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Effect of low doses of FSH and season on the *in vitro* maturation, fertilization and embryo development of bovine oocytes

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ABSTRACT

This study was aimed at determining the effect of follicle-stimulating hormone (FSH) and season on the *in vitro* maturation, fertilization and embryo development of bovine oocytes. Bovine ovaries obtained from a local slaughter house were transported to the laboratory within 2–3 h in a thermos flask containing antibiotic-supplemented physiological saline (0.9%) and at a fixed temperature of 30°C. Bovine oocytes collected in spring and autumn were incubated in culture media containing FSH at concentrations of 0.2 and 0.8 µg/ml. After maturation, oocytes were fertilized. Fertilized oocytes were incubated in CR1aa culture medium for 7 days at 38.5°C for *in vitro* development. The assessment made after the completion of the maturation process revealed that, for both FSH doses, the maturation rates obtained with the oocytes collected in spring were higher than those obtained with the oocytes collected in autumn. The incubation of the oocytes collected in autumn in culture media supplemented with 0.2 µg/ml of FSH resulted in a low level of oocyte maturation. After maturation, oocytes were subjected to fertilization. Fertilized oocytes were incubated in CR1aa culture medium for 7 days at 38.5°C for *in vitro* development. In both seasons, 0.8 µg/ml FSH application was higher than the maturation values obtained with 0.2 µg/ml FSH in terms of fertilization and embryo development rates. The study was repeated 9 times for each season. Although there was no significant difference between fertilizations and embryo development in the seasons, better results were obtained in spring season.

Keywords: Embryo, Fertilization, FSH, Maturation, Oocyte, Season

The *in vitro* production of bovine embryos is a rather costly process, which requires the application of advanced technology. Several factors are influential on *in vitro* embryo production. One of these factors is the season in which the oocytes are collected. In previous research, both the fertilization of oocytes and embryonic development, had been reported to be affected by season (Rust *et al.* 2009). In a previously conducted study, it was suggested that the cleavage rates obtained in winter season were lower than those obtained in other seasons (Pessoa *et al.* 2010). Temperature, season and diet affect reproductive efficiency, follicular development and oocyte quality, and thus have effect on fertility (Wolfenson and Roth 2018). High ambient temperature compromise reproductive performance through reducing feed intake and decreasing nutrient utilization, growth rate and feed efficiency which lead to economic losses in dairy animals (Kumar *et al.* 2019). Gonadotropins (primarily due to the known role of FSH) are frequently added to maturation media for maturation, cumulus expansion and embryonic development (Bahrami *et al.* 2019). Stress is a process stimulant that activates the whole system and produces an organic response that has a negative impact on animal health and production (Mahdy *et al.* 2018). Particularly due to high temperatures in summer, the internal body temperature of the animal's increases and this negatively affects the reproductive performance of the animals (Wolfenson and Roth 2018). Therefore, changes in the molecular and cellular levels of oocytes in response to temperature stress need to be investigated further.

This study was aimed at examining the effect of different doses of FSH on the *in vitro* maturation, fertilization and embryo development of bovine oocytes during different seasons.

MATERIALS AND METHODS

For the study, the oocytes were divided into 4 groups, i.e. in the spring and fall season 0.2 µg/ml FSH was used while in the spring and fall season 0.8 µg/ml FSH was used for oocyte maturation. The ovaries used in the study were obtained from the slaughter house in Çubuk district of Ankara. Cattle whose ovaries were taken were selected from farms with similar feeding characteristics and from same breeds and similar age groups in and around Ankara. Thus, effects on reproduction, except for effect of seasons, such as feeding, environment, race, age have been eliminated. The location of the animal farm is 40°14′ North 33°01′ East and 985 m above the sea level. In the autumn of 2011, when the ovaries were collected and in Ankara where cattle

