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Effect of Embryo Holding on Bovine Oocyte Maturation outside the Incubator

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Maintenance of immature oocytes before the onset of puberty help to do next manipulation. The aim of study was to use Embryo Holding with and without the use of the incubator for transfering oocytes to the laboratory and examining effects on oocytes maturation. Cow ovaries were collected from slaughterhouse animal and 6 groups (EHT) of them were replaced on the Embryo Holding (Syngro@) and kept in darkness in a styrofoam container with thermo packs at 4°C, 22°C, 38.5°C for 12, 24 hours (EHT-12h, EHT-24h).One group (C_1) was replaced on the standard medium IVM. After staining , the highest percentage of oocytes maturation was 92/2 % in the C_1 while it had significantly different from all groups EHT (Pd"0/05). In the EHT-12h numerically had the highest percentage of maturation in 22°C (80%) and they didn't have significant difference with groups EHT-12h (P>0/05). In EHT-24h, the highest percentage of oocytes maturation were in 38.5°C (54.3%) and they had statistically significant difference with 4°C (Pd"0/05). Embryo Holding media in our study showed that can be helpful for maintenance of immature oocytes until 12 hours at room temperature and without incubation.

Keywords: Embryo Holding, Bovine, Oocyte, Maturation, Incubator.

IVP (Invitro Production) is performed to transfer, freeze and other technologies such as cloning, transferring gene from an animal to other animals and also maintaining a genetically superior animals. Invitro production in cattle has four steps which included: taking oocytes from follicular, oocytes maturation in the laboratory or IVM (Invitro Maturation), IVF (Invitro Fertilization) and IVC (Invitro Culture). Obtaining the oocyte and embryo production is used in non-pregnant cows and heifers, pregnant cows to day 110 of pregnancy, cows that do not respond to injections of FSH for superovulation and also cows with reproductive problems such as blockage of the fallopian tubes in the lab[1].

IVF related problems can be cited polyspermia at the time of IVF, large loss of embryos 35 days after embryo transfer that most deaths related to lack of development in allantois[2]. The most serious problem in IVF will be large size and abnormality of fetus. In this regard, there are some reports indicate respiratory problems, sudden death near the birth of calves and disinterested with regard to breastfeeding[3].

The total number of embryos produced by IVP in cattle to 2011 was equal to 453 471 pcs which included embryos from oocytes obtained by ovum pick-up (OPU) and slaughterhouses[4]. The number of oocytes taken from cows ovaries can be affected by genetic, diet and weather conditions[5,6].

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In a new study has emphasized the role of nutrition in the number of oocytes, embryo quality of their[7,8].

There are several ways to collect follicular oocytes that are laparoscopy, surgical removal of ovarian, ovaries collected from slaughterhouse and Ovum Pickup(OPU). For the first time in 1987 in Denmark was raised guiding ultrasonic and ovarian follicular aspiration[9]. In 1988, in Netherlands was the first real OPU[10].

Generally maintenance of immature oocytes before the onset of puberty helps to do next manipulation[11]. Also culture in the maturation inhibitors such as roscovitine and butyrolactone I can be effective in keeping bovine oocyte in germinal vesicle stage[12,13], but it may be reduced blastocyst development[14]. These medias can be toxic if used long. So it is necessary to used in shorter time or environments that Have the least impact on cytotoxicity and best effect in oocyte maintenance[11].

According to the above-mentioned, there is a need for using the environment to maintenance and preservation of oocytes in the process of obtaining oocytes and sending it to the laboratory for IVF and embryo transfer, in the short and long distances. Despite the losses and damage caused by the media holding it is advisable to use them for storing oocytes. So it should be used media holding where have minimal damage and negative impact on the process of sampling and sending to the laboratory for completion. On the other hand it is possible to use accessories like incubators in the process of doing cell work. While the operation is performed on the farm, it is not possible completely. In this research we decided to use Embryo Holding (Syngro@) with and without the use of the incubator for oocyte transfer to the laboratory and examining the positive and negative effects on oocytes.

MATERIALS AND METHODS

Oocyte Recovery

Cow ovaries were collected from slaughterhouse animal and were transported to the laboratory less than 1 hour. Oocyte - Cumulus complex were aspirated of follicles 2-6 mm size by needle 18G and syringe. PBS supplemented with penicillin and 0.2% 0 BSA were used for storage the recovery procedure [11].

