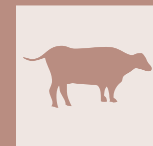
In vitro maturation of bovine oocytes may using royal jelly as protein source in the culture media



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SUMMARY

The present study investigated the effect of using royal jelly (RJ) as protein source for the culture media that would be used in the nuclear maturation stage of bovine oocytes. Bovine ovaries were collected from local slaughterhouse and then the cumulus oocyte complexes (COCs) were recovered from visible antral follicles (2 to 8 mm) by aspiration method. The obtained COCs were examined under an inverted microscope. COCs with uniform cytoplasm and homogeneous distribution of cumulus cells were selected for in vitro maturation. COCs were randomly incubated in tissue culture media–199 (TCM-199) with 10% royal jelly (10RJ, n=179) and 10% fotal calf serum (0RJ, n=172 oocytes) for 22h at 39 ºC under 5% CO_2 in humidified air at 95%. The nuclear maturation stages were determined by examining the oocytes under the inverted microscope. The proportion of oocytes reaching metaphase-I (MI) stage in the 0RJ and 10RJ groups was 19% and 20%, respectively. The rate of oocytes reaching the anaphase-I (AI) stage in both groups was determined as 2%. On the other hand, 1% of the oocytes developed up to the telephase-I (TI) stage in both groups. The maturation rate in 10RJ media (78%) was similar when compared with 0RJ media (77%). Methaphase-II (MII) stage oocytes the 10RJ media did not affect the expansion rates of cumulus cells when compared to 0RJ media. Similarly, the ratios in first polar bodies and the maturated oocytes cleaved to 2- cell 48h post activation and were not affected by the use of 10RJ in the culture media. Therefore, these results suggest that royal jelly (%10) can be used as a protein source in the in vitro maturation (IVM) of bovine oocytes. This study has shown that it will contribute to the studies to be carried out by identifying different protein sources in the in vitro maturation stage.
The present study investigated the effect of using RJ as protein source for the culture media that would be used in the nuclear maturation stage of bovine oocytes.

KEY WORDS

Bovine, oocytes, IVM, royal jelly, parthenogenetic activation.

INTRODUCTION

In recent years, while new biotechnologies have been developed that can help animal breeding, some technologies have started to spread to application areas. These biotechnologies include estrus synchronization, artificial insemination, in vitro embryo production, cloning and Multiple Ovulation Embryo Transfer (MOET). The aim of these reproductive biotechnologies is to increase the number of offspring obtained from selected males and females and to accelerate genetic progression by shortening the interval between generations[1].
The genetic progression rate of males was increased by artificial insemination. Especially in female cattle, the period between generations is longer due to the generation of one generation per year. For this reason, there is a greater need for reproductive biotechnology in increasing the rate of genetic progression by shortening the time between genetics in cattle. One of the technologies developed to obtain more than one offspring per year in females is in vitro embryo production technology[2].

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In vitro embryo production consists of in vitro maturation, in vitro fertilization and in vitro culture stages and this technology is known as a biotechnology to increase the genetic progression rate in females. While this reproductive biotechnology developed to increase the rate of genetic progress in animal breeding is being put into practice, it is also necessary to increase the efficiency of the technologies developed.
Genetic progression rate has been increased with in vitro embryo production biotechnology. It has been shown that the birth weight of calves obtained by in vitro embryo production is higher than those produced in vivo[3, 4]. With the implementation of this biotechnology, which is aimed to increase the rate of genetic progress, various negativities may occur. Due to the increase in birth weight, the incidence of caesarean section, dystocia[5] and large offspring syndrome[6] also increases. In addition, protein sources used in the in vitro embryo production process lead to the transfer of various pathogens and some agents whose effects are not fully known[7]. Therefore, in order to eliminate such negativities affecting the success of in vitro embryo production, it is necessary to determine the protein sources that will not cause such adversities in the culture environment.
For these reasons, this study aimed to investigate whether 10RJ can be used as a protein source in the culture medium for in vitro maturation of bovine oocytes.

MATERIALS AND METHODS

Ovaries and oocytes collection

The research material consists of cattle ovaries obtained from different breeds. Ovaries were placed in a salt solution with phosphate tampon (PBS) at 39 °C, which was prepared prior to the experiment, and then they were taken to the laboratory inside this solution. Intra-follicular fluid was collected from follicles 2-8 mm in diameter on the ovaries using a syringe with an 18 g needle diameter.

