Research Paper Veterinary Science

In Vitro Fertilization in Sheep Using Long Standing Capacitated Spermatozoa (Abattoir sample)

Wafer M. Saleh Department of Surgery and Theriogenology, College of Veterinary Medicine, Baghdad University

Ihsan H. AL-TIMIMI Department of Surgery and Theriogenology, College of Veterinary Medicine, Baghdad University

ABSTRACT

Abattoir fresh genetalia could be regarded as a highly yielding male (spermatozoa) and female (oocyte) samples, in which these samples if prepared well considered as good reservoir for male and female gametes than could be contributed to the advanced reproductive technique (ART). Fresh female and male whole genitalia had been collected from Al-Shoáalla abattoir, west of Baghdad on January 2014 to March 2015, collected samples transported by cool box (4-8) directly to Lab. Sample washed thoroughly with distilled water, ovaries dissected and separated from surrounding tissue, washed with d. water containing antibiotic and antifungal preparation, ovaries bearing large follicles (3-6mm) was inspired by 18 gauge needle, entire ovaries then sliced by scalpel blade to small pieces. Collected oocytes incubated for IVM and IVF. Collected methods showed; 380 oocytes had been collected by aspiration method toward 1570 oocytes in slicing method with a degree of maturation 223 to 752 Oocytes for the aspiration and slicing methods respectively. Seasonality affect the grading and fertilization index ($P<0.05$), good grade showed 364 to 878 (out to within season respectively) toward fair grade 104-252 (out to within season respectively) while fertilization percentage showed 49.55% out of season toward 57.42% within season. Cauda samples were sliced and incubated at (RT) for following period of time capacitated with heparin gave a good fertilizable ability of these spermatozoa even for this prolonged period of time.

KEYWORDS Epididymis, cauda, spermatozoa, IVF, transplantation, ewe, IVM, IVP, ET, ICSI, AI, IVSM, COCs, P4, MEM, ART

Introduction

In mammals, freshly ejaculated spermatozoa cannot fertilize mature oocytes; rather they possess a fertilization-competent in female genital tract through time dependent process of capacitation (Yanagimachi 1994). Capacitation is a complex set of modifications that happened to the spermatozoa inside the female reproductive tract, and gain its ability to fertilize a mature oocyte; many authors demonstrate a great importance of the caudal spermatozoa to preserve the genome of individual male after death in buck (Al-Timimi, 2013). The role of epididymal secretion is to maintain spermatozoa vitality and to permit the development of spermatozoa motility and possibly to protect them from noxious agent (Ezer, 2002). Genetically superior and highly producing genotype rams, that their spermatozoa were used in progeny improvement programs (ET, AI, IVF ICSI, IVSM, etc.) when severely and dangerously affected that interfere with collection of their spermatozoa as: Accidently death, Injuries, trauma and fractures, Damage to the vas deferens, Penile infection and fracture or damage to the accessory glands that may terminated its breeding Career or genetics materials (Roberts, 1986; Arthur, 2008). According to previous serious events that directly interfere and affect the improvement and the preservation of genetic material, studies were directed their goal toward the suitable and more practical methods to serve these rare and most valuable breed by harvesting their genetic material for long period of time and examined their fertilizable ability with mature Oocytes (IVF) to produce embryos. The idea optimally directed to the more available, cheapest and easily manipulated method which is the abattoir samples. For this reason and for the absence or limited studies in Iraq concerning the main factor of this study which is the caudal spermatozoa after death, this study was designed to prove the role of the caudal spermatozoa in preserving and improvement the ovine population in the country.

Materials and Methods

Abattoir male and female genitalia specimens were collected from Al-Shoáalla abattoir west of Baghdad in an average of 2-3 visits per week. Genetalia specimens were obtained from mature local sheep (more than 2 years old), age is determined according to the dental tables (Miller and Robertson, 1959). Abattoir fresh genetalia of rams and local breed ewes were collected directly after slaughtering, entire female genitalia was taken starting from ovary until vagina, specimens of male or female genetalia were transported by cool box at 4-8 from abattoir to the Laboratory of Theriogenology/College of Veterinary Medicine / Baghdad University.

