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Research Article

EFFECTS OF DIFFERENT INSULIN CONCENTRATIONS ON IN VITRO MATURATION OF BOVINE OOCYTE IN THE PRESENCE AND ABSENCE OF CUMULUS CELLS

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A B S T R A C T

This study investigated the effect of various insulin concentrations on the maturation of Bovine oocyte with cumulus and stripped cumulus. In the cumulus group, aspirated oocytes had at least three layers of cumulus. The oocytes were collected from 2-8 mm follicles of the bovine ovary from a slaughter house and transferred to the maturation media after being washed three times in the rinsing medium. The oocytes with and without cumulus were divided into two sets of four groups including the control group, which did not receive any insulin, and groups receiving 0.1, 1, and 10 µg/ml insulin. The maturation media were incubated at 38.5°C with 5% carbon dioxide for 22-24 hours. After incubation, the cumulus around the oocytes was removed in the cumulus-containing groups and evaluated for polar body expulsion. The analysis showed that the oocyte maturation in the groups containing higher concentrations of insulin was significantly higher. Also, in the group where oocyte was kept in the culture medium with cumulus cells, the maturation was significantly higher than the group without cumulus cells. Finally, it can be concluded that increased insulin concentration in the presence of cumulus cells can improve the efficiency of oocyte maturation medium in dairy cattle.

INTRODUCTION

Assisted reproductive technology in animals and humans have dramatically improved over the last few decades. These technologies are used to achieve genetic advances and accelerate the process (Noakes, 2009). Oocyte retrieval and in vitro embryo production are possible for cows, non-pregnant heifers, and pregnant cows, cows up to 110 days pregnant, cows that do not respond to Follicle stimulating hormone for superovulation after birth, cows with a history of poor response to superovulation treatment, cows with reproductive problems such as acquired fallopian tube obstruction, dying cows, and slaughter house cows.

The in vitro embryo production systems are the solution to the genetic progress of high-capacity cows. Many advances have been achieved since the production of the first calf in the laboratory in 1982 by Bracket *et al*. (Farin *et al*., 2007). In vitro production of embryos in cows includes four stages of oocyte retrieval from the ovarian follicles, in vitro oocyte maturation, in vitro oocyte fertilization, and probable zygote cultivation to morula or blastocyst stages.

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The first study on oocyte maturation was carried out around 80 years ago by Pincus *et al*. in rabbits in 1935 and in humans in 1938. Since then, many studies have been conducted in this regard (Kane, 2003). Advances were to the extent that while less than one percent of the in vitro maturated oocytes underwent developmental stages in the 1970s, today 40% of them do. More than a decade of research in the 1990s has determined that despite the use of extensive techniques, many bovine oocytes have defects in the development of embryonic stages after in vitro maturation (Kane, 2003).

There are several methods for collecting follicular oocytes, including laparoscopy, oophorectomy, collecting of ovaries from the slaughter houses, and vaginal ultrasound and ovumpickup (OPU). In the slaughter house method, oocyte can be extracted from ovaries of an animal only once, but in the OPU, which is a non-invasive method, oocytes can be retrieved from the cattle several times. OPU is used in humans, too (Van Wagtendonk-de Leeuw, 2006)

Given the increase in embryonic manipulations and in vitro fertilization of animals, extensive studies have been conducted in this regard. Therefore, many studies focused on the effectiveness and competency of oocytes or embryos (Kane, 2003). The achievement of a viable competent oocyte that is capable of development is important in the development process of in vitro fertilization, embryonic culture, and

reproductive technology (Parks and Ruffing, 1992). Over the past three decades, the focus of studies on mammalian growth processes has increased human knowledge about the routine physiological events during fertilization, growth and early embryo development, in which farm animals were the major models. The result of these studies has finally led to the formulation of different culture media and conditions for culture and transfer of embryos (Shabankareh *et al*., 2012). Many studies have used the combination of insulin with thefollicle-stimulating hormone in maturation culture medium for the growth ability of oocytes with cumulus.

Insulin is a peptide hormone produced by the cells of the islets of Langerhans (Lumelsky *et al*., 2001). Insulin is an important anabolic hormone in cows. The secretion of insulin is stimulated through the intake of food and increased free fatty acids and amino acid concentrations. Increasing food intake increases blood insulin levels (Freret *et al*., 2006). Insulin stimulates the uptake of nutrients by cells, as well as the production of protein and triglycerides (Oskam *et al*., 2004). The plasma insulin level depends on the follicular/luteal phases and varies between 0.1 to 10 ng/ml. The concentration of most metabolites in the follicular fluid is similar to or lower than their plasma concentration, which results in the production of follicular fluid as a result of the plasma filtration. The measured amounts in the follicular fluid are 0.1 to 10 ng/ml.

