EFFECT OF HEPARIN, ESTROGEN ON EPIDIDYMAL SPERM CAPACITATION AND *IN VITRO* FERTILIZATION IN IRAQI SHEEP

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ABSTRACT

This study was designed to study the capacitation of caudal spermatozoa *in vitro* by different levels of heparin, estrogen for *in vitro* fertilization (IVF) in the Iraqi local sheep. Results of sperm capcitation (massive motility percentage) by applying three levels of heparin (50, 100, 150) IU in relation to breeding season showed no significant differences during breeding season with the three levels, which were (82.23 ± 0.58), While results by applying the higher level of heparin (150) IU out of breeding season showed significantly (P<0.05) more and active motility Which were (62.07 ± 0.62) than the other levels (56.85 ± 0.61). At the same time applications result of three estrogen levels (20, 40, 60) mg on sperm capcitation showed Positive relationship between concentrations and (massive motility percentage), which showed the highest concentration gave the best results during and out of breeding season (87.20 ± 0.60) and (65.86 ± 0.62) respectively. with significant differences (P<0.05) between the three levels. While the results of the *in vitro* fertilization (IVF) index which reflected the sperm capcitation were recorded with the highest estrogen concentration during breeding season (22.70 %) compared with the best heparin level (20 %) while she was (10.45 %), (8.70 %) out of breeding season Sequentially, in which the highest estrogen concentration gives a best capacitation and IVF index level compared with the high heparin level over the year.

Key words: fertilization, in vitro, testis, sheep, capacitation, spermatozoa, oocyte, epididymis, motility, abattoir, heparin, estrogen.

تأثير الهيبارين، الاستروجين على تكييف حيامن البربخ ودورهما في الاخصاب الخارجي في الاغنام العراقية

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المستخلص

صممت هذه الدراسة حول تمكين تكييف النطف المأخوذة من ذيل البربخ مخبريا بواسطة مستويات مختلفة من مادة الهيبارين، الاستروجين لزيادة قدرة الاخصاب الخارجي للنطف في الأغنام المحلية العراقية. نتائج تكييف النطف (النسبة المئوية للحركة الجماعية) باستخدام ثلاثة مستويات من مادة الهيبارين (50، 100، 150) وحدة دولية وعلاقتها بموسم التناسل أظهرت عدم وجود فارق معنوي خلال الموسم التناسلي فيما بين المستويات الثلاثة والتي كانت (0.58 ± 82.23)، بينما أظهرت النتائج ان استخدام أعلى مستوى من الهيبارين (150) وحدة دولية خارج موسم التكاثر والتي كانت (0.62 ± 62.07) أن هناك فارق معنوي (P<0.05) في زيادة النشاط الحركي عن باقي المستويين الاخرين واللذين كانت قيمهما متشابهة (0.61 ± 56.85). في نفس الوقت أظهرت نتائج تطبيق ثلاث مستويات من هرمون الاستروجين (20، 40، 60) ملغم على تمكين قدرات الحيوان المنوي وجود علاقة إيجابية بين التراكيز والنسبة المئوية للحركة الجماعية، والتي أظهرت أن أعلى تركيز أعطى أفضل النتائج خلال موسم التكاثر وخارجه (0.60 ± 87.20) و (0.62 ± 65.86) على التوالي مع وجود فروقات معنوية (P<0.05) بين المستويات الثلاث. بينما نتائج مؤشر الاخصاب الخارجي التي تعكس تكييف النطف سجلت باستخدام اعلى تركيز للأستروجين خلال الموسم التناسلي (% 22.70) مقارنة مع استخدام اعلى مستوى للهيبارين 20) (%بينما كانت (% 10.45)، (% 8.70) خارج الموسم التناسلي بالتتابع، بحيث يعطي أعلى تركيز لهرمون الاستروجين أفضل نتائج ومؤشر اخصاب خارجي مقارنة باستخدام اعلى مستوى هيبارين على مدار العام.