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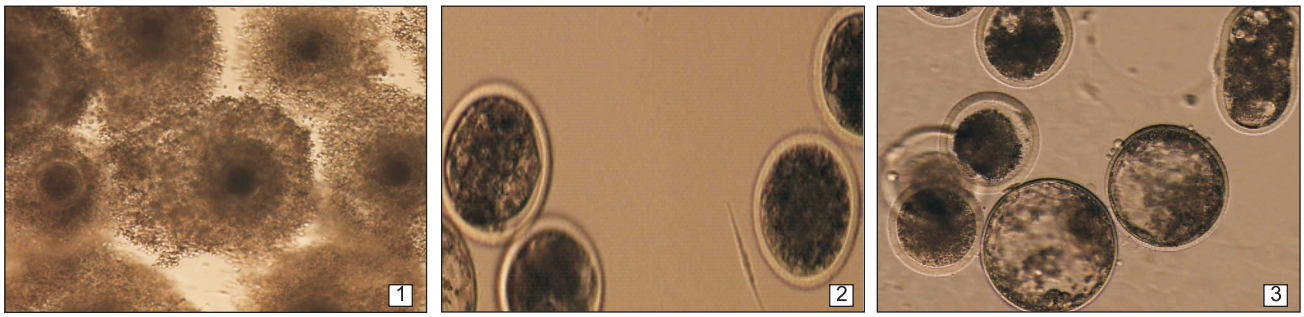

Figs 1–3. **1.** Matured oocytes and expanse cumulus cells. **2.** Compact morula embryos. **3.** Blastocyst embryos.

were raised, the surrounding air temperature was around seasonal norms and the average temperature was 13.4°C. The average temperature was generally around seasonal norms in most of the country during autumn 2011. Autumn rainfall is around 75 mm within the seasonal norms. In the spring season of 2012, when the ovaries were collected, the average temperature in Ankara and its vicinity were above the seasonal norms and the average temperature was 12.1°C. The average temperature of the spring season in 2012 was 0.1°C below with 12.1°C being slightly below the seasonal norms. Spring rainfall remained below seasonal norms and rainfall was around 130 mm.

Oocyte collection and IVM: Cattle's ovaries were obtained from a local slaughter house and transported to the laboratory in a thermos flask containing 0.9% physiological saline supplemented with 0.5 ml/L of antibiotic, at a fixed temperature of 30°C. The follicles on the ovarian surface, ranging between 2 and 8 mm in diameter, were aspirated. The aspiration procedure included 1 ml of Dulbecco's modified phosphate buffer solution (D-PBS) with 3% calf serum, 5 ml inserted into the tip of an 18-gauge needle. The resulting fluid was evacuated to a 90 mm petri dish and screened under a stereo microscope and the oocytes contained were taken to D-PBS in a 35 mm petri dish. Oocytes washed in D-PBS and evaluated for quality were taken to TCM199 culture medium. Oocytes washed 3 times in TCM199 solution were matured. Oocytes with 10% fetal calf serum (FCS) and 0.2 µg/ml or 0.8 µg/ml FSH, TCM-199 medium were incubated for 22 h. Incubation was carried out in an incubator with 5% CO_2, 95% humidity and 38.5°C heat. Maturation of oocytes after a 22 h incubation period, was evaluated according to the state of nuclear maturation and expansion of the cumulus cells.

Sperm preparation and in vitro fertilization: For fertilization of the oocytes, Holstein sperm with equivalent motility, which was frozen on the same day from a bull, was used. After removing 5 straws from liquid nitrogen, it was dissolved in 37°C water. Sperms were treated with Bracket&Oliphant (BO) medium by direct sperm washing method. It was centrifuged in BO wash solution at 1,800 rpm for 5 min. The supernatant was centrifuged again for 5 min at 1,800 rpm. In 35 mm culture petri dishes, four pieces of 100 µl BO solution were prepared and coated with mineral oil. About 18–20 oocytes were placed on each tube. Sperm was treated with a drop of 25,000 spermatozoa per oocyte. Mature oocytes were incubated with spermatozoa for 5–6 h at 38.5°C.

