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In Vitro Production of Ovine Embryo in Non-breeding Season

Abstract

The aim of this study was under taken to show the effect of follicle size and culture media on the results of IVF and IVEP in sheep. A total of 100 ovaries of local Iraqi ewes were taken from abattoir of Fallujah during non-breeding season. The total number of follicles were 256 with a mean of 2.56 follicles per/ ovary. A total number of recovered oocyte were 189 (73.8%). The recovery rate of oocyte from large follicles were 85.8% (98/189) and from small follicles were 64.7% (92/189%). Only grade A and B oocyte have been used with a total number of 148 (78.3%) oocytes. Maturation rate in different media; MEM, RPMI1640, DMEM low glucose and DMEM high glucose were 64.42%, 51.28%, 32.43% and 26.66% respectively. There was a significant difference ($p \leq 0.05$) in maturation rate between different media. The total fertilization rate was 56.7% (38/67). Fertilization rate in different culture media were 66.66%, 55%, 50% and 37.5% in MEM, RPMI1640, DMEM low glucose and DMEM high glucose respectively. There was a significant difference ($p \leq 0.05$) in fertilization rate between different culture media. The cleavage rate was 70% (20/38). The total number of morula and blastocyst stage was /40% (8/20) and 35% (7/20). There was a significant difference ($p \leq 0.05$) in morula and blastocyst production between different media. It was concluded that abattoir was a good source of oocyte recovery, and the large follicle give the best morula and blastocyst stage. MEM was a good diluent of semen. mMEM give the best result as cultured media as compared with the other media used in this study.

Keywords: IVF, IVEP, Local Iraqi breed sheep Non-breeding season.

Introduction

In Vitro Fertilization (IVF) technique provides large scale, low Cost economical production of early and late stage embryo for gene integration and cloning, expected to make efficient utilization of high numbers of ova left in the ovaries (kharche *et*

al., 2011). The embryo obtained from ruminant species have been cultured in a numbers of defined and semi defined or undefined media. A defined medium is a medium which is prepared using Identifiable components prior to embryo culture and that components must facilitate embryo biochemical requirements and the physical environment created must simulate the in vivo environment (Thompson *et al*., 1996). The method or procedure of IVF involves three main steps; Maturation of primary oocytes from Large follicles, fertilization of matured secondary oocytes with fresh or epididymal sperms end culture putative embryos for up to one week until formation of blastocysts that can he transferred to recipient or cryopreserved for future use (wani, 2002; paramio and Izquierdo, 2014). There are many factors plays a role in successful of IVF technology including culture media, breeding season, size of follicles, age of recipient, semen donor and the method of oocyte collection (Majeed, 2012; Davachi *et al*., 2014; Wani, 2002; paramio and Izquierdo, 2014). The aim of the study was designed to establish a reliable method of ovine embryo production and effects of certain factors such as season, method of collection of oocytes, follicular size and culture media on embryo production.

Materials and Methods

Oocytes collections:

Fifty ovine female genital system were collected from abattoir of Fallujah and transported within one hour in cool box containing normal saline at 33 35˚c to the Reproductive Biotechnology Lab., Dept. of surgery and obstetrics, Coll. Vet. Med, Uni. of Fallujah. The ovaries were isolated and subjected to three washing with collecting media (MEM, RRMI 1640, DMEM low glucose and DMRM high glucose). Follicles were calculated wether in right or left ovary and measurement of its diameter with an automatic vernier. Aspiration of follicular fluid of 2-8 mm size follicles using 18 gauge needle attached with a sterile 5ml disposable syringe containing 3ml of collecting media (Wani, 2002). The media with recovered Oocytes were transferred to one well out of 24 wells dish.

Grading of Oocytes:

The Oocytes collected were examined under inverted microscope and graded according to Wani *et al*., (2000) as good (grade A), fair (grade B) and poor (grade c), on the basis of cumulus cells and uniform cytoplasm.