الكلمات الدالة: اخصاب، خارجي، خصية، أغنام، تكييف، النطف، البويضة، البربخ، الحركة، مجزرة، هيبارين، استروجين.
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INTRODUCTION

In vivo, male spermatozoa after ejaculation stay a few hours in female genital tract, with no ability to fertilize oocytes (1). Within the female genital tract, spermatozoa undergo several changes including biochemical, metabolic and structural changes, these events, were known as capacitation (2). The biochemical changes in sperm plasma membrane include an increase in plasma membrane fluidity, increase in Ca+2 ions influx, induction of tyrosine phosphorylation and increase in sperm metabolism and expression of hyperactivity (3) Capacitation process causes sperm to undergo a spontaneous acrosome reaction (AR). Physiological agonists are suggested to be inducer of AR including zona pellucida protein, follicular fluid and oviduct fluid (4). Capacitation involves a series of molecular activities that include reorganization of membrane proteins, metabolism of phospholipids, reduction in cholesterol level, and hyperactivation of sperm cells (3،5). This capacitation associated changes induce acrosome reaction, which is an irreversible exocytotic event and a prerequisite for a sperm to bind and penetrate the zona pellucida to fuse with the oocyte plasma membrane during the process of fertilization in the female reproductive tract (3). Acrosomes are necessary for protecting and releasing the enzymatic contents at the right time and place for effective fertilization. The enzymes stored between the inner and outer membrane of acrosome, when released at the time of acrosomal reaction, act sequentially and specifically on the cumulus, corona radiate, and zona pellucida of the ovum. Furthermore, the plasma membrane undergoes capacitational changes and acrosomal reaction in the uterine environment, which is a prerequisite for successful fertilization (6). However, in vitro capacitation is possible in the absence of reproductive tract fluids with the help of specified media containing various compounds like bicarbonate, calcium, heparin, HEPES, and serum albumin that mimic the oviduct fluid (7) First and (8), proposed that heparin-like glycosaminoglycans remove decapacitation factors from the sperm plasma lemma and play a direct role in calcium uptake. Glycosaminoglycans (GAGs) are essential components of the extracellular matrix, contributing to cell recognition, cellular adhesion and growth regulation. Individual GAGs ($C_{18}H_{32}O_{17}$) are characterized by their sugar residues and other features, such as sulfation. Four main groups can be distinguished: a- the non-sulfated GAG, hyaluronan, b- the sulfated GAG keratin sulphate, c- heparin / heparin sulfate and d- the sulfated galactosaminoglycans chondroitin sulfate/ dermatan sulfate (9). Heparin has been extensively used in vitro to study the endogenous role of heparin-like GAGs secreted by the epithelium of the female reproductive tract. Its capacitating effect on bovine (10) and human (11) sperm has been established. However, its action on pig sperm physiology is still unclear. In cattle, heparin is thought to promote capacitation by binding to and removing seminal plasma proteins that are adsorbed to the sperm plasma membrane, and would inhibit capacitation (12). In this mammal, heparin also produces a rise of sperm intracellular pH and Ca+2 concentration (10), protein phosphorylation and modification of motility parameters (13). Estrogens (E2) are a group of steroid compounds, named for their importance in the estrous cycle. Although estrogens have been considered mainly female reproductive hormones, they also play an important role in regulating male reproductive functions (14). Steroid hormones (17- β estradiol and progesterone) are present in the female reproductive tract. Also, receptors for both hormones are present in ram spermatozoa. Estradiol increased the progressive motility after incubation, whereas progesterone improved the sperm viability, both hormones decreased the non-capacitated sperm rate and increased the percentage of capacitated sperm, and progesterone also increased the acrosome-reacted sperm rate, however, no differences were found in the fertilizing, cleavage or blastocyst rate when treated spermatozoa were used in IVF procedures, steroid hormones can stimulate in vitro ram sperm capacitation and the acrosome reaction (15). Progesterone (P4) and 17-β estradiol (E2) are present in the female genital tract. The concentrations of these hormones in the follicular fluid have been estimated in the

nanomolar range (16), and part of this fluid is released into the oviduct together with the oocyte at the moment of ovulation. Furthermore, after ovulation, the cumulus cells surrounding the oocyte secrete P4 and E2 (11 ، 17), which could reach micromolar levels (18) and diffuse to form a molecular gradient toward the edge of the cumulus matrix and beyond (19). This study was designed to investigate the effect of capacitation on caudal spermatozoa by different levels of heparin, estrogen for *in vitro* fertilization (IVF) in the Iraqi local sheep.

