Cows

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Abstract

The aim of study was to investigate the effect of corpus luteums (CL) size that detected before embryo transfer on conception in recipient Holstein cows. The recipient cows utilized had at least one birth and aged 3-8 years old. PGF2α was injected 2 times space 14 days apart to cows and 87 cows which show estrous symptoms were determined as recipient. Fresh embryos were transferred after estrous in 6^{th}-8^{th} days. Ovaries were examined by ultrasound before transfer and CL sizes were measured. A scale of '++++' were set as a result of measurements. Recipient cows were into 3 groups. The first group was named as CL2+ (n=25) and consisted of cows with '++' (≥15-20< mm) size CL. The second group was named as CL3+ (n=52) and consisted of cows with '+++' (≥20-25< mm) size CL. The third group was named as CL4+ (n=10) and consisted of cows with '++++' (≥25 mm) size CL. The embryos were transferred to the uterine horn on the side of the CL (ipsilateral). Pregnancy rate was respectively 36%, 46.2% and 40% for groups. No statistically significant difference was found between groups in terms of the effect of CL on conception (P>0.05). As a result, it was concluded that the size of the corpus luteum has no effect on the conception of cows utilized as recipients in embryo transfer.

Introduction

Bovine embryo transfers attempt to produce a number of high-quality calves by first selecting a cow (donor) with good genetic creation, a sturdy body conformation and outstanding strength, then artificially inducing the production of a number of fertilized eggs (superovulation) and finally transferring them into the uteri of other cows or heifers (recipients). This helps to quickly improve the breed as well as increase profits (Kanagawa *et al.*, 1995). One of the most important ways providing genetic progress rapidly in dairy cattle and increasing number of selected male and female in the herd is embryo transfer applications (Akyol, 2001; Pabuçcuoğlu, 2013; Seidel and Seidel, 1991; Tekeli, 2010). Furthermore, embryo transfer is the most important modern technique used to increase the success of animal breeding in the most effective way (Bülbül and Dursun 2005). The most important goal of bovine embryo transfer is to obtain oocytes and embryos of high quality from superior dams that will result in the birth of healthy calves (Santos *et al.*, 2008). Normally, while a cow can breed one calf in a year, at least 5 times the number of offspring that can be obtained throughout life can be obtained by embryo transfer (Seidel and Seidel 1991; Tekeli, 2010). The successful transfer of embryos into lactating dairy cattle has shown beneficial effects in improving fertility in dairy cattle, especially during summer heat stress. The transfer of an embryo could bypass certain causes of infertility (i.e., fertilization failure and early embryonic loss) (Bilby, 2010).

After ovulation, the corpus luteum (CL) is formed under the influence of LH from follicle residues. The follicle cavity is filled with blood vessels and the size of the granulosa cells increases. Progesterone is indispensable for normal cycle in cow, and it is primarily responsible hormone for maintaining pregnancy after conception. Around 16 days following ovulation, PGF2α secreted from the non-pregnant uterine endometrium

initiates regression of the corpus luteum and this event is called luteolysis (Ptaszynaska, 2009).

The ovarian follicle wall, consisting of granulosa cells and theca cells, vascularizes and luteinizes after ovulation to form a corpus luteum. This temporary steroid-producing gland undergoes marked structural and functional changes in a short time-span during its development, functional life and regression (O'Shea *et al.*, 1987). There is a high correlation between plasma progesterone concentration and corpus luteum mass, volume and histomorphology (Singh *et al.*, 1997). For pregnancy to be established in cattle, the embryo has to be inhibited the development of the luteolytic mechanism and maintain the secretion of progesterone by the corpus luteum (Silva *et al.*, 2002).

A successful pregnancy depends on complicated interactions between the mother and the conceptus in the genital tract in ruminants. During maternal pregnancy recognition, the viable embryo must be able to interrupt the luteolysis with the production of bovine trophoblastic interferon and promoting the action of a functional CL that synthesizes adequate levels of progesterone (P4), a hormone that plays a vital role in early gestation, since it stimulates and maintains the functions necessary for the growth of the conceptus, implantation and placentation. An ovulatory follicle with appropriate size can become, after ovulation, a functional CL that will secrete P4 with a positive effect on the establishment of pregnancy (Monroy *et al.*, 2018).

