Evaluation of in Vitro Fertilization Index by Caudal Spermatozoa Capacitation with Different Heparin Concentration in Iraqi Sheep

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Summary

Sperm capacitation refers to the physiological changes in which spermatozoa must undergo in order to have its ability to penetrate and fertilize a mature oocyte. Recognition of the phenomenon was quite important to early in vitro fertilization (IVF) experiments as well as to the fields of embryology and reproductive biology. This initial study is designed to study the process of capacitation of ram sperm with three heparin levels (50,100,150 IU). 140 pairs of abattoir testicular samples (70 pairs each season), with 70 ewe genitalia specimens (35 each season). Spermatozoa sample were capacitated with heparin in CO2 incubator for 45 minutes, and evaluated by its total motility and its ability to penetrate oocytes zona pellucida for fertilization. Different heparin level for spermatozoa capacitation in relation to seasonal influences revealed that; in regarding to capacitation index there was an increased spermatozoa activity and motility significantly for the three levels within season, but capacitation index 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. Results of oocytes collection in relation to seasonal influences revealed to; total oocytes number was 2035 oocytes on both season in which 1039 oocytes were obtained within season while a number of 1006 oocytes was collected out of season. Oocytes maturity index within season time is about 40% for the oocytes grade V.good and good grade, fair and poor grade is 25%.Oocytes collected out of breeding time showed range between 25% for the v. good and good grade to 10% for the other. Fertilizable index for embryos development after capacitated spermatozoa adding to the medium containing matured oocytes is range from 35% for the v. good and good graded oocytes within a season to 10% out of season. In conclusion; heparin can induced spermatozoa capacitation in any levels through the breeding season while the high level of heparin gave better results, fertilizable index and embryo production are both gave best result via breeding season.

Keywords: **Capacitation, Oocytes, Heparin, Spermatozoa, Epididymis, Testicle. Sheep.**

Introduction

Successful fertilization depends upon several interrelated physiological processes all of which must take place in a coordinated manner, one such process is sperm capacitation, which involves modifications in membrane composition and fluidity, increases in intracellular cAMP, induction of tyrosine phosphorylation events, and the expression of hyperactivated motility. Sperm undergoes these changes within the female reproductive tract or, in vitro, when incubated in a medium that supports capacitation (1). During the passage of sperm through the female genital tract, the spermatozoa undergo functional and molecular changes which confer the ability to fertilize the oocyte; this process is known as sperm Capacitation (2). Capacitation is a key process through which spermatozoa acquire their fertilizing ability. This event is required for the successful application of assisted reproductive technologies such as in vitro fertilization (IVF) (3). Development of successful in vitro fertilization techniques is essential for the study of the basic aspects of fertilization process. Freshly ejaculated mammalian spermatozoa are not immediately capable of achieving fertilization (4). During residence in the female tract, the sperm cell undergoes a complex and poorly understood set of modifications which confer fertilization

competence, a process collectively called capacitation (5 and 6). Capacitation is believed primarily to involve membrane modifications, including changes in lipid composition, surface properties, fluidity, permeability to calcium and lowered concentration of cholesterol in membranes, most of these alterations are related to changes in the plasma membrane of spermatozoa and have led to the contention that capacitation is a process of membrane maturation (7 and 8). The using of cultured medium with heparin in sperm capacitation and fertilization in vitro was evaluated by (9), providing a highest ability for sperm to capacitate and highest proportion for zona pellucida (ZP) penetration .

Heparin positively aid in facilitating the fertilization ability of spermatozoa in rams, heparin which is a glycosaminoglycan, of highly sulfated trisaccharide (C18 H32O17) with one carboxylic group, preserved in granules of mast cells used as an anticoagulant, is also corresponding in composition with uterine secretions that aid in the sperms capacitation in vitro, is binding to the sperm cells and aid the cells in calcium uptake (influx) which affected the sperm's head plasma membrane permeability(10). Capacitation is a complex process, which appears to be controlled by crosstalk between different pathways (11). The most notable event is an increase in protein tyrosine phosphorylation (12 and 13). Capacitation is a key process through which spermatozoa acquire their fertilizing ability. This event is required for the successful application of assisted reproductive technologies such as in vitro fertilization (14). Many materials have been successfully used to support in vitro capacitation of mammalian spermatozoa. For instance, several hormones present in the uterine and oviductal fluid at the time of in vivo fertilization as well as those controlling the estrous cycle, such as progesterone or estrogens, may be good candidates for triggering in vitro sperm capacitation (15) . In this regard, the addition of progesterone to the capacitation medium exerted a positive effect on sperm membrane cholesterol efflux, hyperactivation and the acrosome reaction (16). However, the effect of estrogens on sperm capacitation has been controversial (17).