Oocytes maturation

COCs were examined under a microscope. A mixture of 100 ml Hepes buffered tissue culture media (HTCM-199, Sigma, M7528), 1 ml penicillin streptomycin and 10 mg L-glutamine (Sigma, G3126) was stored at +4 °C. 19 ml of this mixture was taken and 1 ml of Fetal Bovine Serum (FBS) (5% v/v, Sigma, F4135) was added on it and the temperature was brought to 35 °C. The COCs examined under the microscope were then transferred to this mixture. The COCs, which were then transferred to the oocyte search medium, were morphologically evaluated with the aid of a microscope, and only oocytes with smooth cytoplasm and non-atretic cumulus cells lining the zona pellucida and with enough cumulus cells around were selected for maturation.
These selected oocytes were washed twice in oocyte search medium. Later, two different culture media were prepared. The first of these is bicarbonate buffered culture medium with serum addition. This culture medium is mixed with 1 ml of FBS, 100 µl penicillin (50 IU / ml) -streptomycin (50 µg / ml) and 20 µl sodium puree (22 µg/ml, Sigma, S8636) into 9 ml bicarbonate buffered tissue culture medium (TCM-199, Sigma, M4530). This mixture (0RJ) has been gassed for approximately 2 hours in a humid atmosphere containing 5% CO_2 at 39 °C before use. The second culture medium is a culture medium with RJ added as a protein source. Before this culture medium was prepared, the crude protein analysis of RJ was made according to the Weende analysis method and the crude protein content of RJ was determined as 17.3%. Culture medium supplemented with RJ, 2 g royal jelly is dissolved in 20 ml Hepes buffered medium, then 200 µl penicillin (50 µl / ml) -streptomycin (50 µg / ml) and 40 µl sodium pyruvate (22 µg / ml) are added to stock solution was prepared. After taking 1 ml of this stock solution, 9 ml of bicarbonate buffered culture medium (50 IU penicilline, 50 µg streptomycin, 22 µg sodium pyruvate per ml) was added. Thus, a culture medium containing 10RJ was prepared. The using both maturation mediums (0RJ and 10RJ) were drops (45 µl) prepared. These drops are covered with 2-3 ml of mineral oil (Sigma, M8410). These drops have been gassed for 2 hours at 39 °C, 5% CO_2 and 95% humidity. Morphologically normal oocytes were transferred into drops with 10 oocytes per 5 µl. These oocytes were matured at 39 °C, 5% CO2 and 95% humidity for an average of 22 hours. 172 oocytes in the 0RJ group and 179 oocytes in the 10RJ group were subjected to in vitro maturation.
In order to remove the cumulus cells, oocytes were kept for 5-7 minutes in 1 ml Hepes buffered culture medium containing 100 Units /ml hyaluromidase (Sigma, H3506) enzyme at room temperature. The oocytes from which the cumulus cells were removed, detected 1st polar bodies under the inverted microscope. The oocytes detected as 1st polar body were transferred to each compartment of the four-well culture dishes by placing approximately 1 ml of 3: 1 acetic acid: ethanol mixture and the oocytes were fixed at +4 °C for at least 24 hours.
Oocytes were placed on a slide, stained using 10-15 µl aceto orcein and covered with coverslip. Following these procedures, prepared slides were examined under a inverted microscope. Thus, the nuclear maturation stages of oocytes were determined.

Parthenogenetic activation

Oocytes were subjected to a two-step parthenogenetic activation. In the first stage (ethanol activation), Hepes buffered culture medium (50 µl FBS and 10 µl penicillin-streptomycin per ml) was prepared. This mixture was passed through a 0.20 µm diameter filter. Later, to this mixture, 7% ethanol (152 µl, 92% absolute ethanol) was added. Oocytes were treated with the prepared solution at room temperature for 5 minutes. Following this process, oocytes were then washed two times in the culture medium with Hepes tampon. In the second step (activation with Cycloheximide and Cytochalasin-B), 10 µl of Cycloheximide and 10 µl of cytochalasin-B were dissolved in 1.5 ml of Hepes buffered culture medium (no serum and antibiotics were added, filtered). Drops of 45 µl were prepared from this mixture and they were gassed in the incubator for a certain time after they were covered with mineral oil. Oocytes were transferred to drops following activation with ethanol and incubated for 6 hours at 39 °C, in an atmosphere containing 5% CO_2 and 95% humidity. After 6 hours of incubation, oocytes were washed three times in Hepes buffered culture medium and transferred into previously prepared culture medium containing granulosa cells. Then, the oocytes were continued to be incubated at 39 °C, in an atmosphere containing 5% CO_2 and 95% humidity. The division rates of oocytes were determined at 72 hours following the activation process.