Design of Experiments

Oocyte - Cumulus complex were classified into 7 randomized groups. 6 groups (EHT) of them were replaced on the Embryo Holding (Syngro@_ USA) and kept in darkness in a styrofoam container with thermo packs at 4°C, 22°C, 38.5°C for 12, 24 hours (EHT-12h, EHT-24h). 1 group was replaced on the standard medium IVM.

IVM (Invitro Maturation)

Maturation medium consisted of TCM-199 with Earle·s salt supplemented with 5% estrous cow serum, 3.5 µg/ml FSH, 3.5 µg/ml Hcg (Sigma-Aldrich) and 1 µg/ml estradiol 17². Maturation medium was carried out under mineral oil at 38.5°C in 5% CO_2 for 22 hour[11]. Treatment groups were transferred to the IVM media (mentioned above) after the desired time the Holding.

Determination of Oocyte Maturation

Oocytes of each group were stripped of cumulus cells in working medium containing hyaluronidase (0.01% w/v) under mineral oil and were evaluated for the presence of a polar body under the stereomicroscope. Then oocyte will be fixed in 4% formaldehyde and stain with Hoechst 33342 (Sigma-Aldrich) for their nuclear maturation status[11].

Recording and Analysis of Data

The data was transfered to *spss:19.* The normality of distribution of maturation was checked using tests of normality. One-way ANOVA was used to compare dependent variable (maturation) in different groups with *P* d" 0.05 considered to be significant.

RESULTS AND DISCUSSION

According to the results in table.1, the highest percentage of oocytes maturation was 92/2 % in the control group (C_1) while it had significantly different from all groups experiment (P≤0/05).

EHT-12h numerically had the highest percentage of maturation between treatment groups in 22°C and 38.5 °C (80%,79.5%) and they didn't have significant difference (P>0/05). Also the lowest percentage of oocytes maturation was in 4°C (71/6 %) which had no statistically significant difference from EHT-12h-22°C and EHT-12h-38/5°C (P>0/05).

In EHT-24h, The highest and lowest

percentage of oocytes maturation was in 38.5 °C and 4°C (54.3% vs. 38%) and they had statistically significant difference (P≤0/05). Also, there was no statistically significant difference between EH-24h-4°C with EHT-24h-22°C and EHT-24h-22°C with EHT-24h-38/5°C (P>0/05).

According to table.1 , the highest percentage of immature oocytes and degenerated was observed in EHT-24h-4°C (62%) which had difference statistically significant with C_1 group (7.8 %, P≤0/05). Statistical analysis showed that oocytes maturation in EHT-12h was more than EHT-24h and there was significant differences (P≤0/0001).

The aim of this study was maintenance of oocytes obtained from slaughterhouse in the Embryo Holding (Syngro[@]). As it is clear because of the distance and time from the slaughterhouse to the laboratory , oocytes obtained are changed that can reduce the maturation and ultimately reduce the rate of blastocyst. As well as the purchase and transportation incubator that can keep the oocytes is expensive and hard. Therefore, there is the need for beneficial holder media without the need for incubator.

In the past, various studies have been done to maintain the immature oocytes. In a study by using Butyrolactone I (B) as meiosis inhibition could block meiosis for 24 hours and then compered the oocytes maturation with the control group after IVM. The results showed that 95% of oocytes that were blocked in the first stage, progressed to metaphase II and had no difference from the control group. It also stated that this inhibitor can be used for storing oocytes before IVM. The results are listed in study was not consistent with our results in 12 hours holding at 4°C, 22°C and 38.5°C. But in a last study the rate of blastocyst in treatment group (B) was less than the control group[13].

Ponderato and et al.[16], showed that the use of different doses of Butyrolactone I (B) and Roscovitine (R) have different impact on block meiosis and progressed to metaphase II. Of course, they thought that high doses can have toxic effects on the oocytes.

Table 1. Determination of oocyte maturation after EH treatment (n total = 721 oocytes)

Temperature	Time (h)	n	Mature (%) Telophase I & Metaphase II	Imature & deg. /without chromatin (%)
Embryo holding (EHT) 4 °C	12	102	73 (71.6)$^{a\times}$	29 (28.4)
	24	100	38 (38)$^{\alpha\times}$	62 (62)^{a}
22 °C	12	106	85 (80)$^{a\times}$	21 (20)
	24	91	35 (38.5)$^{\alpha\times}$	56 (61.5)
38.5 °C	12	102	81 (79.5)$^{a\times}$	21 (20.5)
	24	105	57 (54.3)$^{z\times}$	48 (45.7)
Control (C1)	-	115	106 (92.2)$^{b}_{*}$	9 (7.8)^{b}

a vs. b, × vs. $_{*,}$ α vs. β, are statistical difference

Adona and et al.[13], used B and R to suppress and control the acceleration meiosis. Given that these substances can increase the speed of nuclear maturation after the transition to media maturation, so they were added to the TCM-199 with low doses and used for the maintenance of immature oocytes. Before starting IVM in zero hour, in the group which only B was added GV stage was to maintain a higher percentage than the group BR (100% vs. 89%, P≤0/05). They concluded that group B had less effective in blocking meiosis. Also treatment groups with B and BR showed more speed to the resumption of meiosis in maturation than the control group.

