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IN VITRO MATURATION AND VERIFICATION OF SHEEP OOCYTES USING SOYA MILK EXTRACT

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Abstract

The study was carried out investigated the effect the adding of aqueous extract of soya bean to the specific media of sheep laboratory oocytes maturation and oocyte preservation by glazing technology,the study was carried out in postgraduate lab, Animal Production Department , Agriculture Collage, Al-Muthanna University from 22/9/2017 to 7/7/2018. There were two experiment groups : the first group includes the effect of different level of aqueous extract of soya milk (0, 5, 10 and 15%) concentrations on the in vitro maturation of sheep oocytes. The second group includes effect adding of aqueous of soya milk to the verification solutions as (0, 3 and 5%) concentrations on viability and normal morphology of sheep oocytes. The results revealed non significantly differences (P≤0.05) in percentage of sheep oocytes invitro maturation between soya milk concentrations 0, 5 and 10% with calculated superiority to 5% concentration , the values of invitro maturation were 41.43, 41.65 and 40.58, respectively and these concentrations were revealed significantly superiority on 15% concentration which 26.93. In second experiment when using extract of soya milk solvent solutions verification oocytes of normal oocytes morphology revealed significant superiority (P≤0.05) the 3% concentration on 0 and 5% concentrations as values 81.49, 72.78 and 75.84, respectively. The study concluded that adding of aqueous extract of soya milk 5% concentration to the culture media lead to improvement the percentages of sheep oocyte *in vitro* maturation and adding of aqueous extract of soya milk 3% concentration to verification solutions lead to improvement of viability percentage and morphology of oocyte after solving.

Key words : *In vitro* maturation, Verification, Sheep, Oocytes, Soya milk extract.

Introduction

Sheep are considered the most important agricultural animals in the world because they provide basic food such as meat, milk and other dairy products that are important to humans (Fabjan *et al*., 2014). Improvement of sheep reproductive efficiency are a main goal for animal production and in order to achieve this, the researchers have been developed the Assisted Reproductive Technologies (ARTs), which includes super ovulation, embryo transfer, *in vitro* fertilization, Cloning and cryopreservation of oocytes and embryos, for treatment of reproductive problems, increasing the births of animals with distinct genetic characteristics for using in genetic improvement programs (Gordon, 2004 and Wright *et al*., 2008). *In vitro* maturation of oocytes is the first important steps for *in vitro* fertilization. *In vitro* oocytes maturation was used at different culture media containing the necessary substances, growth factors, amino acids and hormones (Warnes *et al*., 1977 and Choi *et al*., 2001). The cryopreservation technique for oocytes and embryos is the backbone of ARTs because it allows for using in the future use (Herriat *et al*., 2008). There are two main methods are commonly used for cryopreservation of oocytes involving the traditional slow freezing and the newly developed technique called vitrification (Kuwayama *et al*., 2005 and Lowson *et al*., 2011). Cryoprotectants, such as dimethyl sulphoxide (DMSO) and ethylene glycolwhich using to prevent the formation of ice crystals that causes damage to cells during cryopreservation (Fuller, 2004). For the importance of the culture media in many reproductive applications which containing the most of the essential elements for growth and maintenance of oocytes as well as containing different sources of protein and many hormones and sugars to provide an environment similar within the body of living

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animals (Son *et al*., 2008). So today researchers are looking for alternatives to animal products used in reproductive technology, and can be used soya milk of plant origin, which is free of pathogens. Therefore, the aim of the present study was to *in vitro* maturation and vitrification of sheep oocytes using soya milk extract.

Materials and Methods

The present study was conducted at the postgraduate laboratory that belonging to the Department of Animal Recourses, Collage of Agriculture, University of Al-Muthanna for the duration from 22/9/2017 to 7/7/2018 including two experiments: The first experiment included the in vitro maturation of oocytes using different concentrations of soya milk after the collection of sheep ovaries from the Samawah slaughter house, 5 km away from the site of Al-Muthanna University. The ovaries were transported to the laboratory within 1 hour using plastic containers containing normalsaline solution (0.9% sodium chloride) supplemented with antibiotics 100 IU/ mLPenicillin and 100 mg/mLStreptomycin) at37°C. In the laboratory, ovaries were washed three times with warmed normal saline solution to remove the blood on the surface ovaries (Rezk, 2009). Then, Soya milk extract was added to the culture medium.

Preparation of soya milk

Soya milk was prepared by weighing 10 grams of soybeans, washed with distilled water and soaked overnight in 100 ml of distilled water. After that, the distilled water was removed and soybeans washed again with distilled water, then 100 ml of distilled water was added and heated at 80-100ºC for 20 minutes. Immediately after heating, they were mixed with a high-speed mixer for 5 minutes until the formation of the slurry. Then, the slurry was heated for 10 minutes with continuous stirring at 80-100º C to stop the effectiveness of the Lipoxygenase enzyme. The slurry was cooled and filtered by a clean gauze cloth, and then centrifugation process at a speed of 4000 cycles per minute for 20 minutes to completely remove the solids and pH was adjusted at 7.