MATERIALS AND METHODS

Abattoir female and male gonads: Specimens were collected from Al-Shoáalla slaughterhouse Located On the western side of Baghdad during November.2017 to October 2018 as:

a-Adult Ram Testes (175 pairs) were collected from mature local rams; age is determined as possible according to the dental tables (Miller and Robertson, 1959) in which; (105) pairs was collected within season from (27-11-2017 to 19-02-2018), while (70 pairs) was collected out of season from (04-04-2018 to 31-07-2018).

b-Adult ewe's ovaries of local breed (162 specimens) were obtained directly after slaughtering, as (72 pairs) was collected within season from (26-11-2017 to 16-01-2018), and (90 pair was collected out of season from (03-04-2018 to 30-07-2018). Specimens of female and male genitalia were washed by tap water and carried from the slaughterhouse to the Laboratory by cool box (4 - 8°C).

Preparation of spermatozoa samples

Testicular samples with attached epididymis of adult rams were collected directly after slaughtering, all collected samples were washed by tape water then kept in cool boxes of (6 - 8 °C), transferred to the lab. Testicles Specimens then washed with distill tap water again, then normal saline with 100 IU/ ml penicillin and 0.1mg/ ml streptomycin antibiotics, then carefully separated and refreshed from surrounding tissues and fascia, its parameters as weight, size and orientation determination by the adjustment of epididymal positioning (head of epididymis mainly laid laterally) as mentioned by (20). Dissected and separated of the epididymis from the entire testicle, length and weight of epididymis, caudal weight and size were measured. Caudae samples were injected with 5-8 ml of the warmed normal saline. Injected Caudae were sliced to small pieces in order to examine under light microscope for mass spermatozoa motility estimation and stained smear by (Eosin and Nigrosine stain) for dead or alive and sperms abnormalities detected. All results were recorded (21). Spermatozoa were evaluated under light microscope, an individual motility more than 50% were incubated in 5% CO_2 incubator at (39 °C) at six hours for further maturation, presence of distal protoplasmic droplet was the criterion of sperm maturation (21). Caudal spermatozoa samples which were prepared previously subjected to capacitation process (pre-fertilization degree) the selective sperms samples were divided into two (2) groups according to the following transactions:

1- Treatment a certain number of sample with three estrogens (Estradiol Benzoate) level according to the concentrations mentioned, level with the best result would be propagated to the experiment. Data should be recorded: a- 20 mg estradiol benzoate. b- 40 mg estradiol benzoate. c- 60 mg estradiol benzoate.

2- Treatment of each sample with different levels of heparin (Heparin Sodium) as a- 50 IU 0f heparin. b- 100 IU 0f heparin. c-150 IU 0f heparin. Each samples of different heparin: estrogen level would be incubated for spermatozoa capacitation in CO_2 incubator for 45-60 minutes, head to head agglutination dissociation of distal proximal droplets were the criterions for capacitation index (22).

Preparation of oocyte samples for IVF

Ewe's ovaries of unknown history had been collected from Al-Shoáalla abattoir during October 2017 to January 2018, preserved in cool box at (6 - 8°C), ovaries were separated carefully from all surrounded tissues, were submerged in a beaker containing normal saline added to it penicillin-streptomycin for (5-10) min, then all samples were sliced to very small pieces in dishes contained (5-8) ml of Minimum Essential Medium (MEM) and left at room temperature for (15 -30 min) for further settlement.Collected ovaries kept in a glass Petri-dishes containing (6 – 8) ml of (MEM), ovaries sliced to small particles and

left to stabilized at room temperature for 15 minutes, then floated medium examined under inverted microscope for the oocytes. Evaluations of collected oocytes were done as mentioned by (23) in regarding to the arrangement of cumulus cell surrounding oocytes and status of cytoplasm as:

a- Very Good grade: the oocytes with a transparent, homogenous and uniform cytoplasm surrounded with high density layers of cumulus cells. (Figure.1).

