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Maturation, Fertilization and Implantation of Cattle Ova Cultured in Different Media

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Abstract: Several experiments have been conducted to study factors affecting the maturation and fertilization of the cattle ova in the laboratory. Ovaries were collected from 77 cow reproductive systems in Al- Shuala Massacre- Baghdad. Oocytes have been obtained from these samples by OCRC method and finishing. Oocytes were classified into several grades from both methods, and then oocytes class A and B only were chosen for maturation, where oocytes were incubated in multiple media including; TCM-199, MEM, SOF, SOF+BCS, SOF+BSA in incubator 5% CO2, 90% humidity at 38.5ºC temperature for 24 hours. Semen has been obtained by extracting of the epididymal tail, and then collected by using a sterile 5 ml syringe containing MEM media. Sperms were incubated with mature oocytes at a concentration of 1 × 10 6/ml of the cultivated media in the incubator for 30 hours for the fertilization and to obtain on zygotes. Zygotes were then isolated in the growing media. The developing embryos were monitored every 24 hours and up to 9 days until the reach blastocyst stage. Results indicate the possibility of obtaining embryos in vitro fertilization in an average 6.31% (111/1604), and the best media was by via using SOF + BCS media. The study also determined appropriate technologies to enhance in vitro fertilization. ORCS method showed a positive effect on increasing the rate of embryos production in vitro.

Keywords: Slicing, ORCS, TCM-199, MEM, SOF, In vitro fertilization

Introduction

For the importance of culture media in most livestock reproductive techniques, many researchers have sought to find a culture that contains most of the necessary elements for the development and maintenance of the oocytes and sperm. Thus, they added multiple sources including; protein, hormones, and sugar, to the media to create an optimal environment for the oocytes and sperms and subsequently obtaining a high quality of embryos (Son et al., 2008). The media composition is the major factor that maintains oocytes and then achieves of the maturation and fertilization(Alofi and Alhimaidi 2004).Several media, including; TCM-199 (Kharche et al., 2006; Amer et al., 2008), SOF (Gandhi et al., 2000), and MEM (Ravindranatha et al., 2001) have been used for oocytes maturation in mammals. Many significant additions that are added to the growing media to contribute in oocyte maturation and fertilization in an environment similar to the living body. These additions help in oocyte development because they contain essential materials for the process of oocyte maturation (Chiamenti et al., 2009). The IVF culture also should be useful in providing sperm the needed movement and adaptation, which eventually leads to its union with the ova and then the beginning of embryonic development (Mahoete, 2010).

In general, the rate of successful embryos in vivo is higher than in vitro fertilization, suggesting that embryonic production conditions are still not at the optimal level for embryos growth and development. Therefore, many studies and efforts have been conducted to improve the capability of fertilized oocytes for growth and development (Angela, 2004). Therefore, noted Duran, (1998) to the importance of presence media close to the

natural environment (in vivo) that provides suitable conditions for the growth and development of the zygote. Several implantable media such as SOF, CR1, and KSOM have been developed specifically for the cultivation of rat, sheep and cow embryos (Lawitts and Biggers 1991). Recently, successive media have been used for the oocyte maturation and fertilization on the basis that in vivo that provides required nutrients for the embryo development and regulate embryo transport to the uterus (Gardner, 1999). Nowadays, many media are available with some differences between their composition that can be adapted to the needs of laboratory or researcher. SOF media is an ideal example of adapting media from its original form (Takahashi and First 1992); Gardner et al., 1994; Holm et al., 1999; Sinclair et al., 1999 ; Gandhi et al., 2000).

Method

The study was conducted in the Laboratory of Graduate Studies / Department of Surgery and Obstetrics / Faculty of Veterinary Medicine / University of Baghdad, for the period from Feb. 1, 2016, to Jun. 1, 2016. The experiment was repeated three times, which 149 bovine ovaries were randomly collected from 77 slaughtered cows in the slaughterhouse. The reproductive status of these cows was unknown before the slaughter, but they were at different stages of age.