Oocytes collection:

Ewe genitalia of unknown history had been collected from February to July2015, transported within one hour to the theriogenology lab. All specimens washed carefully with distilled water then with normal saline, ovaries were dissecting and separating from all surrounded tissues suspended with normal saline containing penicillin- streptomycin combined with an antifungal solution, kept at room temperature for half hour to further settlement (Sofi, 2012), oocytes were collected by 2 methods:

Aspiration Method: Ovaries bearing follicles measured more than 8mm subjected to aspiration using 18G needle connected to 3ml plastic syringe containing 1ml MEM medium, collected oocytes were kept in a glass Petri dishes containing 5-6ml MEM for further examination (Anamaria, 2009).

Slicing Method:

All collected ovaries had been gathered in a glass Petri dishes containing 5-6ml of MEM with antibiotics, using a sterile scalpel blade, ovaries would be incised more and more to very smallest pieces. Petri dishes of the both techniques were leaved to settlement at room temperature for 15 minutes, and then examined under inverted microscope searching for the oocytes (Wani *et al.,* 2000).

Oocytes evaluation:

Evaluations of the collected oocytes were done as mentioned by Rehman, (2008) in regarding to the number of cumulus cell surrounding oocytes and the status of the cytoplasm as Good

grade, Fair grade or Poor grade. Good and fair grades oocytes were subjected to the process of *in vitro* maturation (IVM), oocytes after grading were moved to another Petri dishes by aspiration with an automatic micropipette, re-examining the dishes after these ova were transport to be sure that all selective ova were transport, ova were grading and counting and recorded.

Oocytes Maturation (IVM):
Good and fair grade oocytes were subjected to the next process, maturation medium prepared as mentioned by Wani (2013) and equilibrated for 2hrs in CO_2 incubator before oocytes added. Oocytes were incubated in CO_2 incubator for 24-28hrs supplied 5% CO_2 tension, 90% humidity and at 38.5. Matured oocytes were examined under inverted microscope, degree of maturation evaluated as mentioned by Dulam and Sreenivas (2013) in regarding to the extruding of first Polar body and the degree of COCs expanding in which there were 3 degrees in assessment the oocytes maturation in regarding to these foundlings:

Degree 2: Cumulus cells were spread homogenously and clustered cells not found.

Degree 1: Cumulus cells were spread non- homogenously with the presence of clustered cells.

Degree 3: Cumulus cells were closely adhering to the zona pellucida without expansion.

Both degree 2 and 1 considered as matured oocytes. The graded and selected oocytes were washed many times by the medium; number of matured oocytes were counted and recorded.

Collection of caudal Spermatozoa:
Testicle samples after collecting and transporting to the lab, were washed with distill water firstly, then with normal saline containing penicillin and streptomycin antibiotics by a small scissor we dissected and separated the epididymis from the entire testicle, then cauda separated from epididymis, all surrounding tissue will be removed, washed thoroughly with d. water then hold in becker containing MEM medium with antibiotic and antifungal preparation (Karja *et al.,* 2013).Cauda samples were injected with 5-8 ml of the medium put in glass Petri dishes and sliced for small pieces by blade, spermatozoa evaluated and stained smear for dead and alive were examined and recorded, Petri dishes preserved at refrigerator temperature (RT) for periods of time (Kaabi, 2003).

Spermatozoa maturation and capacitation:
Caudal spermatozoa were evaluated under light microscope, individual motility samples lower than 60% were rejected. Sperm samples incubated in CO_2 incubator at 35 °C for 6hrs for sperm maturation, the presence of distal protoplasmic droplet was an indicator of sperm maturation. Samples leaved for 30 minutes at room temperature, then 1ml of the sample add to 3ml of MEM containing antibiotics and antifungal supplement, washed twice by centrifugation at 2000 rpm for 10 min, supernatant were discard, spermatozoa pellet was suspended in capacitated medium and washed again, then second spermatozoa pellet resuspended in MEM containing 50 IU heparin. Penicillin and Streptomycin with antifungal supplements incubated in CO_2 incubator (38.5ºC, 90% humidity) at sloped positioning for 1-2hr (Wani, 2013).