Heparin may promote both in vitro sperm capacitation and fertilization by binding to seminal plasma proteins that are incorporated into the sperm plasma membrane (18). This induces changes in the properties of the plasma membrane that may stimulate increases in intracellular calcium, pH and cAMP during capacitation. Moreover, when added to the bovine capacitation medium, heparin supported downstream time-dependent increases in protein tyrosine phosphorylation(19).

Capacitation occurs in vivo when sperms are exposed to the female reproductive tract or in vitro after incubation in a defined medium. The assumption that steroid hormones present in the female genital tract may have a rapid effect on ram spermatozoa by interaction with specific surface receptors. Steroid hormones, such as estrogens and progesterone, play a crucial role in the regulation of reproductive events in mammals. It is well established that these hormones regulate gene expression in the hypothalamic–hypophyseal gonadal axis through nuclear receptors (20). 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 (21) , 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 (22 and 23) , which could reach micromolar levels (24) and diffuse to form a molecular gradient toward the edge of the cumulus matrix and beyond(25).

The event of capacitation process is accomplished in two steps, Fast and Low (26), in which, during fast step the activation of sperm motility is employed. Although sperm stored in the cauda epididymis being practically immobile, the flagellum movement starts immediately after sperm are released from the epididymis and contact has been made with seminal plasma, this is due to exposure of sperm to the HCO-3 (27), during slow capacitation, sperm acquire the ability to fertilize, which is preceded by the preparation of the sperm to undergo the acrosome reaction and change the pattern of motility called hyperactivation. Components in oviductal

fluid such as high weight molecular proteins and high density lipoproteins promote cholesterol efflux resulting in an increased capacitation and tyrosine Phosphorylation (PY) using the cAMP signaling pathway/PKA, these slow processes also are achievable in vitro by incubation of spermatozoa in defined media, which contain a protein source (usually bovine serum albumin (BSA), and different ions, including HCO-3 and Ca+2(28). There are two kinds of unsaturated fatty acids which present on the surface membrane of Ram spermatozoa (Lipid Raft Marker) (29), the two lipid markers are Caveolin-1and Ganglioside GM-1, which may affect the capacitation (decrease) and acrosome reaction AR (increase), considered as a characteristic representing marker of ram spermatozoa, and may contribute to better understanding of sperm fertilizing potential acquisition mechanism.

Materials and Methods

Abattoir female and male gonads: Specimens were collected from Al-Shoáalla slaughterhouse located on the western side of Baghdad the capital from November.2017 to October 2018 as : Adult ram Testes (140 pairs) were collected from mature local rams; age is determined as possible according to the dental tables (if possible) in which; (70) pairs were 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). Female Genitalia of the local breed (70 specimens) were obtained directly after slaughtering, (35) were collected within season from (26-11-2017 to 16-01-2018), and (35) were 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 the cool box (4 - 8°C) .

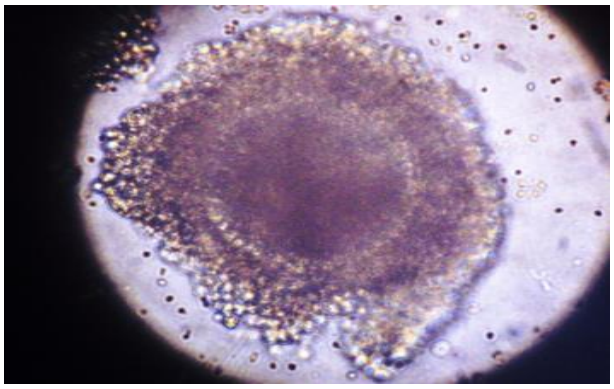
Preparation of testicular sample: Testicles Specimens were washed with distilled water first, and then with normal saline supplement with 100 IU/ ml penicillin and 0.1mg/ ml streptomycin antibiotics. Testicles were separated carefully from the surrounding tissues and fascia, size and weight were recorded by vernia and electrical balance respectively. Epididymis then separated from each entire testicle weight and length were recorded for further analysis, caudal epididymis weight and width were measured, injected with 5-8 ml of the warmed normal saline at (38 C°) which Pre-heated in a water bath before (30) . Injected Caudae were sliced in Petri dish in order to examined under light microscope for mass spermatozoa motility and, stained smear will be prepared with Eosin , Nigrosine stain for dead, alive sperms and abnormalities detected, all results were recorded and connecting with sample age and date as discussed by(31) . Spermatozoa samples with individual motility over 50% were incubated in CO2 incubator at (39 C°), 5% CO2 tension and 90% humidity for six hour for excessive sperm maturation, presence of the distal protoplasmic droplet was the criterion of sperm maturation(31).