In vitro development: Cumulus cells around the oocytes which were subjected to fertilization for 5 to 6 h in the incubator. Oocytes were removed in 35 mm petri dish with mineral oil coated CR1aa solution for removal of the cumulus cells. Cumulus cells surrounding the fertilized oocytes were performed only by pipetting method. Oocytes fertilized after maturation were taken to incubator in mineral oil coated 100 µl Charles Rosencrans (CR1aa) culture drops. About 18–20 oocytes were placed in every 100 µL drops. Cleavage controls of the cells were performed at the 48 h of incubation. It was performed on the 7^{th} and 8^{th} day to find out whether the cells taken into the culture medium came to the morula-blastocyst stage.

Statistical analysis: The chi-square test was used to evaluate the effect of FSH and season on oocyte development. The difference of $P<0.001$ was considered statistically significant. Descriptive statistics for fertilization and embryo development rates were expressed in numbers and percentages. The Z test was used for comparisons of these properties. Statistical significance level was taken as 5% in calculations and MINITAB statistical package program was used for calculations.

RESULTS AND DISCUSSION

It was determined that, the quality of the oocytes did not differ in context of the season they were collected in ($P>0.001$) (Table 1). The effect of FSH administered and season on oocyte maturation is shown in Table 2 (Fig. 1).

In terms of embryonic growth rates, the best rates were obtained in the 0.8 µg/ml FSH in the spring season. Calculated P values for the values examined are given in Table 3 (Figs 2–3).

When the cattle were mostly fed in the pasture, the quality of the oocytes was higher and the maturation and post-development rates were better (Maia *et al.* 2017). This result is similar to the high maturation rate we obtained in the spring and the high maturation rate according to the autumn season. The number and quality of oocytes obtained from ovaries of cattle that are fed in pasture conditions are better. In the spring and autumn season, when the rates of reaching fertilization and morula blastocyst of oocytes obtained with the same dose of FSH, it was observed that the oocytes in

Table 1. Number and quality classification of oocytes obtained in different seasons

	Number obtained		Number of oocytes per ovary		Proportion of oocytes		P
	Spring	Autumn	Spring	Autumn	Spring	Autumn	
Number of ovary	111	87					
A + B quality oocytes	636	488	5.73	5.61	85.14	87.61	–
C quality oocytes	55	37	0.50	0.42	7.36	6.64	–
Degenerated oocytes	56	32	0.50	0.37	7.50	5.75	–
Total oocytes	747	557	6.73	6.40			

P>0.001.

spring showed better results. This situation shows that the oocytes are of better quality because the cattle were slaughtered after spending a long time in the pasture.

The number of oocytes obtained per ovary was lower than that previously reported by some researchers (Faheem *et al.* 2011), higher than that reported by some other researchers (Nematullah and Akther 2005, Wang *et al.* 2007). It is suggested that season has a major effect on follicular dynamics (theca, granulosa, cumulus cells and oocytes) and affects *in vitro* development (Al-Katanani *et al.* 2002). The oocyte source is reported to be the most important determinant factor for the maturation and fertilization of oocytes and embryonic development (Conti and Franciosi 2018). The poor body condition score of the cows slaughtered and the low number of oocytes collected from their ovaries are in support of this finding. The number of oocytes obtained per ovary being low in the present study may have arisen from the use of the aspiration technique alone. The number of high quality oocytes, which showed very small differences for season, was considered to be at an adequate level based on the literature reports (Nematullah and Akther 2005).

The administration of both FSH doses to the culture media of the spring oocytes and the administration of 0.8 µg/ml of FSH to the media of the autumn oocytes yielded maturation rates similar to those achieved in some previously conducted studies, in which high doses of FSH (2–5 µg/ml) were tested (Faheem *et al.* 2011) (92% and 94%, respectively), and higher than those reported in some other studies (Wang 2007, Pandey *et al.* 2009). The administration of 0.2 µg/ml of FSH to the incubation media of the autumn oocytes yielded maturation rates lower than those reported in previous studies, excluding an investigation conducted by Wang *et al.* (2007). In the present study, despite the low number of oocytes obtained per ovary, it was observed that the maturation rates achieved were, in general, high. Although FSH was added to the maturation media at doses lower than those reported in literature (Mondadori *et al.* 2008), the maturation rates achieved were higher than those reported by researchers, who used a similar technique. This demonstrated that the supplementation of maturation media with even low administration doses of FSH would produce satisfactory rates of oocyte maturation.