Our study showed that maintenance of immature oocytes in the EH, for 12 hours at temperatures 4°C, 22°C, 38.5°C made maturation to 71.6%, 80% , 79.5% after IVM which is significantly different from the control group (92.2%, Pd"0/05). Among temperatures tested, 22°C and 38.5°C have similar results during the 12 hours maintenance. But 22°C (room temperature, no incubator) is preferable to 38.5°C (with incubator). In three temperatures, 4°C has the lowest rate of oocyte maturation which could be due to the bovine oocyte sensitivity to temperature reduction. The

results also showed that maintenance of immature oocytes in EH for 24 hours at 38.5°C showed 54.3% maturation that compared with EHT-24h-4°C and EHT-24h-22°C was higher (38%, 38.5%) and compared with control group was so lower (92/2%, P≤0/05).

In 2008, Alm et al.[11], expressed a new idea while without the use of inhibitors meiosis kept immature oocytes at room temperature for 18 hours then entered maturation media. Holding used in their study was 40% TCM-199 with Hanks' salts, 40% TCM-199 with Earle' s salts 25 mM Hepes and 20% FBS (fetal bovine serum). After the transition to maturation media cheched chromatin status at different times. At zero hour, there was no statistically significant difference between treatment groups and control in maintaining GV stage. (79.3% vs. 87.5%, P>0.05). Alm's results had significant differences from this study. Nuclear maturation in this study in EHT-12h, at 4°C, 22°C,38.5°C (71.6%,80%,79.5%) was higher Alm's results at 14 hours holding in room temprature. Therefore, holding media we used has better maturation after IVM while in Alm's results maintenance of immature oocytes up to 24 hours had better maturation than our study for 24 hours at 22°C (69% vs. 38.5%). The percentage of nuclear maturation in EHT-24h-38.5°C was less than Alm's study (54.3% vs. 69%).

As mentioned, few studies have been carried out for immature oocytes maintenance before the IVM. In some cases, oocyte freezing was used as a quick method for long term storage of oocytes, but greatly reduced the development of cow's oocytes.[16,17]Oocyte aging process reduced the activity of MPF (maturation promoting factor), spontaneous activation causingcortical granule exocytosis , granules movement disorder, changes in mitochondrial function and ATP, zona hardening and apoptosis. Finally decline its growth and development the effect.[18,19]

Studies in mouses showed that temperature affected the protection and authority of the metaphaseII, and the right temperature to preserve mature oocytes in mouse is 25-27°C[11]. In addition there are various differences between species in sensitivity to store oocytes between cattle and mouse. Cytoplasmic features and microtubules lipid affect between two species[21]. Microtubules have a key role in the mobility and distribution of mitochondria in the cytoplasm. They are the main structure of meiotic spindle in oocytes.[22] Studies showed that bovine oocyte serves more sensitive to reduce the temperature by virtue of having large amounts of fat compared to mouse. In the present study we were able to record 71.6% maturation in EHT-12h-4°C. In this study was observed less impact of cold on oocytes and can be considered as one of the advantages of embryo holding.

The groups EHT-12h-22°C (80%) and EHT-12h-38.5°C (79.5%) had more maturation than 4°C (71.6%). We concluded that using EH for immature oocytes maintenance at 22°C and 38.5°C during 12 hours coused acceptable maturation in oocytes.

The researchers argued high phosphate levels involved during the cells of PH and calcium homeostasis. Therefore, the use of TCM-199 is superior to PBS due to lower phosphate levels. However, inhibitors meiosis like B and R coused maintenance of GV stage but high doses can have toxic adverse effects on the oocytes and ultimately the rate of blastocyst.[11]

CONCLUSION

EH media in our testing showed that phosphate and harmful substances can be helpful for maintenance of immature oocytes until 12 hours at room temperature, but most importantly, there is no need for incubation in experiment.

It is good to be considered IVF treatment and the rate of blastocyst at the time listed in other studies while we did not discuss it.This results in the future could be a great help in the process of IVM, IVF and embryo production.

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