b- Good grade: the oocytes with a transparent, homogenous and uniform cytoplasm surrounded with few layer of cumulus cells. (Figure.2).

c- Fair grade when the oocytes with transparent, less homogenous (Small granules exist) and uniform cytoplasm and less compact cumulus cells partially surrounded. (Figure.3).

d- Poor grade when the oocytes with mild or absent cumulus (denuded) with dark and granular cytoplasm (Figure.4). Very Good, good and fair categorized oocytes were subjected to the following process of *in vitro* maturation (IVM) with maturation medium (Table.1), oocytes after grading were transited to another Petri-dishes containing maturation medium by Pick up with an automatic micropipette, the final dishes were re-checked it after these selected ova transport, and to verify that all selective ova were transport. Ova were grading and counting and recorded.

Table 1. the supplements that added to the MEM

Supplements	Once. %
Minimum Essential Medium (MEM)	10 ml
Bovine Serum Albumin (BSA)	10%
Fetal Calf Serum (FCS)	10%
Follicular Stimulating Hormone (FSH)	10 µg
Luteinizing Hormone (LH)	10 µg
Penicillin& Streptomycin (PS)	50µg per ml.
Antifungal solution (Nystatin)	50µg per ml.

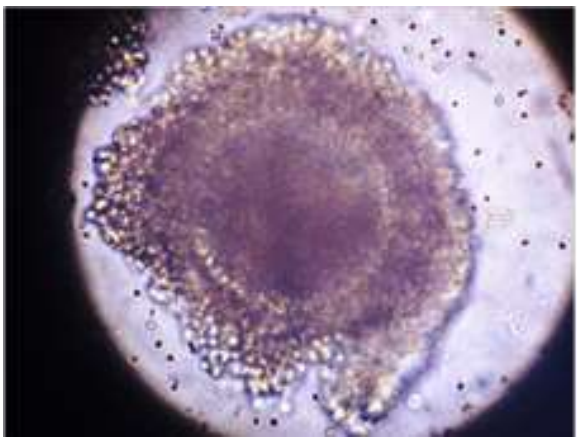

Fig.1 Very Good grade oocyte

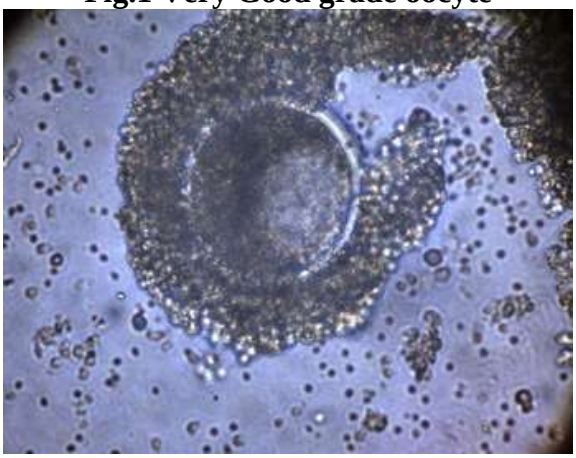

Fig.2 Good grade oocyte

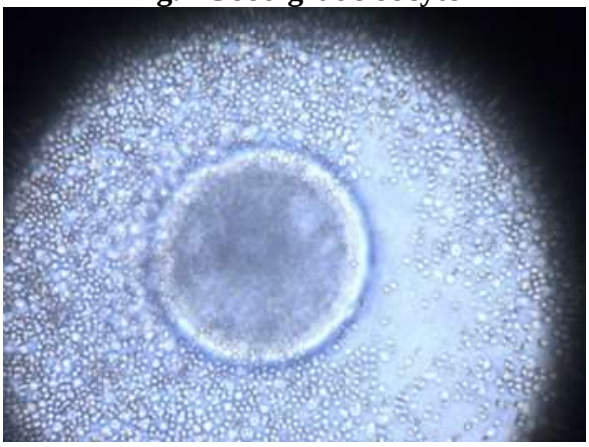

Fig.3 Fair grade oocyte

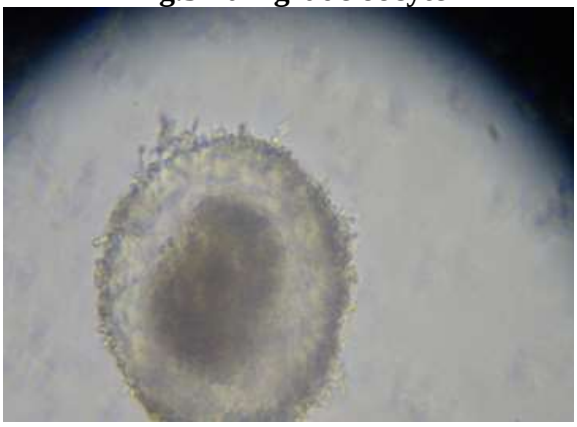

Fig.4 Poor grade oocyte

***In vitro fertilization* (IVF)**

Matured oocytes of very good, good and fair grades were only subjected to the process of fertilization (Figure.5), matured and capacitated spermatozoa (1-2X 10^9 ml) were added to the maturation medium in advance containing matured oocytes. Mixture of two gametes was incubated at 5% CO_2 level at 39 ℃ and 90% relative humidity for (26-30) hours. Fertilization media supplemented with, LH, FSH, BSA, FCS, antibiotics and antifungal preparation, signs of fertilization achievement is indicated by dissociation of second polar body and cell splitting to two (Figure 6), four (Figure 7) and…. etc. cells (embryo).

RESULTS AND DISSCUSION

1- Effect heparin concentrations on spermatozoa capacitation in regarding to breeding season