Ovaries Collection

Reproductive organs collected from Al- Shuala Massacre- Baghdad. They placed in a cooling box containing a brine solution at 35-33 ° C, and transferred within 1-2 hours to the laboratory. The ovaries were collected from these organs by sterile scissors and cleaned from all the surrounding ligament tissues. All were washed for the first time in a protective phosphate solution (PBS،P-5493،Sigma) to remove blood clots and remaining dirt, then wash twice with SOF media, and finally put them in a sterile glass container containing the SOF, Nystatin and Penicillin Streptomycin. Oocytes were collected from ovaries in a biosafety cabinet via the following methods:

ORCS and Slicing

The ovaries were collected by long and sterile forceps, placed in a PETRI medium containing 10 mL SOF. The surface of each ovary was sliced with a sharp blade. The ovaries then transferred to a sterile glass container containing SOF, Nystatin and Penicillin Streptomycin. All contents were transferred to test tubes and spun them for 5 minutes in centrifuge 1200 cycle /minute. Remove all floating fluid from the tubes and the deposited contents were transferred into PETRI dish, containing 5 ml culture media. Oocytes were assessed under the microscope to select mature oocytes to conduct our research.

Oocytes Evaluation

Collected oocytes were examined via inverted microscope, and the quality of oocytes was classified according to into Wani et al., (2000) as Good (Class A), Fair (Class B) and Poor, based on of cumulus cells and cytoplasmic homogeneity.

Oocytes Maturation

After collection, assessment and classification of oocytes, oocytes from class A and B were selected. Oocytes were washed twice in SOF and then incubated in appropriate media at temperature of 38.5 ° C, 5% CO2 and 90% humidity for 24 hours. At the end of the maturation period, plates were examined via inverted microscope, and appearance of the first polar body is a good indication of oocyte maturation. The numbers of mature oocytes were calculated.

Sperm Collection and Preparation

Sperms were obtained from local bulls slaughtered in the slaughterhouse, where testicles were taken and placed in a cooler box, and then transported within one hour to the laboratory. The epididymis was taken by sterile scissors, and the epidermis tail is washed with a protective phosphate solution (PBS, P-5493, Sigma) to remove the blood clots and dirt. The epididymis is placed in a sterile PETRI dish. MEM was injected into the epididymal tail by using a G-18 needle. The surface of the epididymis tail was removed via sharp scalpel, and epididymis contents were withdrawn by using a sterile syringe of 5 ml Lone et al., (2011). Samples were tested for semen quality, as well as for the assessment of the individual and collective movement of sperm. Samples with an individual motion of less than 60% were rejected. The sperm-containing syringe kept in the incubator at 35 ° C, 5% CO2 for 6 hours for sperm maturity. The presence of the protoplasm droplet at the end of the sperm tail is evidence of sperm maturity. Heparin is then added to the sperm and incubated at 38 ° C for 45 minutes in according to Palamo et al., (1999) in order sperm adaptation.

In Vitro Fertilization

After oocytes maturation period, the pH level of the oocytes-containing medium is adjusted at 7.4 - 7.8 by withdrawing 50% of the medium by pipetting and replaced by media has all required additives for fertilization. After confirming of maturation of the sperm, 3 drops of sperm were added to 10 mL of the medium containing 20 oocytes and then incubated at 38.5 ° C, 5% CO2 and 90% Humidity for 30 hours.

Zygote Evaluation

Zygotes were evaluated after 24 hours of fertilization based on the presence of the second polar body or presence of the sperm head within the oocyte cytoplasm. The number of zygotes was counted.

In Vitro Culture

Zygotes were implanted in different media mentioned above that used in this study. These media containing zygotes were incubated at 38.5 ° C, 5% CO2, and 90% Humidity. The embryonic development was monitored every 24 hours with the replacement of 50% of the media with a new sterile medium every 24-hour. During the monitoring time, undeveloped zygotes were removed and maintain only developing embryos in the media.

Statistical Analysis

The Statistical Analysis System SAS, (2012) was used to analyze our results according to the complete randomized design (CRD) and the mathematical models below. Comparisons between treatments mean measured by Duncan, (1955) multidimensional test.

Results and Discussion

Effect of Culture Media on the Bovine Oocyte Maturation

Results indicate that SOF + BCS media exhibited higher maturation rate of oocytes (60.91%) but did not differ significantly between TCM-199 and SOF + BSA media groups (57.31%) and (55.65%) respectively. SOF also did not differ significantly with SOF + BCS and TCM-199 media, (51.57%). However, MEM showed a significantly lower maturation rate (37.02%) in comparing with SOF + BCS ($P<0.01$).