***In Vitro* fertilization (IVF):**
Matured oocytes of good and fair grade were subjected to follow the process of fertilization. Matured oocytes were washed twice with medium supplied with antibiotics and antifungal before transferred to a glass Petri dishes containing medium with same supplementation. Capacitated spermatozoa sample were prepared after diluted to yield 1-2×10^6/ml sperms (Wani, 2013). The mixture of gametes was incubated in CO_2 incubator at 38.5 and 90% relative humidity for 26-28hrs.

***In Vitro* Embryo Production and Evaluation:**
Fertilized oocytes were removed from cultured medium after diagnostic by inverted microscope. Fertilization rate was evaluated based on pro-nucleus formation, presence either sperm head in the vitelline space, emission of 2nd polar body and cleavage. Embryos were evaluated according to Angela (2006) depending on the cytoplasm and its contents, blastomers and Zona pellucida. Embryos were examined each day for further development according to Dulam, (2013), zygotes or embryos do not show further development or cleavage discarded from the medium, 50% of this medium replaced at each 24hrs. According to Edivaldo, (2013) 2-4 cells embryo was developed at first 48hr, 8-12 blastomers at 96hrs, morulla stage at 120 hr, and for the blastocyst development, not less than 6-8 days.

Recipient ewes:
Three recipient ewes were brought from the local animal selling yard in Al-Shoáalla area with no previous history concerning its reproductive status, for this reason a protocol designed to cover this unknown reproductive history in which it involved the following after applying ultrasonography examination of reproductive stat and a protocol of vaccination and anti-parasitic therapy:

Hormonal assay:
Hormonal assayed was demonstrated as discussed by Naderipour, (2012), blood samples from recipient ewes were subjected to analyzed level of progesterone (P_4) and the estrogen (E_2). Sample had been taken from recipient's jugular vein using 18 gauge needle. Samples were collected first time when recipient ewes were presented to the experiment, 2nd samples took before synchronization with progesterone sponge inoculation, and 3rdsamples took after sponge removal.

Induction of abortion:
Premature parturition, pregnancy termination or abortion induction in which, it was conducted to get non pregnant recipient ewes qualified to the experiment and be able to occupy embryos inoculation or transferred, recipient ewes must had its uterus empty with health situation. Induction of abortion was performed by two protocols;

$PGF_2\alpha$ protocol which was conducted as discussed by Tyrrell (1981), each recipient had received 2 injection of 2ml cloprostenol (250µg) 9 days apart, all results obtained was recorded.

Dexamethasone protocol which was conducted as discussed by Webster, (1981), a single intra-muscular injection of 16 mg dexamethasone was given to each recipient ewe. Recipient ewes were routinely observed after 48-72hrs for hormonal effects, all results were recorded.

Estrus\synchronization:
Induction of estrus\synchronization was conducted as discussed by Dogan, (2006), each recipient ewe was loaded with 20mg medroxyprogesterone acetate (MAP) intravaginal sponge for 14 days, day before sponges removal, an intramuscular injection of 400 IU eCG. Ram testing after 2-3 days for estrus detection, positively reacted ewes was subjected to 2-3 embryo inoculation (transferred)/ewe surgically at day six after showing signs of estrus.

Pregnancy confirmation:
Pregnancy confirmation after embryos inoculation was conducted as discussed by Ishwar, (1995), 20-22, 60 days after embryos inoculation, blood samples were obtained for P_4 assay. Between day 60-80 from inoculation recipient ewes were subjected to ultrasonography test, animal before examination laid on its back with an elevated anterior end to keep its genetalia available, abdominal and flank region were cleaned and washed carefully lubricated and then examined (abdominal examination). For rectal examination which gains more accuracy on the beginning, rectum must be evacuated carefully before probe intromission; examination must be done to investigate all area carefully searching for the attached embryo and pregnancy confirmation.