Statistical analysis

Statistical analysis of data obtained from the experiment was conducted by using ki-square method (Minitab 13.0).

RESULTS

In vitro maturation rates

The maturation rates obtained in this study conducted on the effect of adding RJ as a protein source to the culture medium on the in vitro maturation parameters of bovine oocytes are also given in Table 1. The effect of adding 10RJ as a protein source to the culture medium on the rate of oocytes reaching MII ($\chi 2 = 0.836$) was found to be insignificant ($P > 0.05$). In vitro maturation rates of bovine oocytes were found to be 77% for 0RJ group and 78% for 10RJ group.

Cumulus expansion rates

The effect of adding 10RJ to the culture medium as a protein source on the cumulus expansion of bovine oocytes is given in Table 2. The effect of adding 10RJ as a protein source to the culture medium on the rate of in vitro cumulus expansion ($\chi 2 = 0.215$) was found to be insignificant ($P > 0.05$).

Table 1 - Effect of adding RJ as a protein source in culture medium on in vitro maturation of bovine oocytes.

Culture	No of oocytes	Nuclear maturation degree (%)				Unidentified
		MI	AI	TI	MII	
0RJ (n/n)	172	20 (28/139)	2 (3/139)	1 (1/139)	77 (107/149)	33
10RJ (n/n)	179	19 (27/141)	2 (3/141)	1 (1/141)	78 (110/141)	38

Unidentified: Number of oocytes for which it is not determined at which stage of nuclear maturation.
MI: Metaphase-I, AI: Mainphase-I, TI: Telaphase-I, MII: Metaphase-II.

Table 2 - Expansion rates of cumulus cells in culture media using royal jelly and serum as protein sources.

Culture	No of Oocytes	No of Cumulus Expansion	Expansion Rate (%)
0RJ	172	167	97
10RJ	179	169	94

Table 3 - Polar body numbers that can be observed in oocytes matured in culture media using 10RJ and 0RJ as a protein source.

Culture	No of Oocytes	No of Cumulus Expansion	Expansion Rate (%)
0RJ	172	69	40
10RJ	179	69	39

Table 4 - Cleavage rates following parthenogenetic activation of bovine oocytes matured in vitro.

Culture	No of oocytes analysed	PB* number	No of oocytes divided
0RJ	117	59	25
10RJ	122	62	22

PB*: Number of oocytes for which polar bodies (1st polar body) are determined.

The impact that RJ as protein source to the culture environment, on the expansion of in vitro cumulus cells ($\chi 2$ = 0,215) created was found to be insignificant (P>0.05). Following the in vitro maturation of bovine oocytes, rates of in vitro cumulus expansion were found to be 97% for 0RJ group and 94% for 10RJ group.

Ratio of first polar body

1st polar body numbers detected in bovine oocytes matured in vitro by adding serum 0RJ and 10RJ as protein sources to the culture medium are given in Table 3. There was no difference in 1st body numbers observed between 10RJ and 0RJ groups (P> 0.05). Observable ratio of 1st polar bodies was 39% (69/179) in the 10 RJ group and 40% (69/172) in the 0RJ group.

Parthenogenetic activation of bovine oocytes

The findings obtained in this study to determine the effect of royal jelly and serum (fetal calf serum) used as protein sources in culture medium in in vitro maturation on the division rates following parthenogenetic activation of bovine oocytes are given in Table 4. The effect of adding royal jelly as a protein source to the culture medium on the cleavage rates determined at the 48th hour in granulosa monalayer co-culture following in vitro maturation ($\chi 2$ = 0.437) was found to be insignificant (P> 0.05). Following in vitro maturation, the division rate of bovine oocytes subjected to parthenogenetic activation was 36% in the 10RJ group and 42% in the 0RJ group.

DISCUSSION

In vitro maturation rates

In this study, it was determined that adding royal jelly to the culture medium had no negative effect on in vitro maturation of oocytes.