The level of follicular fluid insulin level is consistent with that of the plasma, which indicates the systemic effect of nutrition and subsequent insulin levels in the ovaries (Spicer *et al*., 1995). Insulin levels decrease during the first week after delivery because of a negative energy balance. It takes an increasing path after 2-7 weeks due to the body's performance in order to overcome the negative energy balance. Hence, insulin levels can be used as a useful indicator of the body performance (Fratrić *et al*., 2013). Therefore, using factors that affect oocyte maturation such as insulin can be effective in improving in vitro culture systems.

MATERIALS AND METHODS

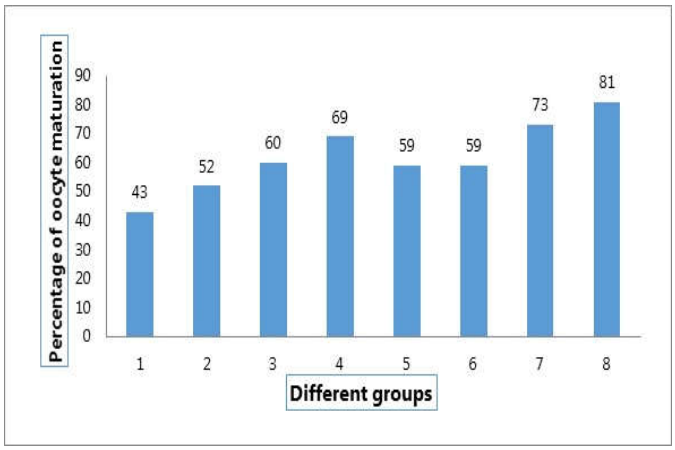
This study was conducted on slaughterhouse cow ovaries. All ovaries were collected from a local slaughter house by an expert. The ovaries were transferred to the lab in flasks containing normal saline (9 g of salt in 1 liter of water), antibiotic 100 IU/ml, penicillin 100µg/ml, streptomycin sulfate, in less than 2 hours at a temperature of 35°C. Tissue fragments were removed from the ovaries. The appropriate ovaries were selected for oocyte collection with cumulus oocyte complexes (COCs) and oocytes without cumulus. The ovary was placed in a beaker, and then a 10 ml syringe (needle gauge 18) containing aspirating fluid (containing 1 ml heparinized solution of H-TCM+10%FBS, which was previously placed incubated at 38.5°C for 20 minutes) was used to slowly aspirate follicles with a diameter of 2 to 8 mm in ovarian cortex and poured in 50 ml falcon tubes from each ovary. They were then transferred to water bath for 20 minutes. After removing the COCs-containing falcon tubes from water bath, the COCs were separated from the follicular fluid by removing the waste tissue and cells contained in the suspension. The sediments of the falcon tube were transferred into a dish using a Pasteur pipette, observed under a stereo microscope with proper magnification, and examined. A pipette was used to gently separate COCs with uniform cytoplasm and at least a few layers of cumulus cells. They were washed in a dish containing 200µl/droplet of H-TCM+10% FBS medium, with sodium pyruvate coated with mineral oil to remove heparin. They were washed at least 3 times). In order to perform the maturation stages, COCs were washedwith mineral oil to remove H-TCM in dishes containing 200µl/droplet of maturation medium, sodium pyruvate, Gentamicin, BSS, NAHCO3, ESTRADIOL, FOLLTROPIN, and TCM199.