Results of different heparin level for spermatozoa capacitation in relation to seasonal influences revealed that; in regarding to capacitation index showed as an increased spermatozoa activity and motility with no significant difference in the massive motility percentage between the three levels of heparin during breeding season, capacitation index is the same for all three levels. But results showed significant variations between the three heparin levels out of breeding season in which the highest heparin level gave the best capacitation index than the other two (Table.2)

Table 2. Effect of heparin level under season influences on capacitation index

Season	Heparin conc. (IU)	Mean ± SE
With season	50	82.23 ± 0.58
	100	82.23 ± 0.58
	150	82.23 ± 0.58
out of season	50	56.85 ± 0.61
	100	56.85 ± 0.61
	150	62.07 ± 0.62 *
LSD		9.317

* (P<0.05).

2-Effect of estrogen (17-β estradiol) additional to the capacitation medium upon spermatozoa parameters in regarding to breeding season

applications result of three estrogen levels (20, 40, 60) mg on sperm capcitation showed Positive relationship between concentrations and (massive motility percentage), which showed the highest concentration gave the best results during and out of breeding season (92.23 ± 0.56) and (65.86 ± 0.62) respectively with significant differences (P<0.05) between the three levels. It's mean that estrogen as a steroid hormone modulates the capacitation process in related to the spermatozoa parameters with significantly variable with an increasing concentration limit, even under influences of seasonal factor. Estrogen reacted very well upon spermatozoa capacitation when its concentration is being elevated even during or out of breeding season (Table.3).

Table 3. Effect of estrogen conc. on mass motility in within and out of season

Season	Estrogen conc. mg	Mean ± SE
Within season	20	82.14 ± 0.59
	40	87.23 ± 0.56 *
	60	92.23 ± 0.56 *
out of season	20	56.85 ± 0.61
	40	62.86 ± 0.61 *
	60	65.86 ± 0.62 *
LSD value	---	9.468 *

* (P<0.05) .

3-Effect of season upon ovum collection and evaluation: Results of the oocyte collection showed that; total oocytes number is not significantly variable between both season, but when oocytes quality is concerned very good and good quality oocytes showed significant and variable account, both quality oocytes are quiet elevated within season not its account only as shown in (Table.4).