Collection of oocytes

Sheep oocytes were collected from the ovaries by aspiration method. Oocytes with follicular fluid were aspirated using 23-gauge hypodermic needle attached with a sterile disposable 3 ml syringe contain 0.5 ml of RPMI culture medium supplemented with 5% BSA and 20 IU/ml heparin to prevent clotting in follicular fluid. After oocyte retrieval, content of each syringe was poured into a Petri dish. Then, this content examined under dissected microscope for oocyte collection using modified pasture pipette or automatic micro pipette and washed for three times with RPMI 1640 medium (De Smedt *et al*., 1992).

Preparation of Trypan blue dye

Trypan blue is the stain most commonly used to distinguish viable from nonviable cells. The stain was prepared with 0.4% trypan blue by adding 0.4g of Trypan blue powder to 80 ml PBS, agitate well to dissolve then complete the volume to 100 ml PBS, filtered through filter paper, store in dark bottle in refrigerator. Trypan blue has been used in vital staining of various tissues. Staining facilitates the visualization of cell, morphology. Live (viable) cells do not take up the Trypan blue dye, while dead (non-viable) cells do take up this dye (Nandi *et al*., 2002).

Viability test

All oocytes were examined for viability using the trypan blue exclusion test. Unstained oocytes were classified as live and fully stained oocytes as dead .The viability test for oocytes was done post-aspiration and immediately post-thawing and 2 hour post thawing. Oocytes and embryos were placed in trypan blue stain for 30 second and then removed (Abd Allah, 2010).

***In vitro* maturation of oocytes**

After washing the oocytes, maturation medium (RPMI-1640) was supplemented with some hormones (10 IU/mL hCG, 5 IU/mL PMSG and 1 µg/mL Estradiol) for *in vitro* maturation of oocytes. The culture media were divided into four groups. The first group was considered as a control group without addition soya milk. Soya milk extract was added to the second (5%), third (10%) and fourth group (15%) respectively.Oocytes were washed three times in RPMI-1640 culture medium containing 5% BSA to remove substances in follicular fluid. Then, the 5-7 immature oocytes were directly placed in overnight incubated droplet (0.5mL) of culture medium. IVM of oocytes was done using two treatments were two concentrations of sucrose 0.05 M and 0.1 M and control group. Maturation medium was supplemented with 10 IU/ml hCG, 5 IU/ml eCG, 1µg/ml Estradiol and 10 µl/ml penicillin - streptomycin antibiotic and cultured in four well Petri dish, covered with paraffin oil and incubated for 24 h in CO2 incubator (5% CO2) at 38.5°C with high humidity (95%) (De Felici and Siracusa, 1982).

Oocytes vitrification solution

vitrification solution (VS) : The equilibration solutions (ES) consisted of 7.5% (v/v) dimethyl sulphoxide (DMSO) (Scharlau, Spain) and7.5% (v/v) ethylene glycol

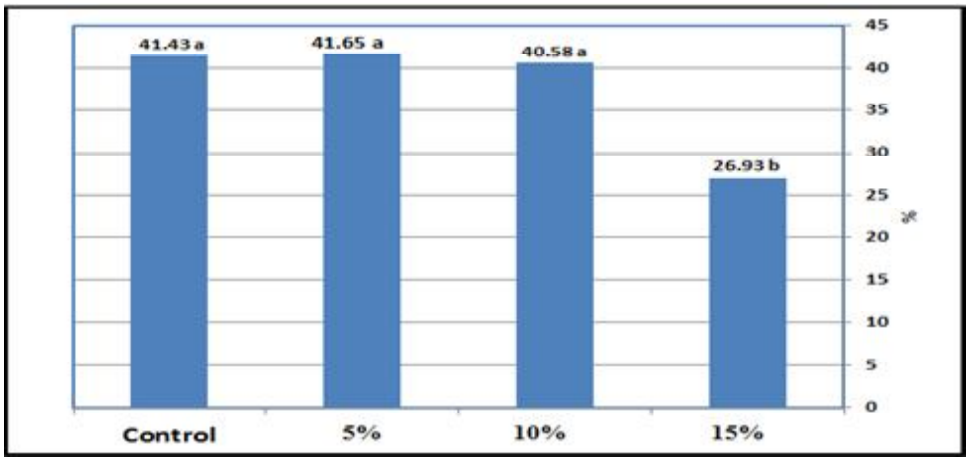

Fig. 1 : Effect of adding soya milk extract on *in vitro* maturation of sheep oocytes ± Standard error.
(a, b, c) The different small letters indicate a significant differences between the meansat level of significance 0.05.

(EG) (Scharlau, Spain) were prepared by adding the corresponding volume of CPA to culture medium containing 10% BSA. Vitrification solutions (VS) consisting of 15% (v/v) DMSO with 15% (v/v) EG, which were added to culture medium supplemented with 10% BSA.