Table1. Effect of culture media on maturation rate (%) of bovine oocyte

Culture media	No. of cultured oocytes	No. of mature oocytes	maturation rate (%)
TCM-199	328	188	57.31 ± 3.68 ab
MEM	316	117	37.02 ± 2.71 c
SOF	285	147	51.57 ± 3.08 b
SOF + BCS	348	212	60.91 ± 3.44 a
SOF + BSA	327	182	55.65 ± 2.32 ab
Significantly	N.S	N.S	0.01

Oocyte maturation rate was reduced in MEM medium compared to the other media groups, and this is consistent with several studies indicating that TCM-199 improves the maturation of buffalo oocyte in vitro compared to MEM (Roushandeh et al., 2006). The low rate of IVM in MEM media can be explained by the fact that it contains a higher level of glucose and glutamine than TCM-199, and it has been known for many years that these molecules are poor sources of energy for cumulus cells of rodent (Downs and Verhoeven 2003). The reasons for differences in oocyte maturation rates may be due to the difference in components between media groups and percentage of serum added to the maturation media, as well as to the oocyte quality. The positive effect of TCM-199 and SOF in the maturation of oocytes due to the presence of certain factors in their composition such as essential amino acids and glutamine, which may stimulate DNA and RNA synthesis and subsequently promoting cell division (Pawshe et al., 1996; Gandhi et al., 2000; Gordon , 2003). Serum additions to the maturation media may provide energy sources, amino acids, growth factors, and vitamins. The positive effect of the serum on the oocyte maturation rate might occur due to the presence of growth factors through improving embryo growth and development after in vitro fertilization. Eppig et al., 1992).Furthermore, it is important to add serum into maturation media to prevent hardening of the transparent area that can adversely affect fertilization (Downs et al., 1986).

Effect of Culture Media on the Bovine Oocyte Fertilization

The study results showed a significant increase in the percentage of fertilization ($P < 0.01$) in SOF + BCS compared to the other cultivars (69.81%), followed by TCM-199 with fertilization rate (61.70%). Whereas, there are no significant differences between SOF + BSA, MEM, and SOF in fertilization percentage of oocytes with 53.84%, 52.13%, and 51.70%, respectively.

Table 2 Effect of Culture media on fertilization rate (%) of bovine oocyte

Culture media	No. of mature oocytes	No. of fertilized oocytes	Fertilization rate (%)
TCM-199	188	116	61.70 ± 3.47 b
MEM	117	61	52.13 ± 2.85 c
SOF	147	76	51.70 ± 2.72 c
SOF + BCS	212	148	69.81 ± 4.06 a
SOF + BSA	182	98	53.84 ± 2.17 c
Significantly	N.S	N.S	0.01

Increase fertilization percentages in TCM-199 and SOF + BCS group's media may be due to added serum, which greatly helped to enhance the proportion of oocyte fertilized, and this agreed with what Kharche et al., (2009) referred to. It is observed that addition of serum, fatty acid and bovine serum albumin to the media helps in adaptation and fertilization of oocytes. It is important to note that the superiority of MEM media, which was selected as the medium of activation, maturity, and adaptation of sperm, has helped to increase fertilization rates because it contains lactate and pyruvate as an energy source. High fertility rates can be attributed to the vitality of the sperm obtained by the method of slicing of the epididymal tail. These results agreed with what Klinc et al., (2005) indicated that semen quality is positively associated with fertility. In this study, it can be concluded that there is a high possibility to achieve IVF in cows via using sperm from the epididymal tail.

Effect of Culture media on the bovine embryonic development

Table (3) showed a significant superiority (P <0.01) for SOF + BCS in the division ratio of 67.56%, and did not differ significantly with TCM-199 and SOF + BSA, where the division rate was 62.06% and 65.30% respectively. SOF group showed similarity (59.21%) to both groups TCM-199 and SOF + BSA, Whereas, MEM group exhibited lower fertilization percentage (45.90%). Morulae rate was higher (P <0.01) for SOF + BCS group (43.24%) compared with other groups. Morulae rate for SOF+BSA was not different from either groups TCM-199 and SOF groups 29.31% and 28.94% respectively. Morulae rate was reduced (P <0.01) in MEM media group compared to SOF + BCS group (11.47%). A significant superiority (P <0.01) was observed for SOF + BCS, SOF + BSA, TCM-199 and SOF media clusters in the rate of blastocysts conformation 27.70%, 25.51%, 23.27%, and 22.36% respectively compared to MEM medium 1.63%. Results indicate that blastoderm ratio being similar between TCM-199 and SOF + BCS.