Selection of recipient ewes and preparation for embryo transferred:

Ewes recipients were submitted to the surgically embryos transferred synchronized previously. Synchronization of the recipients began (before the process of oocytes collections), by vaginal sponges, it is very important to arrange the interval between embryological developments and transferring with the time of synchronization, in which, the tolerance in estrus synchronization time is ±1 day (Gibbons and Cueto, 2001). Recipient estrus synchronization accomplished as mentioned by Gibbons and Cueto (2011) in which, a medroxy progesterone acetate vaginal sponges introduced by plastic introducer inside ewes vagina. The external genitalia were thoroughly washed with disinfectant soap, the introducer was well lubricated and intromission was directed upward and forward toward the cervix, vaginal sponges were located and leaved for 14 days. One day before the sponges' removal a 400 IU of eCG were injected. Two days after sponges' removal, ewes were exposed to the ram for estrous detection and the cyclic ewes were subjected for the surgical operation at the same time of the embryological development date was. On the day 5th (time of operation) synchronized recipients were received a dose of Xylazin (5ml), abdominal area were clipped, washed and disinfected, wiped with Bovidone iodine and prepared for midline surgical operation. These recipient ewes are evaluated as possible to be ready to accept 16-32 cells embryos or 5 days of development (Arthur, 2001).

Para-median surgical embryos transfer:

Recipient lay on its back over the surgical disc, well restrained and dressed with the surgical drape. A surgical incision performed lateral to the midline (lina-alba) anterior to the mammary gland, incision then dilated manually to give a suitable space for the intromission of the surgeon's hand toward the ewe genitalia. Surgeon's hand gently introduced via the incision pick up the genitalia and droop it outside were the two horns clearly observed to the surgeon, in which the embryos were inoculated inside the horn epsilateral to the ovary with the CL (Cseh and Seregi, 1991).

Loading of embryos:

Five ml plastic syringe connected to 18 gauge needle were subjected to embryos loading, each syringe first, has 1ml of a medium then 2-3 embryos were inspired with another 1ml medium to be ready for transferred.

Embryos inoculated:

Needle is directly injected inside the horn wall of synchronized recipient ewe epsilateral to the ovary with CL were embryos inoculated inside the horn, it is better to inject the embryos slightly below the utero-tubal junction, genitalia firmly retarded inside abdominal cavity were the incision sutured .

Results

Oocytes collections:

Fresh abattoir samples are an excellent source for oocytes in regarding to the time of collection (seasonality). Aspiration of oocytes was conducted to the follicles more than 3mm, results showed that, this follicles size was found mainly through the breeding season, while samples collected out of season being poorly bearing large follicles. As shown in table 1, the results obtained by aspiration and by slicing in breeding season were significantly higher (P<0.05) than those obtained out of the season.

Tab. (1): Methods of ova collecting according to the seasonal variations.

Methods of ova Collection	Out of Season	Within Season	Total ova number
Aspiration	110 (28.9%)	270 (71.1%)	380
Slicing	450 (28.67%)	1120 (71.33%)	1570
Total	560	1390	1950

Evaluation and grading the Ovum:

Results of ova collection in the 2 methods reveal the ability to obtain a high number of good quality ova. Abattoirs genitalia specimens are the perfect reservoir for ova soon after slaughtering in which the aspiration methods found to yield a considerable ova number with an elevated good quality index, while, the slicing methods has the ability to give more ova account with different grading. Season played a direct effect upon the ova quality in which the elevated good numbers were conducted mainly to the breeding season (Table 2).

Table (2): Grading and number of collected ova by different methods in concerning the breeding season.

Grading of collected ova	Out of Season		Within Season		Total number	
	Aspiration (n)	Slicing (n)	Aspiration (n)	Slicing (n)	Out of Season	Within Season
Good +	36 (32.72%)	165 (36.6%)	85 (31.4%)	425 (37.9%)	201	510
Good	25 (22.72%)	120 (26.6%)	65 (24%)	303 (27.05%)	145	368
Fair	27 (24.54%)	77 (17.1%)	75 (27.7%)	177 (15.8%)	104	252
Poor	22 (20.00%)	88 (19.5%)	45 (16.6%)	215 (19.9%)	110	260
Total	110	450	270	1120	520	1390

Results can be easily describe that; harvesting of good quality ova with considerable grading result but of moderate number; 459 during non-breeding season, toward 1130 within the breeding season, there is a direct effect of the seasonality in regarding to the number of ova collected and its quality.