In vitro maturation:

Only good and fair quality oocytes were selected. The oocytes were washed twice in a maturation medium either MEM or DMEM low glucose. They were incubated in maturation media at 38◦c temp., 5% co_2 and 90% relative humidity for 24-27 hrs. The incubated petri dish was examined under inverted microscope. The presence of first polar body was indicative for oocytes maturation in vitro (IVM). The numbers of matured oocyte were counted.

Semen collection and preparation:

Semen was collected in fresh state from two rams of good fertility, Presented in the farm of college of veterinary Medicine, University of Fallujah, via electro ejaculator (India Instrument, 620 Lesher place, Lansing, MI 48912., USA) and transported within 5 minutes to the Reproductive Biotechnology Lab., at 30-33◦c. Semen samples were examined under light microscope to evaluate semen quality (mass and individual motility). Semen Samples were warmed in a water bath at 35◦c. The semen then diluted 1:20 with MEM solution. Heparin sodium (Sundent, China) 10Mg/ml were added to diluted semen for sperm capacitation.

In vitro Fertilization:

Diluted capacitated sperm in a concentration of 1×10^6 sperm/ml were added to fertilization medium containing mature oocyte Kept in a group of 5-8 oocyte in one well out of 24 wells petri dish and incubated at 38◦c, 5% co_2 and 90% relative humidity for 24-27 hours (Kharche *et al*., 2011). At 24 hours after insemination, oocytes were evaluated as fertilized Oocyte when having the Second polar body or oocyte with sperm head in the cytoplasm. The numbers of fertilized oocytes were calculated.

In vitro culture:

Culture of fertilized oocytes (zygotes) were performed in different cultural media (mMEM, mRPMI 1640, mDMEM low glucose and mDMEM high glucose) incubated at 38-38.5◦c temp., 5% co_2 and 90% humidity. Embryonic development were observed every 24 hours and half of culture media were replaced with fresh one at 24 hours intervals. Proportions of fertilized oocytes reached 2 to 4 cells stage were recorded at 48 hrs. Morula were observed at 120 hours and blastocyst at 168 hours after fertilization.

Statistical Analysis:

Student t-test and chi-square test were used for analysis of data according to schefler (2000).

Result and discussion

Follicles numbers counted in 100 ovaries was 256 with a mean of 2.56 follicles for every ovary. The numbers of follicles found in the right ovary was 104 with a mean of 2.08 follicle per ovary. While that found in the left ovary was 152 with a mean of 3.04 follicles per ovary. (Table -1). There was a significant difference ($p \leq 0.05$) between the left and right ovaries in the numbers of follicles present. Similar observations have been reported by several authors (Greyling, 2000, Hafez and Hafez, 2000, Al-jumaily, 2004). This result might be due to the fact that the left ovary in ewe mere active than the right one (Noakes *et al*., 2009). Table-2 showed the effect of follicle size on oocyte recovery and their grade. The total numbers of Small follicles (2-4mm) observed was 142 (55.46%) with a mean size 3.9±0.6.

Table -1: Effect of type of the ovary and the numbers of follicle/ovary and the number of oocyte recovered

Type of ovary	Percent of follicles/ovary	No. of oocyte	Percent of follicles
Right ovary (50)	2.08±0.2	104	40.6%[a]
Left ovary (50)	3.04±0.4	152	59.4%[b]
Total (100)		256	

Values: mean ± SE.
Different small manuscripts mean significant difference ($p \leq 0.05$).

Table -2: Effect of follicle size on recovery rate of oocyte and their grade

Follicle size	No. of follicles	Size of follicle	No. of oocyte recovered	Recovery rate	Grade of oocyte		
					A	B	C
Small follicle 2-4mm	142[a] (55.4%)	3.9±0.6[a]	92	64.7%[a]	18[a] (19.5%)	42[a] (45.6%)	32[a] (34.7%)
Large follicle 5-8mm	114[b] (44.5%)	6.6±0.8[b]	97	85.8%[b]	39[b] (40.2%)	49[b] (50.5%)	9[b] (9.2%)
Total	256		189	73.8%	57 (30.1%)	91 (48.1%)	41 (21.7%)