Table 2. Effect of the administration dose of FSH and season on oocyte maturation

Season	Dose of FSH	Number of oocytes	Number of mature oocytes	Maturation rate %
Spring	0.2 µg/ml	336	324	96.43 (324/336)[a]
	0.8 µg/ml	300	290	96.67 (290/300)[a]
Autumn	0.2 µg/ml	267	236	88.39 (236/267)[b]
	0.8 µg/ml	221	208	94.12 (208/221)[a]

Different letters within the same column are significantly different (P<0.001).

Table 3. Effect of different doses of FSH and season on *in vitro* embryo division and development rates

Comparison	48 h cleavage rate % P value	7 day morula-blastocyst rate (%) P value
0.2 µg/ml Spring – 0.8 µg/ml Spring	0.029	0.175
0.2 µg/ml Spring – 0.2 µg/ml Autumn	0.815	0.225
0.2 µg/ml Spring – 0.8 µg/ml Autumn	0.062	0.401
0.8 µg/ml Spring – 0.2 µg/ml Autumn	0.020	0.016
0.8 µg/ml Spring – 0.8 µg/ml Autumn	0.797	0.644
0.2 µg/ml Autumn – 0.8 µg/ml Autumn	0.044	0.058
Spring – Autumn	0.792	0.326

The heat stress that occurs in the summer months negatively affects the ovaries of dairy cows and the oocytes which are closed over the ovaries not only in the summer season but also in the fall season (Roth 2008, Gendelman and Roth 2012). In the autumn season, the rate of division according to the spring season and the lower rate of reaching to the Morula-blastocyst support these theses. As Gendelman and Roth (2012) say, the biggest cause of this pause is that the harmful seasonal effects induced during the Germinal Vesicle phase are passed to the following stages of embryonic development and decrease the quality of developed blastocysts. In several researches, the heat stress in summer has a very clear and obvious effect on

oocyte quality. Al-Katanani *et al.* (2002) reported that the negative effect of the heat stress continues for 42 days despite keeping cows in a cold environment in order to reduce heat stress in summer. Again, the same researchers have obtained a higher rate of division from the oocytes they obtained from April to September, compared to the oocytes obtained from October to March. In our study, the rates of cleavage obtained using the same FSH dose were higher in the spring. The lower results obtained in the fall may be due to the fact that the temperature stress is still slightly higher after summer. The rate of morula-blastocysts obtained was higher in the spring season as opposed to what these researchers had reported. The state of delayed embryo cleavage, in the later stages, slows the development of embryo development (Gendelman *et al.* 2010). Pavani *et al.* (2015) indicates that the biggest cause of this decrease as the higher rate of meiotic arrest of oocytes in telophase I stage in hotter seasons compared to oocytes in the warmer seasons. The rates of cleavage obtained and the rates of morula-blastocyst were in agreement to the results obtained by Gendelman *et al.* (2010). The data obtained from the oocyte quality related to the season and from the phases of obtaining embryos from these oocytes support previously done researches in our study (Pavani *et al.* 2015). *In vitro* bovine oocyte maturation and subsequent embryo development are known to be affected by many factors such as hormones and growth factor (Hansen *et al.* 2014). One of these factors is the amount of FSH involved in the maturation environment. The results show that even low-dose FSH in bovine oocytes in the *in vitro* maturation environment is sufficient for the optimal maturation level of oocytes in the spring, when the animals are well fed. In the autumn period, when the nutritional level is poor, it is observed that low doses of FSH could not provide the desired level of oocyte maturation. The results obtained in our study show that the season has an obvious effect on the development of *in vitro* bovine embryos.

The results obtained in the present study demonstrated that, the addition of even a low dose of FSH to the *in vitro* maturation medium of bovine oocytes proved to be sufficient for obtaining an optimal maturation level in the spring season, during which animals are well fed. This is thought to be due to the higher quality of oocytes of animals fed in pastures and not exposed to heat stress in the spring season. On the other hand, it was determined that, optimal oocyte maturation levels could not be achieved with low FSH doses in the autumn season, during which animals are poorly fed.

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