***In Vitro* oocytes maturation (IVM):**

Matured oocytes were examined after incubation for 27-30hrs, the presence of 1st polar body or even a protrusion of the ovum cytoplasm (ooplasm) toward vitelline space were regarded as matured, results in table 3 showed that; for starting of maturation process incubated ovum need about 24-27hrs for complete maturation process, some ova lost its cumulus cell surrounding (denuded) other showed dens, more compact and transparent color cumulus cells. The great number of the oocytes that showed signs of maturation are those which surrounded tightly with cumulus cells.

Table (3): Maturation rate of collected oocytes in relation to the Breeding Season which influencing the maturity index.

Collection Method	Out of Season		Within Season		Total Maturation Rate
	Oocyte		Oocyte		
Aspiration	88	58 (65.9%)	225	165 (73.3%)	223 (71.2%)
Slicing	362	168 (46.4%)	905	585 (64.7%)	753 (59.4%)
Total	450	226 (50.22%)	1130	750 (66.37%)	976 (61.77%)

Results of oocytes maturation reveal that maturation index can be altered according to the seasonality in Iraqi ewes. The aspiration method tried for oocytes collection, although, it yields a low oocytes count but the maturity index is elevated (65.9-73.3%). when slicing method was tried for oocytes collection, it yielded an elevated oocytes count but the maturity index was low (50.22-66.73%). Maturation time under Iraqi environments slightly prolonged than 24hrs and reached about 27-30 hr. Washing of the ovaries with saline before processing and additional of antibiotics and antifungal to the maturation medium minimized contamination that interfered with

maturation process and yielding an elevated matured oocytes count.

Spermatozoa Capacitation:
Results showed that; Heparin which is a high sulfated trisaccharide has a positive effect upon ram spermatozoa capacitation, and this capacitated ram sperms still gain its ability for fertilization after this period of maturation. The more obvious sign of the capacitation is the forward motility which is an indication of increasing sperms activities and this is, mainly, due to the function of heparin upon facilitating the fertilization ability of the spermatozoa in ram. Results also showed that, time needed for complete capacitation is so limited to that time of incubation (45-60 minutes) which ended by the acrosomal reaction. Spermatozoa when is added to the medium containing the matured oocytes, directly and furiously, swim toward the matured oocytes seem to be its targets.

***In Vitro* fertilization:**
Spermatozoa still gaining the ability to fertilize matured oocytes and produced good quality embryos (IVP). The way of choosing this oocytes category (grade) or the good and fair to embryos production is a suitable method for gaining good quality embryos (Table 4).

Tab. (4): Showed the effect of collection methods and season upon fertilization index.

Collection Method	Out of Season			Within Season		
	Total Ovum (n)	Good (n)	Fair (n)	Total Ovum (n)	Good (n)	Fair (n)
Aspiration	58	19	12 (20.60%)	165	75	68
Slicing	168	48	33 (19.60%)	585	153	133
Total	226	67	45 (19.91%)	747	228	201

There was a seasonal influence the fertilization incidence among the Iraqi ewes that affects the quality and number of embryos produced; the methods of oocytes collection altered the quality and the number of embryos production. According to the number of embryos obtained, preserved caudal spermatozoa still gain the fertilization ability.

Hormonal analysis:
These results were depending upon the time that the samples were taken and shown in table 5.

Table 5: described the hormonal analysis of the six recipients;

Number of Recipient	Before P_4 treatment *		After P_4 treatment	
	P_4 ng\ml	E_2 ng\ml	P_4 ng\ml	E_2 ng\ml
1	0.1	35	2.7**	126
2	0.2	48	12.4***	110
3	0.1	22	0.3	67
4	0.1	3.0	0.3	88
5	0.1	32	0.6	77
6	0.1	25	0.4	155

*Elevation E_2 level may be due to follicular stage of the cycle.
**Early pregnancy.
***Pregnant recipient (progress).

Induction of abortion:
$PGF_2\alpha$ protocol:
The result showed that; this regime is effective mainly when ewe was at the stage of early pregnancy, in which, only one early pregnant recipient ewe was aborted in regarding to the aborted small lamb.