Cauda spermatozoa samples then subjected for capacitation process with three levels of Heparin sodium (50, 100, 150 IU) and for each additional incubation for 45-60 minutes to evaluate the capacitation index by evaluation of individual motility for each level before adding to the oocytes samples for IVF index.

Preparation of oocyte sample :Ewe's genitalia (ovaries) were separated carefully from the rest of all genitalia and surrounding tissues, then hold in a beaker containing normal saline with penicillin-streptomycin for (5-10 min), for more settlement, ovaries will be transferred to other dishes containing MEM medium; sliced to small pieces and leaved at room temperature for (15 -30 min).

Evaluations of collected oocytes were done as mentioned by (32) in regard to the arrangement of cumulus cell surrounding oocytes as well as the status of cytoplasm as:

Very Good grade when the oocytes with a transparent, homogenous and uniform cytoplasm surrounded with high density layers of cumulus cells. Good grade when the oocytes with a transparent, homogenous and uniform cytoplasm surrounded with few layers of cumulus cells. Fair grade when the oocytes with transparent, less homogenous (Small granules exist) and uniform cytoplasm and less compact cumulus cells partially surrounded.4. Poor grade when the oocytes with mild or absent cumulus (denuded) with dark and granular cytoplasm



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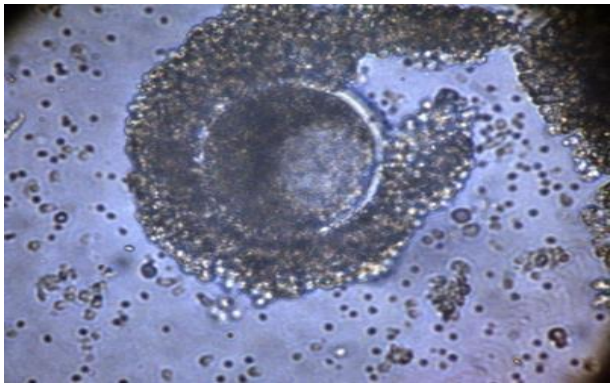

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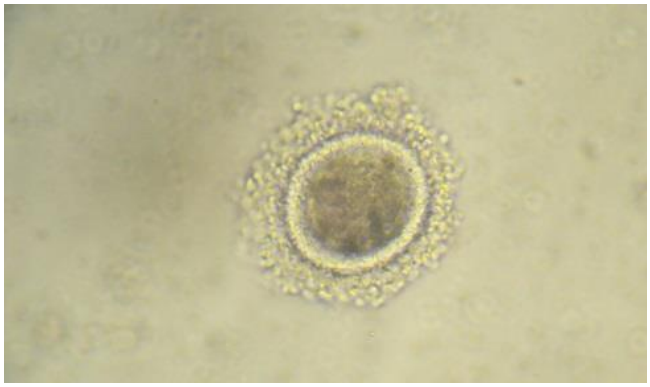

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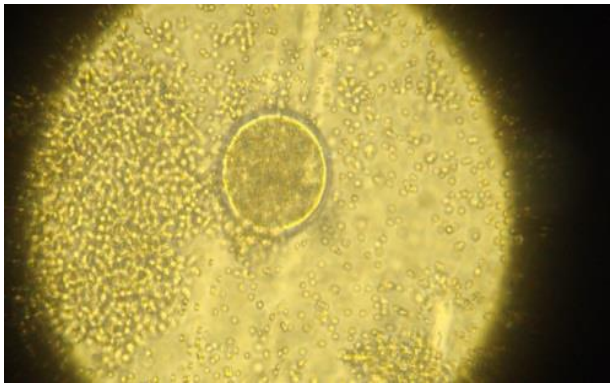

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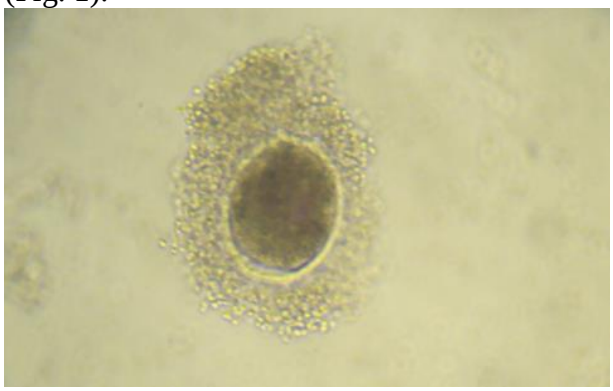
Figure,1: Very good, good, fair and poor grade oocytes.