Each 6-8 washed oocytes in the maturation medium were transferred into dishes containing 100 µl of maturation medium with various concentrations of insulin (0, 0.1, 1, 10 mg/ml) covered with mineral oil. They were then incubated at 38.5°C with 5% CO2 and 95% humidity for in vitro maturation stages for 22-24 hours. The cumulus cells surrounding the oocytes separate 22-24 hours after culturing COCs in the maturation medium. This is one of the symptoms of oocyte maturation. A total of 980 oocytes were retrieved in this study, which were randomly assigned to 8 groups. Group 1 was the control group, which received no substances had no cumulus cells. In the groups 2, 3, and 4, the oocytes had no cumulus cells and received 0.1, 1, and 10 mg/ml of insulin, respectively. Group 5 was the COC group, which had cumulus cells and no insulin.Groups 6, 7 and 8, had COCs, in which different concentrations of 0.1, 1, and 10 mg/ml were added to the medium, respectively. Twenty-four hours after culturing the oocytes, the cumulus cells were expanded. The oocytes were examined by an inverted microscope for comparison of different treatments, the amount of dispersion, and growth of the cumulus layers. Changes were calculated (Fig1). Twenty-four hours after the culture ofimmature oocyte in the maturation medium, the culture dish was removed from the incubator and examined under a stereo microscope. In order to prepare maturated COCs for the assessment of maturity level, first, the maturated COCsmust be separated from high concentrations of sialin and mucin, as well as from fallen and extra cumulus cells. This step involves the transfer of maturated COCs from maturation container to a washing dish containing 80µl/droplet of hyaluronidase. Moving the oocytes drop by drop using a mouth pipette in the washing media and the effect of hyaluronidase remove the cumulus layers and prepares the oocytes for maturation assessment. The perivitelline space can be detected in well-matured oocytes. The polar body is also detectable in case of a complete maturation (Fig 2). Vacuolated oocyte cytoplasm indicates fluctuations in the medium pH or the presence of toxic agents in the medium. After assessment of oocytes with detectable signs of successful maturation, they were counted and statistically evaluated.

Table 1 Percentage of oocyte maturation.

Percentage of oocyte maturation	Number of oocytes	Groups
43	132	1
52	123	2
60	117	3
69	118	4
59	132	5
59	123	6
73	117	7
81	118	8

Figure Legends



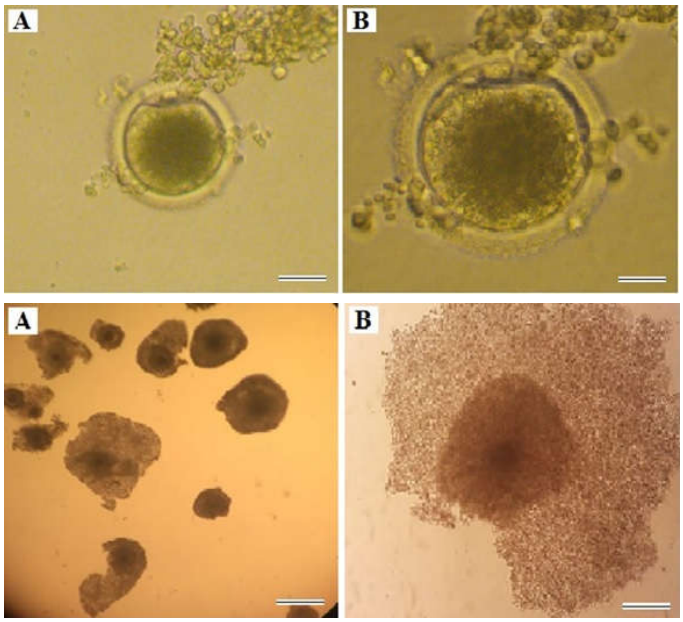


Fig 1 Cumulus oocyte cells mass expanded after 24 h of culture (The size of the scale bar in image A is 400µm and in image B is 200 µm).
Fig 2 Mature oocytes with polar body and detectable perivitelline space. (The size of the scale bar in A image is 50µm and in B image is 25 µm).
Fig 3 Percentage of oocyte maturation.

RESULTS

The oocyte maturation was assessed microscopically, and oocytes were classified into mature and immature groups after examination. The results were recorded first manually and then in the computer. All data were statistically analyzed in SPSS-21 software using the ANONA test. The differences between groups were assessed using Tukey and Duncan tests. Table 1 shows the percentage of oocyte maturation in groups 1 to 8. Fig 3 shows the significant difference between group 4 (10 µg/ml) and groups 1 (control) and 2 (0.1 µg/ml) that had no cumulus. It also shows the significant difference between group 8 (10 µg/ml) and groups 5 (control) and 6 (0.1 µg/ml) that had cumulus.