The uterine microenvironment provides suitable medium for embryos developing only during the luteal stage of the estrus cycle. Therefore, the harmonization of the physiological state of the donor and the recipient in the embryo transfer operations, that is, synchronization, is the basic principle in the success of the method (McGeady *et al.*, 2011).

Accordingly, our hypothesis is that different corpus luteum sizes will affect the conception rate. Therefore, the aim of the study was to evaluate the CL size affect or not the conception rate on recipient cows.

Materials and Methods

Materials

In this study, embryos obtained from lactating cows as selected donors in Eastern Mediterranean Agricultural Research Institute-Adana, Turkey were transferred freshly to cows in this institute. The cows were Holstein cows, and the age of the recipient cows used in the study ranged from 3 to 8 years. 87 cows selected from the farm were utilized in the study. While selecting the animals to be used in the study, the genital examinations were performed by transrectal ultrasonography using a portable ultrasound with a 5 MHz linear probe (Ultrasonic scanner, HS-101V, Honda, JAPAN) to determine if there is a pathological condition.

In the farm, cows are housed in open barns and fans are used with water spraying as cooling systems to minimize the heat stress, which is an important effect of the Mediterranean climate. Wheat straw, alfalfa, corn silage and vetch as roughage and concentrated feed (it contains barley, whaet, corn, sunflower meal, soybean meal and mineral-vitemans) with 18% protein produced in the enterprise was used in ration. Water was provided with automatic waterers as ad libitum.

Methods

The ovarian examinations of the recipient cows in the study were performed with ultrasound. CLs were evaluated on the scale of '++++' in terms of quality and were calculated by size in millimeter. The corpus luteum size was determined firstly by measuring 2 furthest point from right to left and 2 furthest point from top to bottom. Then the average of these two measures was taken. The cows to be utilized as recipients in the study were divided into 3 groups. The first group was named as CL2 + (n = 25) and was consisted of cows with CL of '++' (≥15-20< mm) size. The second group was named as CL3 + (n = 52) and cows with CL of '+++' (≥20-25< mm) size were used as recipients. Finally, the group consisting of group 3 carriers was named as CL4 + (n = 10) and was selected of cows with a size of '++++' (≥ 25 mm).

The cows that could be included in the study were injected PGF2α twice with an interval of 14 days. These cows were followed and those who showed estrus symptoms were determined and recorded. The presence of quality CL in these cows detected was determined by ultrasound examination on the day of transfer. The transfers were applied to the recipients with high quality and suitable CL. In ovarian examinations, CLs were evaluated in terms of diameter, quality, presence of antrum and on which side it was located (Figure 1). The fresh embryos were transferred to the horn on the side where CL was detected (ipsilaterally).

Statistical Analysis

SPSS package program version 25 (SPSS Inc., Chicago, USA) was used for statistical analysis of the study. Pregnancy status of the groups was statistically compared by Chi-square test.

Results and Discussion

Twenty-eight days after embryos were transferred to recipient cows (35 days after estrus), pregnancy examinations were performed using the recto-vaginal method using ultrasound. 37 of 87 (42.5%) recipient cows that were embryo transferred were diagnosed to be pregnant. The relationships between the groups' corpus luteum sizes and conception are shown in Table 1. When the pregnancies were evaluated as a group, 9 pregnancies were obtained from 25 transfers in the CL2 + group and a 36% pregnancy rate was obtained. 24 pregnancies were diagnosed from 52 transfers in CL3 +

Table 1. Pregnancy rates in groups according to different corpus luteum diameters (P>0.05).