Dexamethasone protocol:
One advanced pregnant recipient ewe was aborted (immature lamb delivered), the small and weak delivered lamb died after three days. This result described the effectiveness of the dexamethasone mainly with the advanced (later) pregnancy to establish an abortion in ewe.

Induction of estrus \synchronization:
Intravaginal sponges for 14 days with an eCG injection last day seems to be a positively induced estrus in ewes, P_4 levels after sponges withdrawal seem to be decreased as the E_2 levels start to increase as showed in table 6.

Table (6) described the P_4 and the E_2 levels after vaginal sponges' withdrawal.

No. of Recipient ewe	P4 ng\ml	E2 ng\ml
1	1.1	48
2	1.1	60
3	1.3	45
4	1.2	43
5	1.5	68

No. of recipients was five only, one advanced pregnant recipient stayed as control.

Three of elevated E_2 level recipients were conducted to the embryos inoculation.

Pregnancy confirmation:
Results showed that; one of the three recipients had an elevated P_4 at first analysis with positive confirmation by ultrasonography at 65 days followed inoculation.

Discussion
Oocyte collection
Fresh abattoir female genital specimens regarded as one of the main sources or reservoir for oocytes .Oocytes were collected by two methods, aspiration, for good quality oocytes, with less time and moderate number, mainly applied within season when follicular size reached more than 3mm (Mermillod *et al.,* 2006), while slicing method, were applied with no seasonal influences, more oocytes number of good , fair and poor quality, time consumed by this method was slightly prolonged than the first one but it is so simple and effective (Mistry and Dhami, 2009).

Evaluation and oocytes grading:
The more important target of this step is to collect a good+, good and fair grade oocytes that moved to the next one which is the IVM Grading system used by this study in depending on cumulus cell arrangement with the morphological pictures of the oocytes cytoplasm and its contents lead to get best results, and the use of good+ and good grading point for the first time will reflect on improving these results, Petrean (2009) and Al-Timimi (2013) found the same results and put the same grading method. Wang and Sun (2007) mentioned same result that; the traditional methods for the evaluation of the oocytes quality are based on morphological classification of the follicles, cumulus-oocyte complex (COCs) and cytoplasmic contents. Kristina, (2009) used the same method for oocytes evaluation in depending on the arrangement of the cumulus cell complex, polar body shape and size, zona pellucida structure and thickness and the vitelline shape are also used. Davachi, (2012) share the same result, in which, season may affect abattoir oocytes quality and quantity, collection out of season altered the final results.

In Vitro oocyte maturation (IVM)
Time needed for complete maturation under Iraqi environment is about 27-30hr; this altered index might be due to the donor ewe maturity, seasonality or the healthy index of donor ewe. Oocytes maturity index as 1st polar body development or even the beginning of a protrusion inside the vitelline space

were developed safely and co ducted as positive, aspiration method was yielding elevated matured oocytes more than the slicing one. Cahill, (1981) mentioned same effects of season on sheep in developing large size follicles (more than 3mm) in which the antral follicles grow rapidly under influence of gonadotropins within the breeding season. Lasien , (2009) agreed that maturation of oocytes includes two interrelated processes, maturation of nucleus and cytoplasm, in which, under stimulation of anterior pituitary luteinizing hormone (LH) *in vivo*. Muhammad-Baqir and Al-Moussawi (2013) mentioned same result in regarding to oocyte maturity in depending on the presence or absence of the first polar body and \or the compacted or expanded of the COCs is the method for oocyte evaluation

Sperm Capacitation

Result showed that the forward motility is the more obvious sign for capacitation and good indication for spermatozoa activity. Al-Timimi, (2013) and Majeed, (2013) mentioned the same signs that related to spermatozoa capacitation.

Heparin used to facilitate and induces fertilization ability of ram spermatozoa, Parrish (1988) found same result concerning the effect of heparin on ram spermatozoa capacitation. Gliedt (1996) found that; glycosaminoglycan (heparin) affect the cleavage increased of the zygote within 24hr. Isabelle (2003) study the additional of progesterone (P_4) with heparin and mentioned that this additional appeared to stimulate sperm capacitation with acrosomal reaction.