It has also been demonstrated that royal jelly can be used as a protein source in culture systems in the in vitro maturation of bovine oocytes. In most in vitro maturation studies, serum and serum albumin are used as protein sources in culture medium. Protein sources of animal origin such as serum

and serum albumin may contain various pathogens in their bodies[7]. Therefore, the use of these protein sources of animal origin in the culture medium is limited. In addition, when serum is used as a protein source in culture media, due to various abnormalities in the metabolism and development of embryos can occur large offspring syndrome[8]. For these reasons, commercial (CPRS-3 and Ultroser-G) products obtained from serum by various methods have been developed. Even when these commercial products were used as a source of protein in culture media, the results were not exactly as expected[9]. In addition, culture media without protein were developed, but the desired results were not obtained from these culture media. In this study, the use of royal jelly, which is a natural protein source, in in vitro maturation in culture medium was tried for the first time. It was observed that the in vitro maturation rates obtained in culture media using royal jelly as a protein source were similar to the in vitro maturation rates obtained in culture media using protein sources such as serum and serum albumin. In addition, royal jelly does not contain various risks such as serum and serum albumin. The in vitro maturation rates obtained in our study were similar to the in vitro maturation rates in culture media using other protein sources. For these reasons, it can be said that royal jelly can be used as a protein source instead of serum and serum albumin in in vitro maturation. In addition, the in vitro maturation rates obtained in different studies using serum as a protein source in culture media are similar to the in vitro maturation rates obtained in our study[10, 11, 12]. In summary, these results show that royal jelly can be used as a protein source in culture media.

Cumulus expansion ratios

It has been determined that there is no difference in the use of 0RJ or 10RJ as a protein source in the culture medium in terms of cumulus expansion.

Bovine oocytes are surroundered by few rows of cumulus cell masses[13]. These cells nourish the oocyte and provide key products for it to develop. The small number or absence of the cumulus cell mass surrounding the oocyte has a negative effect on the embryo after fertilization[14]. The expansion of the cumulus cells, which is an indicator of the maturation of the oocyte, is seen at the 18th hour of the culture, and this expansion has a significant effect on the oocyte reaching MII[15]. In our study, since it was determined that bovine oocytes matured in culture media using serum and royal jelly as protein sources have similar cumulus expansion rates, it has been an indicator that royal jelly can be used as a protein source in culture medium instead of serum in vitro maturation. In addition, it was observed that the cumulus expansion rates obtained in studies using serum as a protein source in culture media were lower than the cumulus expansion rates obtained in our study[16]. These results showed that it is possible to use royal jelly as a protein source in culture medium in terms of cumulus expansion, which is effective in the development of oocytes to the maturation stage.

Number of first polar body observable

It was determined that the use of 0RJ or 10RJ in the culture medium did not cause a difference in terms of polar body number.

The oocyte undergoes a number of structural changes as it progresses towards the MII stage[17]. One of these structural changes is that the 1st polar body becomes apparent. The 1st polar body usually forms between 18-21st hours of in vitro maturation[18]. Some changes seen in the oocyte maturation process are related to the time required for the release of the 1st polar body. Chromosomes localize to the peripheral space following the release of the first polar body[15]. Following this process, chromosomes once again flow towards the dense areas and develop towards the MII stage. The ratios of 1st polar body obtained in a study using serum as a protein source in the culture medium are similar to the rates obtained in our study[19]. Considering the number of 1st polar bodies detected in our study, it has shown that royal jelly can be used instead of serum as a protein source in the culture medium.

Parthenogenetic activation of bovine oocytes

It was determined that bovine oocytes matured in culture media using 0RJ and 10RJ as protein sources had similar division rates following parthenogenetic activation. The resumption of development of oocytes waiting at the MII stage is provided by fertilization or parthenogenetic activation[20, 21]. Oocyte activation is regulated by specific calcium signal[22]. It was determined that oocytes matured in culture media using royal jelly as a protein source and oocytes matured in culture media using serum as protein source have similar division rates. The division rate obtained in a study[23] conducted on the activation of bovine oocytes was found to be similar to the division rate obtained in our study. As a result, it has been shown that the addition of royal jelly as a protein source to the culture medium in the in vitro maturation of bovine oocytes can be divided up to 2-cell and later stages as in the serum addition. According to the results obtained, it has been shown that in vitro matured oocytes can be divided as in vitro mature oocytes in culture media where serum and serum albumin are used as protein sources in culture media where royal jelly is added as protein source.

CONCLUSION

In conclusion, the successful maturation of bovine oocytes in culture media using royal jelly instead of serum and the detection of their cleavage following parthenogenetic activation is a first in this field. It is necessary to investigate the growth potential until the blastocyst stage and pregnancy capacity of oocytes that are matured using royal jelly as a protein source in the culture medium.

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