Values: mean ± SE.
Different small manuscripts mean significant difference ($p \leq 0.05$)

While the numbers of large follicles (5-8 mm) observed was114 (44.54%) with a mean size of 6.6±0.8. There was a significant difference in the size of the follicles ($p \leq 0.05$) between small and large one. Similar results have been reported by several investigators (Crozet *et al*., 1995; Cognie *et al*., 1996; Wani, 2002; Al-jumaily, 2004; Wang *et al*., 2007; Hoque *et al*., 2011; Kharche *et al*., 2011; Majeed *et al*., 2019a). It has been noted that follicular size is affected by several factors; reproductive status of the animals, breeding seasons, age, hormonal stimulation and nutritional state of the animals (Hafez and Hafez, 2000) The results showed that a higher recovery rates were obtained with fair oocyte (grade B) 48.1% (91/189) fallowed by good oocytes (grade A) 30% (57/189) and poor oocytes (grade c) 21.7% (41/189). There was a statistical difference ($p \leq 0.05$) between different grades of oocytes. Similar observations have been made by Wani *et al*. (2000) and Majeed *et al*. (2019b) in sheep and Rahman *et al*. (2009) in goats. It has been reported that high quality oocytes recovered by aspiration in sheep and goats (Sogorescu *et al*., 2010). The low quality oocytes obtained might be due to slaughtering of bad quality ewes.

In vitro Maturation:

The embryo development influenced the event occurred during oocyte maturation and for the success of IVM, the oocytes must undergo nuclear and cytoplasmic maturation. Only grade A and B oocytes (148/256) 57.8% of the recovered oocytes were cultured (Table-3). The maturation rate was 45.2% (67/148). Similar recored have been observed by other workers (Wani, 2002; Majeed, 2012; Majeed. *et al*., 2019b). Maturation rate from small follicles was 35.0% (21/60), while it was 52.27% (46/88) from the large follicle.

It has been reported that oocytes recovered from large diameter follicle showed successful development (Arlotto *et al*., 1996; Wani *et al*., 2003; AL-jumaily, 2004; Majeed *et al*., 2019b). (Fig-1) showed mature follicle with 1st polar body. Effect of culture Media on Maturation rate showed in Table-4. The total Maturation rate in different media was 45.52% (67/148). High maturation rate was observed in MEM media 64.42% (27/42), followed by RPMI 1640 51.28% (20/39), then in DMEM Low glucose media 32.43% (12/37) and in DMEM high glucose media 26.66% (8/30). Similar results have been made by several investigators (Majeed, 2012; Menchaca *et al*., 2018; zhu *et al*., 2018). It has been noticed that maturation medium and the selection of protein supplements and hormones play a role in IVM and subsequent IVF and in vitro development (paramio, 2010; Majeed *et al*., 2012;

Majeed *et al*., 2019c). There was a significant difference ($p \leq 0.05$) in maturation rate between different media.

Table-3 : Effect of follicle size on maturation rate %

Follicle size	No. of oocyte	No. of mature oocyte	Maturation rate
Small follicle	60	21	35.0%[a]
Large follicle	88	46	52.2%[b]
Total	148	67	45.2%

Values: mean ± SE.

Table-4 : Effect of culture media on oocyte maturation rate %

Type of media	No. of oocyte	No. of mature oocyte	Maturation rate
MEM	42	27	64.42%[a]
RPMI 1640	39	20	51.28%[b]
DMEM low glucose	37	12	32.43%[c]
DMEM low glucose	30	8	26.66%[d]
Total	148	67	45.52%

All media supplemented with 10% fetal bovine serum as a complete medium.
Different small manuscripts mean significant difference ($p \leq 0.05$).