In vitro Fertilization (IVF)

Fertilization ability is the final and more accurately index in confirming integrity of abattoir caudal spermatozoa, which preserved for long time and still gains its ability to fertilize a mature oocyte and produces a good quality embryo. Results showed that; under Iraqi circumstances abattoir genitalia specimens (male and female) representing very good sources in obtaining oocytes and spermatozoa and when prepared well they produced good quality embryos. Season, health status of donors (ram or ewe) in which it is unknown (abattoir samples) and some technical issues affect the quality and quantity of the final target (embryo). This study is confirmed tightly that; there is ability to produce good quality embryos without any hurt to the donor male or female. Cognie, (2003) and Al-Timimi, (2013) mentioned this ability in embryos production without any harm effect to the donors. Cox, and Alfaro, (2007) and Freitas, (2010) try to minimize the hurt effect of the surgical oocytes collection by using the Laparoscopy pick-up method in oocytes collection for IVF.

References

1. **AL-TIMIMI, I. H. (2013).** Using of Epididymal Sperms of Local Iraqi Bucks for *In Vitro* Fertilization. **Al-Qadisiyah Sci. J. Vet. Med. Vol. 12. No.2.**
2. **Anamaria, LP. Groza, I., Bogdan, L., Simona, C., and Borzan, M. (2009).** Morphological evaluation of mature sheep Oocytes collected by median laboratory. T he Euro-Mediterranean Student Research Multi- conference.9 June.
3. **Angela, S., Prefac, G., PAUL, T., Alexadru, α and Dana, T. (2006).** Evaluation of sheep embryo quality by morphologic methods advantage and disadvantage. Journal of Tekirdage Agricultural Faculty 3 (1) p; 68-70
4. **Arthur, G.H. Veterinary Reproduction & Obstetrics (theriogenology). 2008.**
5. **Cognie, Y., Baril, G., Poulin, N. and Mermillod, P. (2003).** Current status of embryo technologies in sheep and goat**.** Theriogenology 59(1): 171-188.
6. **Cox, J.F., and Alfaro. (2007).** In vitro fertilization and development o OPU derived goat and sheep oocytes**.** Report. Domest. Anim. 42(1): 83-87.
7. **Davachi, ND, Zeinoaldini, Saeed and Kohram, H. (2012).** A novel ovine oocyte recovery method from slaughterhouse material. Small Ruminant Research. Vol.106 (2-3) p: 168-172.
8. **Dogan, Z. NUR (2006).** Different estrus induction methods during the non-breeding season in Kivircik ewes. Veterinarni Medicina 51(4); 133-138.
9. **Dulam, S., Kaladhar, DS.V.GK. Nagendra, SY. Thomas, VM. and Palnisamy, A. (2013).** Effect of protein supplementation on *invitro* Maturation of sheep Oocytes and *in vitro* culture of Preimplantation with -Tocopherol supplementation in CRIaa medium on sheep embryo to the blastocyst stage. Journal of Aller.Ther. 4:133.
10. **Ezer, N. and Robaire, B. (2002).** Gene expression is differentially regulated in the epididymis after Orchidectomy. Endocrinology 144: 975-988.
11. **Farag, I.M., Girgis, S.M., Zowail, ME. And Abd El-Hafez, M.A.M. (2013).** *Invitro* maturation of camel (camelus dromedaries) Cumulus-Denuded oocytes. World applied Science Journal 26(3): 352-359.
12. **Freitas, VJ. de Figueiredo, and Melo, LM. (2010).** In vitro embryo production in small ruminants. R. Bras. Zootec. V39 p; 409-413.
13. **Gliedt, DW. Rosenkrans, CF.Jr. Rorie, RW.and Rakes, JM. (1996).**Effects of Oocytes maturation length, sperm capacitation time and heparin on bovine embryo development**.** J. Dairy Sci. 79(4): 532-535.