Table 4. Oocytes account and quality grading within and out of season

Season	Oocyte grade				Total number
	Very good	Good	Fair	Poor	
Within season	388 (37.3%)	331 (31.8%)	198 (19%)	122 (11.7%)	1039
Out of season	137 (13.6%)	200 (19.8%)	305 (30.3%)	364 (36.1%)	1006
Total number	525 (25.6%)	531 (25.9%)	503 (24.5%)	486 (23.7%)	2045

Results of IVF index in relation to seasonal factor: While the results of the in vitro fertilization (IVF) index which reflected the sperm capcitation were recorded with the highest estrogen concentration during breeding season (22.70 %) compared with the best heparin level (20 %) while she was (10.45 %), (8.70 %) out of breeding season Sequentially, in which the highest estrogen concentration gives a best capacitation and IVF index level, with significant differences ($P<0.05$) compared with the high heparin level over the year. (Table.5)

Table 5. IVF index (Embryo production) Different medium

Season	Estrogen %	Heparin %
Within season	22.70	20.00
Out of season	10.45	8.70
Chi-Square	4.529 *	4.284 *
P-value	* (P<0.05).	

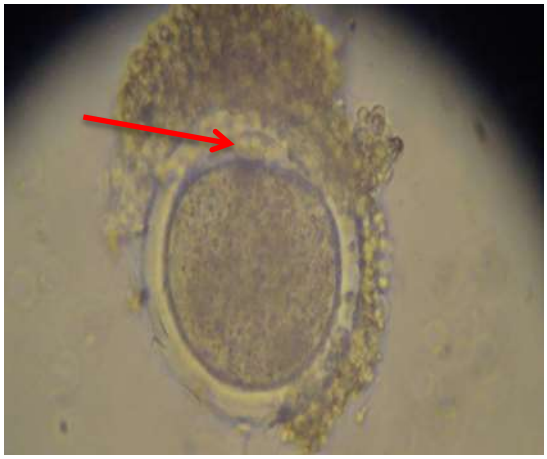

Figure 5. Very good grade mature sheep oocyte (red arrow) denoted first polar body