Groups	n	Pregnant	Non-Pregnant	Pregnancy Rate
CL2+ (Group I)	25	9	16	36.00%
CL3+ (Group II)	52	24	28	46.20%
CL4+ (Group III)	10	4	6	40.00%
Total	**87**	**37**	**50**	**42.50%**

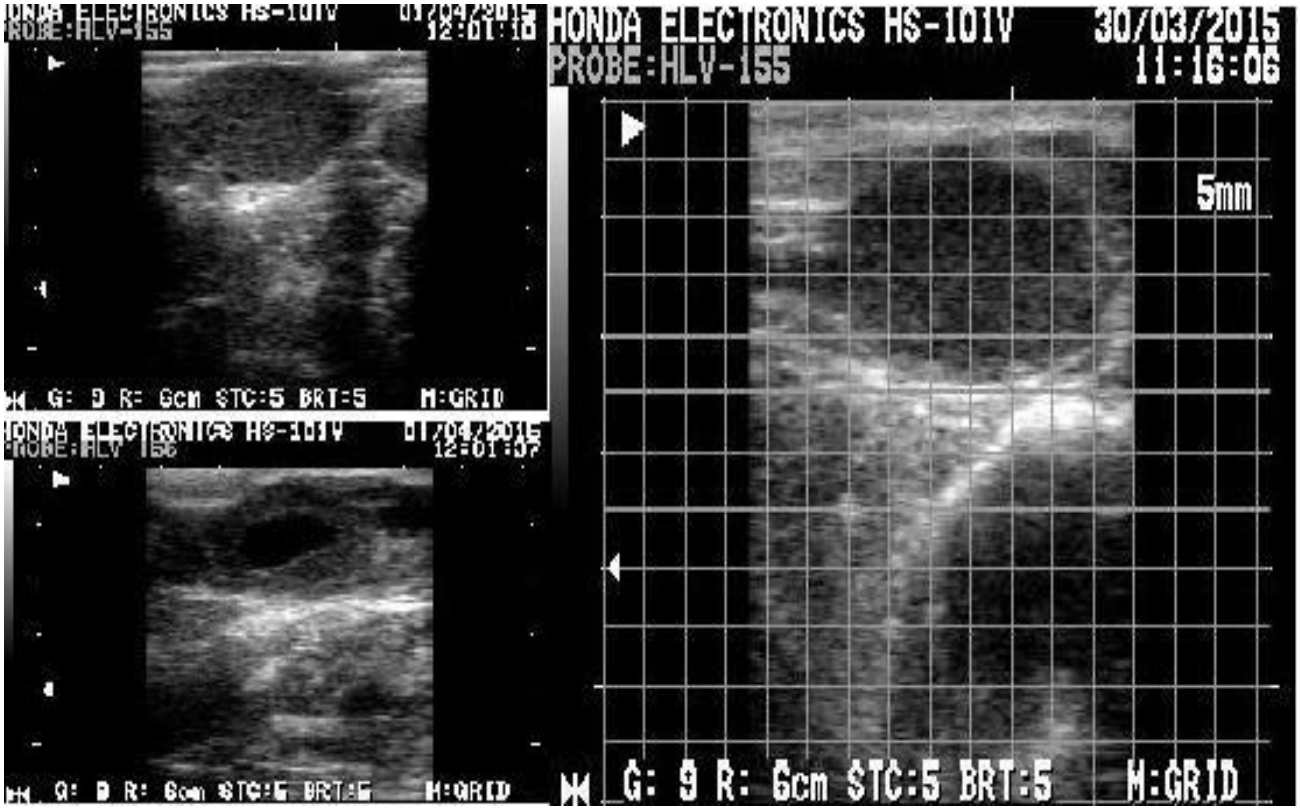

Figure 1. Ultrasound image of the corpus luteums.

group and the pregnancy rate was determined as 46.2%. Finally, in the CL4+ group, 4 pregnancies in 10 cows were determined and the pregnancy rate was found to be 40%. When the pregnancy rates were compared, there was no statistically difference between the groups (P>0.05). However, even an extra pregnancy is important in high-cost applications such as ET.

This study was carried out to search whether corpus luteum size of recipient Holstein cows which were transferred fresh embryo has an effect on conception rate. In our study, corpus luteum size did not affect the conception rate in recipient cows. The relationship between corpus luteum size, progesterone concentrations, and pregnancy rates in recipients is still uncertain, and a lot of research has been made on this subject (Hasler *et al.*, 1980; Ramsen and Roussel, 1982). There are different ideas regarding whether high (>6.0 ng/mL) or low (<2.0 ng/mL) blood progesterone concentrations affect pregnancy rates (Siqueira *et al.*, 2009).