In vitro Fertilization:

Table -5 showed fertilization rate in different complete fertilization media (media supplemented with 10% fetal bovine serum (FBS): The total fertilization rate was 56.7-% in different culture media. Similar findings have been made by sogorescu *et al*. (2010), Hoque *et al*. (2011), Menchaca *et al*. (2018) and Zhu *et al*. (2018). Fig-2 showed fertilized oocyte with the second polar body. It has been reported that there are several factors plays a role in successful IVF, Such as oocyte collection technique, follicle size, season, age, semen preparation with capacitating agent and cultural media (wani, 2002; Majeed, 2012; Menchaca *et al*., 2018; zhu *et al*., 2018). Fertilization rate was 66.66% for oocytes cultured in complete MEM, while those cultured in complete RPMI 1640, complete DMEM low glucose and complete DMEM high glucose were 55%, 50% and 37.5% respectively. There was a significant difference ($p \leq 0.05$) in fertilization rate among different media. These

findings might be attributed to the inclusion of FBS in the media (shirazi *et al*., 2012) that may give the best results. The percent of fertilization were in the acceptable limit in this works and agreed with zhu *et al*. (2018). Also the difference in fertilization rate in different media might be attributed to the components of the media (Menchaca *et al*., 2018; Zhu *et al*., 2018).

Table-5 : Effect of culture media on oocyte fertilization rate %

Type of media	No. of mature oocyte	No. of zygot	Fertilization rate
MEM	27	18	66.66%[a]
RPMI 1640	20	11	55.0%[b]
DMEM low glucose	12	6	50.5%[c]
DMEM low glucose	8	3	37.5%[d]
Total	67	38	56.7%

All media supplemented with 10% fetal bovine serum as a complete medium.
Different small manuscripts mean significant difference (p≤0.05).

In Vitro Culture and Blastocysts production:

Table -6 showed the results of in vitro Culture of fertilized oocytes (zygot) in different Stages (2-cells, 4-cells, 8-Cells and Morula stage) till Blastocyst production. The proportion of Cleaved zygot was 70% (20/38) fig-3, 4-cells fig-4 was 55% (14/38), 8-cells fig-5 40% (11/38) and Morula was 40% (8/38) fig-6, the blastocysts production was 35% (7/38) (fig-7). These results agreed with several investigators (Wani, 2002; paramio and Izquierdo, 2014; Menchaca *et al*., 2018; zhu *et al*., 2018). It has been observed that the expected Blastocysts percent under in vitro fertilization conditions is around 30 to 40%. (vilarino *et al*., 2012; Menchaca *et al*., 2018). Blastocysts production in a complete MEM medium was 22.2% (4/18), while it was 18.1% (2/11) in a complete RPMI 1640 media, 16% (1/6) in a complete DMEM low glucose and Zero in a complete DMEM high glucose. There was a significant difference (p≤0.05) in blastocysts Production between different media. These variations might due to various factors like, age, breed, technique, media, PH and temperature. Also oocyte quality plays as an important factor for embryonic development (Schultz, 2002; Jyotsana *et al*., 2019). The study suggested that MEIM media give a better result than other media used in this work. Also the study were

carried out on non-breeding season so the results show a decrease in development of blastocyst production. So this partially may explain the reason for the variation of the efficacy of ovine embryo production during different season (zhu *et al*., 2018).

Table-6 : Effect of culture media on blastocyst production

Culture medium	No. of zygot	Stage of cleavage (Cleaved zygot)				
		2-cells	4- cells	8-cells	Morula	Blastocysts
MEM	18	10 (55.5%)	7 (38.5)	6 (33.3%)	5 (27.7%)	4[a] (22.2%)
RPMI 1640	11	6 (54.5%)	5 (45.4%)	4 (36.3%)	3 (27.2%)	2[b] (18.1%)
DMEM low glucose	6	3 (50.0%)	2 (33.3%)	1 (16.6%)	1 (16.6%)	1[c] 16.6%
DMEM low glucose	3	1 (33.3%)	0	0	0	0[d]
Total	38	20 (52.6%)	14 (70%)	11 (50%)	8 (40%)	7 (35%)

All media supplemented with 10% fetal bovine serum as a complete medium.
Different small manuscripts mean significant difference (p≤0.05).