Thawing solution (TS) : Two concentration of soya milk extract (5% and 3%) was added to the three thawing solution at as following:(TS1) RBMI 1640 culture medium up to 10ml with 10% bovine serum albumin and1M of sucrose (3.42 g) (TS2) RBMI 1640 culture medium up to10ml with 10% bovine serum albumin and 0.5 M of sucrose (1.71 g) (TS3) RBMI 1640 culture medium up to 10ml with 10% bovine serum albumin and 0.25 M of sucrose (0.86 g). It is important to shake the solution well to dissolve the sugars cryoprotectants (CPA). The final pH of the above solutions was adjusted to 7.2–7.4, and then filtered through Millipore filter (0.22ìm), keep in refrigerator until use.

Vitrification and thawing techniques

The vitrification and warming procedures were performed according to Kuwayama *et al.* (2005) and Kuwayama *et al.* (2007). Normal and viable immature oocytes were transferred to 0.5 mL of ESat room temperature to equilibrate for 15 min. Then, oocytes were placed into 0.5 mLof vitrification solution (VS) for 1 min. Then the oocytes were loaded on the cryotop strip and directly immersed into LN_2. Then, the strip was covered with the plastic tube in LN_2 to protect it during storage.

For thawing, the straws were taken out from the LN2 after two months and immersed in thawing solution 1 (TS1) at 37ºC for 1 min. Then oocytes and were transferred into thawing solution 2 (TS2) at room temperature for 3 min and thawing solution 3 (TS3) at room temperature for 3 min, and then washed twice with RBMI 1640 medium and tested for viability and morphology. The thawed oocytes were considered abnormal when some change occurs in shape such as breakage of zona pellucida, uneven granulation or leakage of oocyte contents (Trigos, 2011).

Statistical analysis

Data were analyzed at a one-way design by the statistical program (SAS, 2001), Averages were compared by Duncan Multilayered Test (Steel and Torrie, 1980) at a probability level (0.05).

Results and Discussion

Effect of adding soya milk extract on in vitro maturation of sheep oocytes

The results in fig. 1 showed no significant differences (P> 0.05) in the percentage of *in vitro* maturation of oocytes between concentrations of 5% and 10% of soya milk and control group which were 41.65 ± 3.50, 40.58 ± 3.88 and 41.43 ± 2.87, respectively. The percentage of in vitro maturation of oocytes was significantly decreased as in the 15% concentration, the percentage of ripening compared with the above concentrations, where the percentage was 26.93 ± 3.34. The results of the present study showed that the percentages of in vitro maturation of sheep oocytesare inversely proportional with the concentration of soya milk in the culture medium of maturation. This is in line with Whitehead *et al*. (2002) when feeding the sheep on high concentration of soybean. It has been shown that soybean lead to prevention or inhibition the production of steroids because of soybean contains isoflavin which containing phytoestrogens, including genistein which lead to inhibition of estrogen action.

Table 1 : Effect of adding soya milk extract on the morphology and viability of vitrified oocytes post thawing.

Soya milk concentration	Morphology	Viability
Control	85.91±2.79 b	72.78±2.12b
3 %	91.69±3.29 a	81.49±2.43 a
5 %	85.08±6.16b	75.84±1.79ab
Level of significance	P≥0.05	P≤0.05

Effect of adding soya milk extract on the morphology and viability of vitrified oocytes post thawing

The results in table 1 showed significant differences (P ≤0.05) in the morphology of vitrified oocytes post thawing where the concentration of 3% was superior on the control group and 5% of the soya milk and the percentages were 91.69±3.29, 85.91±2.76, 85.08±6.13, respectively. On the other hand, no significant differences (P≥ 0.05) were found in the morphology of oocytes among 5% and 10% concentrations of soya milk. For viability of oocytes, the results revealed significant differences (P ≤0.05) where the concentration of 3% of soya milk was superior on the control group and the percentages were 81.49 ± 2.43, 72.78 ± 2.12, respectively. No significant differences (P≥ 0.05) in the viability of oocytes among 3% and 5% of soya milk. The results of the current study showed the highest percentage of viability and morphology post thawing of vitrified oocytes, which were 81.49% and 91.69%, respectively. While the lowest percentage of viability and morphology post thawing of vitrified oocytes was 72.78% and 85.08%, respectively. These results were similar to those found by Atiyah (2018), where it was reported that the percentage of viability and normal morphology of vitrified immature oocytes was 80.21% and 90.74%, respectively. On the other side, The results of this study differed with Al-Saadoon (2014), when reported that the percentage of viability and normal morphology of immature oocytes was 91.11% and 82.22%, respectively. The differences with our study may be due to different concentrations of cryoprotectants, vitrification and thawing solutions and the procedure used in the study. In the present study, the oocytes were loaded onto the Cryotop tool with a minimum volume of vitrification solution (less than 3 microliters). This volume is very necessary for reducing the time ofvitrification and thawing the oocytes and also to reduce the intracellular and extracellular ice crystals formation that cause the death of the oocytes (Ali *et al.*, 2014).

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