Good, bad and cystic corpus luteum quality (good is big size, bad is small size) in cows had no effect on pregnancy rate in embryo transfer (Ramsen and Roussel, 1982). In our study, the corpus luteum size had no effect on pregnancy rate similarly Ramsen and Roussel (1982) study. They also transferred embryos to recipients that have cystic corpus luteum and found no different.

In a study investigating the effects of estrus detection and development of corpus luteum on conception for embryo transfer in recipient cows, it is evaluated the rates of conception by transferring the embryos at the age of 6.5-9 days between November 1983 and May 1984 by non-surgical method. Embryos were transferred to 19 herds in 10 states of the USA. The estrus of recipients was synchronized using PGF2α. The estrus determinations of the recipients were recorded within ± 60 hours (before and after the donors showed estrus) simultaneously with the donor estrus.

The corpus luteum was rated as 1) normal, 2) suspect or 3) non-palpable during transfer. When the pregnancy rates of the groups were compared, it was observed that the size of the corpus luteum had no effect on conception as in the current study (Nelson and Nelson, 1985).

There are studies evaluating corpus luteum number, size, and serum progesterone concentration. There are different situations regarding blood progesterone levels. It has been determined that high P4 concentration does not increase conception rate in recipients with embryo transfer, on the contrary, a decrease in pregnancy rates in animals with the highest P4 levels (Siqueira *et al.*, 2009; Nogueira *et al.*, 2004).

Conclusion

In this study, it was determined that the quality (size) assessment of corpus luteum did not differ in terms of the rate of pregnant and non-pregnant cows in accordance with the literature, in recipient cows which have different sizes corpus luteum for embryo transfer. But a recipient with a CL size of 20-25mm with a numerically higher pregnancy should be preferred.

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Ethic Committee Report was given by Cukurova University Local Ethic Committee of Experimental Animals for this study. The committee met on 25.08.2015. This study is a part of doctoral project named 'Searching of Pregnancy Rate in Repeat Breeder Cows by Embryo Transfer Practices'. And this study is a part of the mentioned work. So, we utilized the same committee report for this study too.

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RESEARCH PAPER

LIVESTOCK STUDIES

The Effect of Epidural Application of FSH on Superovulatory Response in Eastern Anatolian Red Cow

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Abstract

This study aimed to evaluate the superovulation response in Eastern Anatolian Red (EAR) cows treated with a single epidural injection or traditional administration of follicle stimulating hormone (FSH). Eight cows were randomly divided into two groups, as control (CG) and experiment (EG). Four replications were applied for each animal in the groups. A total of 16 studies were carried out in each group. In this study, 300 mg intramuscular (i.m) FSH injection was applied with decreasing doses twice daily for four days to the cows in the CG. (70:70, 50:50, 20:20, 10:10 mg). On the other hand, 300 mg FSH was administered as 150 mg epidural and 150 mg intramuscular injection to the cows in EG. The number of untransferable embryos and the total CLs were found statistically significant ($p<0.05$). However, the number of transferable embryos and unfertilized oocyte (UFO) were found statistically insignificant among the groups ($p>0.05$). According to the obtained data, it was observed that the epidural administration of FSH for superovulation in EAR cows did not provide sufficient follicle and oocyte development. In conclusion, different studies with different superovulation protocols and FSH doses in local breed EAR cows will be needed for further studies.

Introduction

In recent years, in the world, there has been a significant increase in studies for animal genetic resource conservation. Strategic priority issues have been determined in line with the Global Plan of Action for Animal Genetic Resources and, many countries have accepted that they have the priority protection task. In almost all economically developed countries, the genetic resources have been taken under preservation by carrying out national projects for animal genetic resource conservation. (Hiemstra *et al*., 2004). Many countries have established gene banks to protect animal genetic resources, and in this bank, they have started to conserve the genetic materials such as semen, embryos, and DNA of the endangered native breeds (Bailey *et al*., 2000; Medeiros *et al*., 2002).

Some technologies have been used for animal genetic resource conservation and obtaining genetic materials. Embryo transfer technology is one of the technologies used to obtain the genetic material to be frozen. Embryos obtained from animals whose genetic materials are frozen (in vivo) or from gamete cells of the animals in the laboratory (in vitro) are frozen and stored. (Machaty *et al*., 2012; Kaymaz *et al*., 2015).