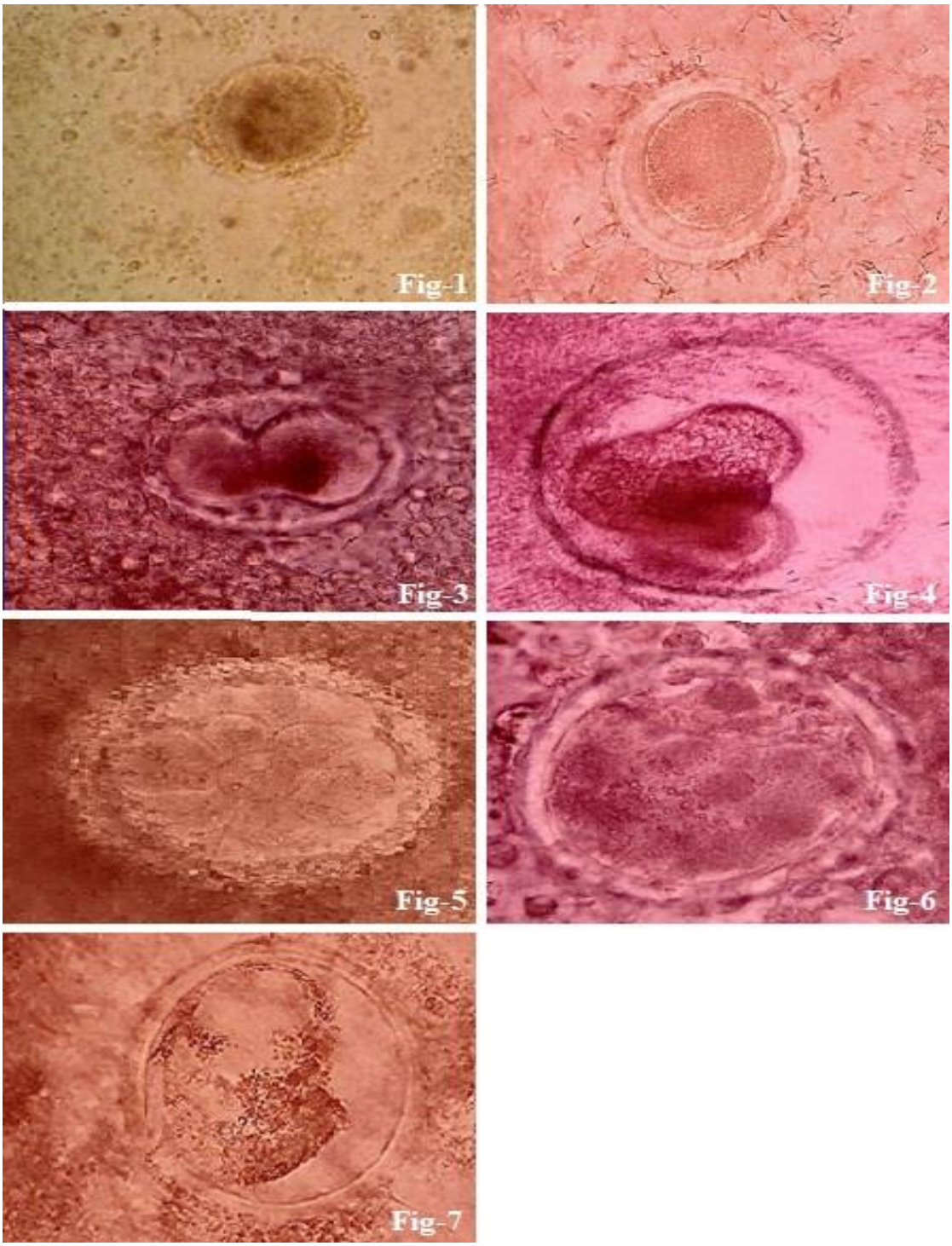

Figure-1: (Mature oocyte), Figure-2: (Fertilized oocyte "zygot"), Figure-3: (2-cells stage), Figure-4: (4-cells stage), Figure-5: (8-cells stage), Figure-6: (Morula stage), Figure-7: (Blastocyst stage).

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