Table (3) Effect of Culture media in bovine embryonic development rate (%)

Culture media	No. of fertilized oocytes	Division ratio (%)	Morula rate (%)	Blastocysts produced rate (%)	Haching Blastocysts produced rate (%)
TCM-199	116	62.06 ± 2.57 ab (116/72)	29.31 ± 1.38 b (116/34)	23.27 ± 1.44 a (116/27)	1.72 ± 0.37 (116/2)
MEM	61	45.90 ± 1.82 c (61/28)	11.47 ± 0.76 c (61/7)	1.63 ± 0.61 b (61/1)	-
SOF	76	59.21 ± 2.66 b (76/45)	28.94 ± 1.49 b (76/22)	22.36 ± 1.73 a (76/17)	-
SOF + BCS	148	67.56 ± 3.19 a (148/100)	43.24 ± 2.52 a (148/64)	27.70 ± 1.46 a (148/41)	2.70 ± 0.41 (148/4)
SOF + BSA	98	65.30 ± 2.92 ab (98/64)	33.67 ± 1.72 b (98/33)	25.51 ± 1.33 a (98/25)	-
Significantly		0.01	0.01	0.01	NS

The positive effect of SOF+BCS (P<0.05) in improve rates of embryonic development may be attributed to presence BCS. BCS promotes oocyte maturation, fertilization, and subsequently increase blastocytes rates resulting simply accelerates rates of cell division and reach embryonic stage earlier with a better development efficiency and quality compared to those that delay reaching division stage (Dinny´es et al., 1999; Salumets et al., 2003).As a result, bovine blastocysts at day 7 have higher numbers of cells due to increasing rates of cell division (Lonergan et al., 1999). Bovine zygotes cultured in SOF media showed 8 cells more than zygotes cultured CR1 media after 48 hours of fertilization (Nedambale et al., 2006). The results of this study support the idea that embryos that have reached an early stage of division in SOF media are more likely to reach earlier the blastocyst stage as well as getting more blastocysts compared to TCM-199 and MEM. Also, there are some differences in embryonic development and conformation in SOF media compared to TCM-199 and MEM, and this can be attributed to the presence of citrate and Myo-inositol in the SOF composition. Citrate and Myo-inositol are essential sources for DNA synthesis in rabbits during blastocyst stage when they begin to increase their cell and protein content (Fahy and Kane 1992; Gray et al., 1992).This study also provides evidence that the optimal embryo development in the laboratory depends

partially on the presence of amino acids, cysteine, glycine, and glutamate, for the synthesis of GSH which is a component of the SOF that is important for embryonic development. Glutathione synthesis is essential for the metabolism and can be considered as a sign to assess the efficiency of oocyte development, which refers to the biochemical and molecular state that allows mature oocyte to be fertilized naturally and develop into the fetus (Furnus et al., 2008). Development of blastoderm was significantly observed when TCM-199 with BCS and SOF + BCS was used as a maturing medium, indicating that TCM-199 enhances maturation, fertilization, and embryonic development. These data allow understanding the early stages of embryo development. However, the relationship between total cell numbers, blastoderm, and vital or embryonic development remains unclear and more researches are needed.

Conclusion

The study is the first kind in Iraq from use the culture media SOF with adding and comparing then with other culture media that use in the country. Possibility to use the culture media SOF as medium for maturation and fertilized oocyte and embryo development rather than standard culture media not because it cheap material only but because its provides environment similar to environment living body.Add the bovine calf serum (BCS) to the culture media enhance maturation and fertilized rate and embryo development .

Recommendations

Use the modern culture media for maturity and sperm activity which contain lactate and pyruvate as sources of energy and not to use activation media container glucose as an energy sources .Conduct more studies about invitro fertilization and development it up to embryo transfer stage which is one of the most modern methods successful to spread good genetic structure which contributes in improvement the reality animal production in Iraq quantity and quality .

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