Embryo transfer technology consists of various processes. Superovulation protocol, which includes the utilization of hormones, is one of the most significant steps of the process series (Hasler, 2006; Machaty *et al*., 2012). Different hormones and administration protocols are used to achieve superovulation (Mapletoft *et al*., 2002). FSH is one of the most common hormones used in ovarian

stimulation in cattle (Sağırkaya, 2009). However, FSH is a hormone with a very short half-life (about 5 hours or less). For this reason, FSH is treated twice daily at 12 hours intervals (Monniaux *et al*., 1983; Machaty *et al*., 2012; Bó & Mapletoft, 2014).

Since FSH has a short duration of action, multiple applications increase stress in animals and labor. Different protocols are studied to extend the duration of FSH action and reduce the number of injections. In a study, when FSH was mixed with a 30% polyvinylpolypyrrolidone (PVP) solution and administered as a single intramuscular injection, similar results to the classical method were found. (Yamamoto *et al*., 1994). Another study reported that when a mixture of FSH, PVP, and equine chorionic gonadotropin (eCG) was administered as a single injection, a super stimulatory response similar to the traditional FSH protocol could be achieved (Bó & Mapletoft, 2014).

In recent years, there have been studies in which gels containing aluminum hydroxide are used as vaccine adjuvants to ensure the slow release of FSH (Baylor *et al*., 2002; Lindblad, 2004). Kimura *et al*. (2007) reported that an aluminum hydroxide gel and FSH mixture application, as a single injection, provides sufficient effect in cattle. However, it was reported that aluminum hydroxide gel intramuscular injections cause lesions in the injection areas, thus causing economic losses in meat production (Kimura, 2016).

Another study showed that FSH suspension diluted in 2% hyaluronan provided sufficient super stimulatory response. However, obtaining a homogeneous mixture with FSH was reported as problematic due to the high viscosity of hyaluronan. Therefore, it was illustrated that the suspension obtained with lower concentrations of hyaluronan has better results when administered as two injections at 48 hours intervals (Tríbulo *et al*., 2011; Tríbulo *et al*., 2012). In a study on Anatolian Black cow, Taşdemir *et al*. showed that a single epidural injection with intramuscular injection of FSH application could provide acceptable results compared to twice-daily injection of FSH for super stimulatory response (Taşdemir *et al*., 2012).

Eastern Anatolian Red cows are one of our local breeds. Moreover, EAR cows and their genetic materials (sperm, embryo, DNA, and cells) are preserved in the gene bank within the scope of genetic resources. However, there are not enough embryos of these cows in the gene bank. This study aims to determine the efficiency of single epidural injection compared with traditional administration of FSH on superovulation response in EAR donor cows.

Materials and Methods

Eight cows 3-4 years old were used in this study. The cows had the same treatments and feeding conditions in the International Center for Livestock Research and Training. 400 mg FSH (FOLLTROPIN® Bioniche, Ireland), 1.55 g. Progesterone (Eazi Breed, CIDR®, Pfizer, Australia) and PGF2α (ESTRUMATE®, Intervet, Turkey) were used for superovulation protocol. Lactated Ringer's Solution (Polifarma, Turkey), Dual-way balloon catheter (Bioniche) were used for uterine flushing of donors. A 75 microns emcon filter (Agtech) was used to collect the flashing solution.

Animals were randomly divided into two groups, as control (CG) and experiment (EG). Four replications were applied for each animal in the groups. After each study, animals were kept for two months without any treatment. In addition, each animal was taken into a different group in the next repetition and, the study was carried out. Thus, the consequences arising from individual differences

Table 1. Application of FSH to the control group (CG) (Imai, 2005; Taşdemir *et al*., 2012).

First (0) Day	7th Day		8th Day		9th Day				10th Day		11th Day		18th Day
08:00 Am	08:00 Am	08:00 Pm	08:00 Am	08:00 Pm	08:00 Am		08:00 Pm		08:00 Am	08:00 Pm	08:00 Am	08:00 Pm	08:00 Am 10:00 Pm
CIDR Placement	FSH (70 mg)	FSH (70 mg)	FSH (50 mg)	FSH (50 mg)	FSH (20 mg)	PGf2a (3 ml)	FSH (20 mg)	CIDR Remove	FSH (10 mg)	FSH (10mg)	AI		USG/Flushing (Emb. Recover)

Table 2. Application of FSH to the experimental group (EG) (Imai, 2005; Taşdemir *et al*., 2012).