The results of this study in different groups showed that if the set of groups 1 to 4 which were naked, and the set of groups 5 to 8 which had COCs are examined separately, it can be concluded that among the naked groups, group 4 (10 mg/ml) had the highest maturation, such that it was significantly different from groups 1 and 2. Thus it had the best results. Therefore, the highest maturation rate in naked oocytes was obtained at an insulin concentration of 10 µg/ml. The results in the second set of groups including groups 5 to 8 which had COC showed that group 8 (10 mg/ml) had a significant difference between with groups 1 and 2 (Table 1). Therefore, in this set, the highest rate of maturation was in the group with the highest insulin concentration. Comparing both sets of with and without COC, it can be concluded that the set with COC is generally higher in maturation, regardless of its significant difference with the groups without COC. In both sets, the groups with a concentration of 10 µg/ml (groups 4 and 8) had a significant difference compared to the control groups of their own set and groups 1 and 5 (Fig 3). The results of this study showed that oocytes with COC in the presence of 10 µg/ml of insulin can be used to optimize bovine oocyte maturation media in order to obtain acceptable results compared to other concentrations.

DISCUSSION

In vitro maturation and fertilization of the oocyte is one of the methods of assisted reproductive technology, which has created a major evolution in reproductive science (Mota, 2013). This study investigated the effects of different concentrations of insulin in TCM199 culture medium and bovine serum in the presence and absence of cumulus cells on the amount of in vitro oocyte medium. The results of this study showed that the use of high insulin concentrations (10 µg/ml) and oocyte containing cumulus result in proper oocyte maturation. The results of this study showed that the presence of cumulus cells increased the rate of germinal vesicle oocytes reaching the MII phase. Although the germinal vesicle oocytes without the cumulus have the potential to reach the MII phase, other studies have shown that the germinal oocyte with COCs has more potential to reach the MII phase, fertilization and fetal development compared to the germinal vesicle oocyte without COCs because the presence of a gappedjunction between the oocyte and the cumulus cell is necessary for the transfer of essential substances for the development and fertilization of the oocyte (Trounson *et al*., 2001).

The presence of cumulus cells induces meiosis and cytoplasmic maturation of the oocyte, which is essential for the development of the fetus after fertilization (Trounson *et al*., 2001). In this study, the maturation rate in oocytes with COCs was higher in groups with 10 µg/ml of insulin, suggesting that insulin applied its effect through COCs. That is due to the presence of some insulin receptors on COCs.In 2005, Purohit *et al*. (2005) reported increased oocyte maturation in the presence of growth factor, too.

The complex culture medium of TCM199 is buffered with bicarbonate or HPES, supplemented with different serums, gonadotropins or steroid hormones, which is widely used to study bovine oocyte maturation. Insulin is used in a variety of media, such as the maturation culture medium, ovarian and follicular culture medium, and intracellular fertilization culture media. The results of the studies on various insulin concentrations in thedifferent culture media of antral follicles in different animal species are as follows. Aguiar *et al*. (2016) found that insulin improves the follicular growth and survival of in vitro culture of thepre-antral follicle of thehorse, and reduces oxidative stress.

Lindgren (2014) examined the effect of insulin during oocyte maturation in vitro on bovine early embryo development and showed the destructive effect of insulin on the growth and

development of blastocyst compared to the control group without insulin, which contradicts the results of the present study on the maturation of oocytes with and without COCs. This might be due to the excessive amount of insulin in the culture medium and its consumption by the oocyte and embryos. Furthermore, according to the Lindgren report, insulin-treated groups tended to have more initial growth stages, and insulin damaged the blastocyte growth during the onset of in vitro maturation on days 7 and 8. Regarding the IVM efficacy, it has been observed that 30 to 40 percent of the in vitro maturated and fertilized bovine oocytes reach the blastocyst stage. In fact, using the IVM technique, more than 60 percent of the oocytes reached the MII phase and then released the first polar body (Lonergan *et al*., 2003). Although the cultivation of embryos after fertilization is the most important period affecting the quality of blastocyst, the maturation and fertilization conditions of oocyte should not be ignored. Indeed, before IVM becomes a principle and efficient method, the major problem is managing the immature oocytes, that is, until the development of the IVM culture systems, there will be differences between the in vitro and in vivo maturated oocytes (Piquette, 2006). Mota(2013) examined the effects of insulin on the reduction of the oxidative gene expression in the bovine oocyte maturation medium and found that insulin causes these genes, which was consistent with the present study. Insulin had also increased the maturation rate of bovine oocytes. It can be concluded that insulin decreases the destructive and stressful effects in the culture medium. The effects of human and animal insulin were examined on the maturation rate, fertilization and division of bovine oocytes in vitro. Studies have reported that adding human or bovine insulin increases oocyte maturation and subsequently fertilization and division. The comparison with this study is possible due to the similar concentrations and media, and our results were consistent with this study indicating the effect of insulin on maturation rate.

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