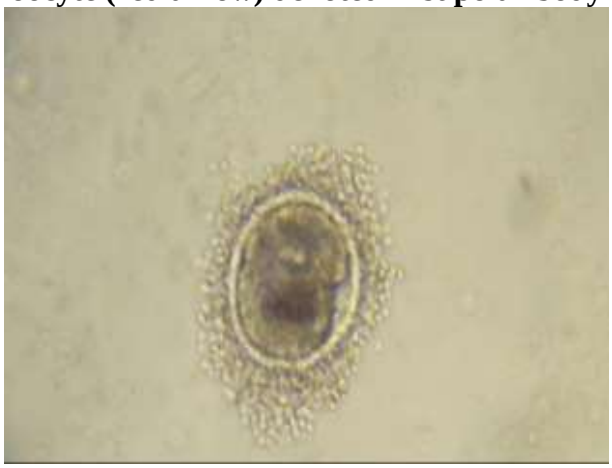

Figure 6. (24-36) hrs. two cells embryo

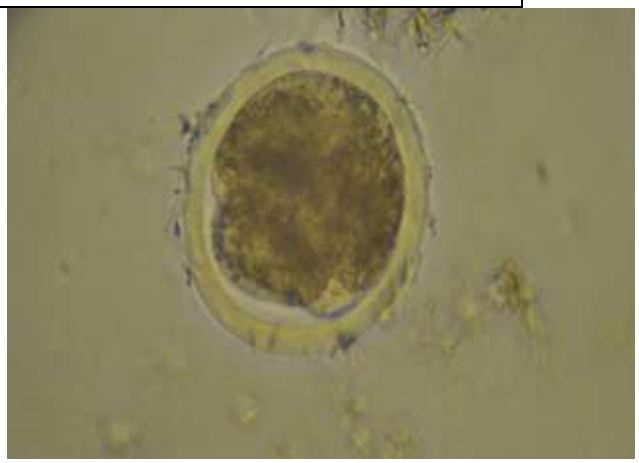

Figure 7. four cell embryo

Effect of heparin level on spermatozoa capacitation under seasonal influence

Results of this study showed no significant variation concerning the usage of heparin as a capacitation inducer in different levels, the degree of spermatozoa massive motility is regarded to evaluate the capacitation index, but when this results are influenced by seasonal factors mainly out of breeding season this results significantly ($P<0.05$) showed variation, low heparin level showed moderated capacitation index, this in turn would be increased toward elevated index as heparin level elevated. (24) agreed with this results and showed that sperm capacitation with different heparin levels mainly showed variable index when seasonal factor influenced the final result. (10) revealed that; the in vitro effect of heparin described indicates that sulfated glycosaminoglycans, which are normally present in the female reproductive tract, might

play an important role in the fertilization process, which was further increased by the addition of heparin due to its effect on capacitation, so heparin enhances in vitro capacitation of sperm only under capacitating conditions. Additionally, we observed that heparin binding sites were located mostly on the sperm acrosomal region in a specific and saturable manner. (25) upon his research concerning the action of heparin on sperm capacitation and acrosome reaction in which; sperm acrosomal status (capacitation and acrosome reaction) and viability were evaluated by heparin. The cleavage and blastocyst rates were significantly increased It was founded a satisfactory model to estimate the cleavage and blastocyst rates by AR induction test. Therefore, it can be concluded that the induction of the AR test is a valuable tool to predict the IVP in cattle and sheep. (26) approved that; even the capacitated medium may have contained many proteins supplement for in vitro capacitation, heparin in the incubation media was necessary for the capacitation and tyrosine phosphorylation in a way that affect the IVF and IVP.

Effect of estrogen level on sperm capacitation influenced by seasonal factor

Results of this study expressed that; estrogen has a direct effect on ram spermatozoa capacitation even with seasonal influences. Season controlled the hormonal effect on capacitation but level of these hormones (estradiol) has more incidence than seasonality, the high hormonal level gives more capacitation index than the low one within and out of breeding season. Estrogens are a group of steroid compounds, named for their importance in the estrous cycle, (27) agreed with this; although estrogens have been considered mainly female reproductive hormones, they also play an important role in regulating male reproductive physiological functions. (28) approved these results in which; ovine spermatozoa have steroid (estrogen) receptors and that steroid hormones are related with the induction of the capacitation and acrosome reaction. The addition of E2 to *in vitro* capacitation medium resulted finally in a higher acrosome-reacted sperm rate, and in turn this may elevate the sperm fertilizable ability and more embryos production. (15) put his agreement in which; estradiol increased the sperm progressive motility after incubation, and decreased the non-capacitated sperm rate and increased the percentage of capacitated sperm, although, no differences were found in the fertilizing, cleavage or blastocyst rate when treated spermatozoa were used in IVF procedures, but in conclusion, steroid hormones can stimulate *in vitro* ram sperm capacitation and the acrosome reaction. Abattoir genitalia specimens (male and female) comprised as a very good sources for gametes utilization if tolerated well due to its easy collected, cheapest, more samples present, always available upon request, easily preserved, easily accessible and easily transferred. Ram caudal spermatozoa after capacitation and Ewes oocytes can be propagated to the Assist Reproductive Technology (ART) as IVM, IVF, IVP, and ICSI with high efficiency. Donor ewe and ram age, ram testicular orientation, breeding season, time between slaughtering to processing and preservative temperature upon specimen's collection affect the final parameters results.Testicular orientation (left and right) has no effect upon spermatozoa massive motility, viability (dead or alive) and abnormalities as breeding season do. Spermatozoa capacitation is well improved with heparin and more modulated when heparin mixed with estrogen. There is no need to elevate heparin level within season but it is very important to increase its level out of breeding season for accomplish sperm capacitation. The Statistical Analysis System (SAS 2012) program was used to effect of difference factors in study parameters least Significant Difference – LSD test was used to significant compare between means in this study.

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