14. **Isabella, T. and Puttaswamy, M. (2003).** Effects of Progesterone on bovine sperm capacitation and acrosome reaction1. Biol. Reprod. 69 (4): 1408-1415
15. **Ishwar, A.K. (1995).** Pregnancy diagnosis in sheep and goat: a review. Small Ruminant Research V 17(1) P; 37-44.
16. **Kaabi, M., Paz, P., Alvarez, M., Anel, E., Boixo,JC, (2003).** Effect of epididymis handling condition on the quality of ram spermatozoa recovered post-partum. Theriogenology 60(7): 1249-1259.
17. **Karja, N.W.K., Fahrudin, M. and Setiadi, M.A. (2013).** *In vitro* fertility of post-thawed Epididymal Ram spermatozoa after storage at 5 before cryopreservation. Media Peter nakan, April: 2013 pp; 26-31.
18. **Lasienα, K., Vitkus, A., Valanαiutė, A. and Lasys, V. (2009).** Morphological criteria of oocytes quality. Medicina (Kaunas) 45(7); 509-515.
19. LIMA; MARCOS ANTÔNIO LEMOS DE OLIVEIRA**. (2013).** LIMITS AND PERSPECTIVE. 2006 Symposium COA/INRA Scientific Cooperation in Agriculture, Tainan (Taiwan, R.O.C.), November 7-10
20. **Mermillod P., Locatelli, Y., Dalbiès-Tran, R., Uzbekova, S., Baril, G., Guignot, F., Perreau, C., Poulin, N., Touzé, J. L., Pennetier, S., Schmaltz, B. and Cognié, Y. (2006).** IN VITRO PRODUCTION OF RUMINANT EMBRYOS: RESULTS,
21. **Miller, Y. and Robertson, S. (1959). Cited by; Sisson, S. (1975)** the anatomy of the domestic animals.
22. **Muhammad-Baqir, M-R Fakhrildin and Raghad, H. Al-Moussawi. (2013).** Effect of two types and two concentrations of Cryoprotectant on ovine Oocytes morphology and viability post- vitrification. Iraqi J. embryology and fertility Researches. Vol.3 No. 6 .
23. **Parrish, J.J., Susko-Parrish, J., Winer, M.A. and First, N.L. (1988).** Capacitation of bovine sperm by heparin. Biol. Reprod. 38: 1171-1180
24. **Rehman, A.N.M.A., Abdulla, R.B. and Wan-Khadijah, (2008b).** *In vitro* maturation of Oocytes with special reference to goat. AREVIEW. Biotechnology, 7: 599-611.
25. **Roberts, S.Y. (1986).** Large animal theriogenology. SUANDERS. ELSEVIER.
26. **Sofi, KA., (2012).** In vitro maturation of ovine oocytes. MVSc Thesis. Submitted to faculty of post Graduate Studies, SKUAT-Kashmir. P: 49.
27. **Tyrrell, RN., Lane, JG., Nancarrow, CD. And Connell, PJ (1981).** Termination of early pregnancy in ewes by use of a prostaglandin analogue and subsequent fertility. Aus. Vet. J. 57(2):76-78.
28. **Wang, Z.G., Yu, S.D. and Xu, Zi-rong (2007).** Effect of collection Methods on Recovery Efficiency, Maturation Rate and subsequent embryonic development competence of oocyte in Holstein cow. Asian Aust. Animal Sci. Vol. 20(4): Pp: 490-500
29. **Wani, A.R., Khan, M.Z., Sofi, K.A., Malik, A.A., Lone, F.A. and Bhat, F.A.(2013).** Effect of follicular size on in vitro maturation, fertilization and culture of sheep embryos. Iranian Journal of Veterinary Research, Shiras University, vol14 No. 4 Page; 299-304.
30. **Wani, N.A., Wani, G.M., Khan, M.Z. and Salahudin, S. (2000).** Effect of oocyte harvested techniques on *in vitro* maturation and in vitro fertilization in sheep. Small Ruminant Research 36: 63-67
31. **Webster, G.M. and Haresign, W. (1981).** A note on the use of dexamethzone to induce parturition in ewe. Animal Reprod. vol.32 (3) p; 341-344.
32. **Yanagimachi. (1994).** Mammalian fertilization. In: Knobil, E., Neil, JD., (Eds). The physiology of Reproduction 2nd edn. New York: Raven Press Ltd. 189-317