First (0) Day	7th Day		9th Day		11th Day		18th Day
08:00 Am	08:00 Am	08:00 Am	08:00 Am	08:00 Pm	08:00 Am	08:00 Pm	08:00 Am 10:00 Pm
CIDR Placement	FSH (Epidural 150 mg)	FSH (Im 150 mg)	PGf2a (3 ml)	CIDR Remove	AI		USG/Flushing (Emb. Recover)

between the groups were minimized. CIDR containing 1.56 g progesterone was inserted into the cows on a random day of the cycle. This application day was accepted as day 0. Cows were divided into two groups on the 7th day of CIDR application according to FSH dose and route of FSH administration (Folltropin). In the CG group, 300 mg intramuscular (i.m) FSH injection was applied with decreasing doses twice-daily for four days. (70:70, 50:50, 20:20, 10:10 mg; n: 4 Table 1). However, 150 mg epidural FSH and 150mg i.m FSH injection applied to the EG on day 7 of the cycle (n: 4; Table 1). 9 days later the beginning of the superovulation program, PGF2α injection was administered to both groups in the morning, and the CIDR was removed in the evening. After the observation of oestrus symptoms on day 11 and day 12, artificial insemination was performed twice at 12 hours intervals by using frozen semen of the EAR bulls.

Results

In the study, as a result of superovulation with decreasing doses of FSH in CG animals; the number of UFOs, the number of untransferable embryos, the number of the transferable embryos, the number of CL, the number of total ovum/embryo, and the embryo recovery rate were 1.75±0.479, 2.11±0.588, 1.50± 0.289, 5.54 ± 0.666, 2.92 ± 0.571 and 41,4 %, respectivel. As a result of superovulation with single epidural and intramuscular FSH application in EG animals, the number of UFOs was 1.67 ± 0.667, the number of untransferable embryos was 1.00 ± 0.000, the number of transferable embryos was 1, the number of CL was 3.25±0.675, the total number of ovum/embryo was 1.13 ± 0.441, and the embryo recovery rate was 43,3 % (Table 3).

The ratio of the total number of the obtained ovum/embryos to the CLs was 41.1 % (7/41) in CG and 43.7 % (7/16) in EG. In addition, the average number of total ovum/embryo obtained per donor was 1.06 (17/16) in CG and 0.43 (7/16) in EG. The ratio of the number of transferable embryos to the total number of ovum/embryo was 0.23 (4/17) in CG and 0.14 (1/7) in EG.

According to the results, there was no statistically significant difference between the CG and EG groups in the number of UFOs, the number of untransferable embryos, and the embryo recovery rates (P> 0.05). However, It was determined that the total number of CL and ovum/embryo obtained from the control group was higher than the experimental group (P <0.05).

Discussion and Conclusion

Currently, the successful results in the superovulation protocols applied with FSH can not be achieved in local breeds as obtained in the exotic breeds. Responses of the applied oestrus synchronization and superovulation programs can still vary. While superovulation responses of some local breeds to are adequate, some may lack such potential. Studies on superovulation protocols specific to local breeds and FSH doses are needed (Kelly *et al*., 1997; Bó *et al*., 2002; Bó *et al*., 2003; Baruselli *et al*., 2006). Some researchers have reported that it may be possible to achieve higher results in local breeds with the appropriate superovulation protocol selection (Bó *et al*., 1996; Bó *et al*., 2002; Carvalho, 2004)

In this study, the CL numbers counted by USG were found to be higher in CG animals than in EG animals. However, the results in both groups are low compared with the exotic breeds. In a similar study on local breed White Yak cattle, Yu reported that they received a low number of CL in a superovulation program that included twice-daily FSH administration in decreasing doses for four days. Thus, the results of our study are similar to Yu *et al*.'s study (Yu *et al*., 2007).

The number of UFOs, untransferable and transferable embryos, and the embryo recovery rate were not statistically different between the CG and EG groups. However, the total CL and total ovum/embryos obtained from the CG were higher

Table 3. Number of UFO, untransferable embryo, transferable embryo, CL, total ovum / embryo and embryo recovery rate in groups.

Groups	n $\overline{X} \pm S_{\overline{x}}$	Number of unfertilized oocyte	Number of untransferable embryo	Number of transferable embryo	Number of CL	Number of total ovum/embryo	Recovery rate %
CG	16	4 (1,75±0,479)	9 (2,11±0,588)	4 (1,50±0,289)	41 (5,54±0,666(a)	17 (2,92±0,571)(a)	41,4 % (17/41)
EG	16	3 (1,67±0,667)	3 (1,00±0,000)	1 (1)	16 (3,25±0,675)(b)	7 (1,13±0,441)(b)	43,3 % (7/16)
*p value		0,844	0,267		*0,036*	*0,036*	

a, b: The difference between different letters in the same column is significant (P <0.05).

than the EG. The results in both groups are lower compared with other studies (Taşdemir el al., 2012; Satılmış *et al*., 2017). It is considered that genetics, environment, nutrition, breed, season, age, the condition of the ovary at the time of application, and the effects of repeated superovulation can be the reasons for the differences in the ovary responses. Also, it is considered that the dose of the administered hormone, the timing of administration, and the use of additional hormones in superovulation protocols can be the other factors causing the differences (Sugano, & Watanabe, 1997; Son *et al*., 2007). There are various studies on determining the appropriate dose and FSH administration methods to eliminate the stress due to multiple FSH injections in cattle (Alvarez *et al*., 2010; Bó *et al*., 2010). It was reported in these studies that similar results were obtained by intramuscular or subcutaneous (SC) applications of a single FSH treatment (Kanitza *et al*., 2002; Alvarez *et al*., 2010).

The number of total ovum/embryos obtained per animal was 1.06 (17/16) in CG and 0.43 (7/16) in EG. It can be claimed that these rates are low compared to the averages of both cultural and local breeds (Karasahin *et al*., 2016). It is considered that these low embryo rates may be due to the low reproductive performance in EAR cows. It was stated in a study on the Anatolian Black cows that the results were similar for the superovulation induced by intramuscular administration with FSH applied to the epidural space compared with FSH treated in decreasing doses. However, transferable embryo rates were found low in both groups. The results of our study and the study on the Anatolian Black cows are similar (Taşdemir *et al*., 2012).

In the study, the ratio of the UFO (23.5 % in CG; 42.8% in EG) was similar between the groups. It has been stated that the UFO rate varied between 23.5% and 39% in previous studies conducted with different breeds (Karasahin *et al*., 2016). The UFO rates we obtained in our study are similar to previous studies (Taşdemir *et al*., 2016).

In the study, the ratio of the total number of embryos / ovums and the total number of CL was 41.4 % (17/41) in CG and 43.7 % (7/16) in EG. Although it was higher in the control group, it was lower than the studies on other local breeds and culture breeds. It is considered that these low rates are due to the breed and superovulation protocols (Kelly *et al*., 1997; Kim *et al*., 2000; Sugano *et al*., 2001; Barati *et al*., 2006). After more than 30 years of studies in Bos indicus, it is emphasized that the superovulation response in these animals, in which ovarian and follicular dynamics have been largely clarified, is better than Bos Taurus. However, this response also depends on environmental conditions such as care and feeding (Sağırkaya, 2009).

In this study, the rates of transferable embryo in both groups were (23.5 % in CG; 14.2% in EG). This ratio was lower than other local breeds (Sistani: 49%, Angus: 52%, Finnish Ayrshire: 61%, Korean Native: 62%, and Japanese Black: 53%) (Sugano *et al*., 2001; Barati *et al*., 2006). However, the results of the studies conducted in Nelore cattle yielded were similar to the results we obtained in our study (Alvarez *et al*., 2010). It was reported that the difference and lowness of these results in local breeds could be due to the decrease in the number of growing follicles (Lopes *et al*., 2007).

As a result, it was concluded that the Eastern Anatolian Red cattle breed does not have the potential to provide a superovulation response with a high success rate compared with culture breeds. However, advanced studies are needed to reveal the reproduction parameters of this breed to increase the number of transferable embryos. Moreover, the superovulation protocol approaches need to be improved in light of these studies.

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