Very Good, good and fair grade oocytes were subjected to the in vitro maturation (IVM), the maturation medium with its additional materials is mentioned in (Table,1), in which oocytes after grading were incubated in CO2 incubator at (39 C°), 5% CO2 tension and 90% humidity (Saleh 2016), for (30) minutes. The dissociation of the first polar body is a criterion for oocytes maturation, (Fig. 1).



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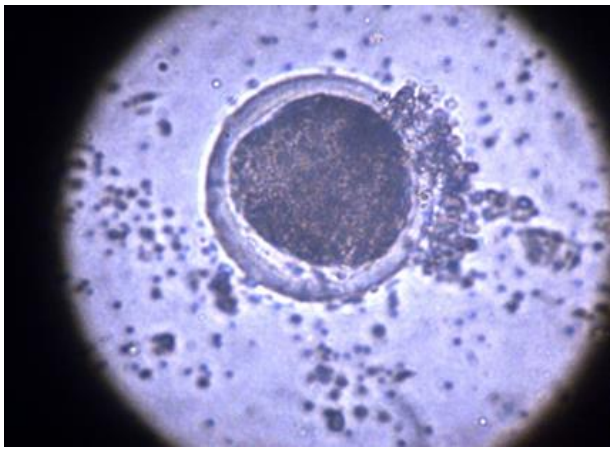

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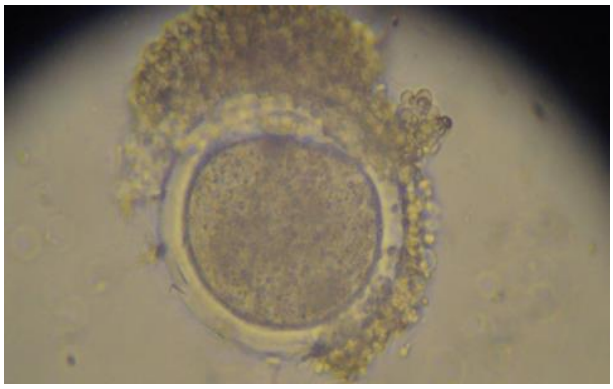

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Figure, 2: Three mature oocytes where the red arrows denoted the dissociation of the first polar bodies.

Matured oocytes then prepared to be mixed with capacitated spermatozoa as discussed (33), Matured oocytes were washed twice by medium supplied with antibiotics and antifungal before transferred to a glass Petri-

dishes for IVF process. Capacitated spermatozoa sample were prepared to yield 1-2×106 sperms. Gametes mixture was incubated at 5% CO2 level at 38.5 ℃ and 90% relative humidity for 27-30 hrs. Fertilization media supplemented with, LH, FSH, BSA, FCS, antibiotics and antifungal preparation, produced embryos then evaluated and all results were recorded (30) .

Results and Discussion

The results of testicular and epididymal parameters shown by (Table,1) as the effect of season significantly (P<0.05) affects those parameters, in which parameters within season were more of higher values than those out of season. Results showed the testicular orientation (left &right) significantly (P<0.05) affect testicular and epididymal parameters influenced by the seasonal factor, in which parameters of right testicles showed the variable result if compared with the left one on both breeding seasons (Table,2)

Results showed that, the effect of testicular orientation significantly (P<0.05) not affect the results of spermatozoa mass motility, dead spermatozoa and abnormalities percentage as the season effect. Season significantly (P<0.05) affect those parameters in which variable results obtained within season confirmed that mass motility, dead sperm and abnormalities percentage were (almost) with the same values on both seasons, while the incidence of the season had clear effects on those parameters (Table,3).

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, there were no significant effect(P<0.05) upon breeding season within or out of breeding season in all heparin level, capacitation index is the same for all three levels. But results showed significant variations (P<0.05) among the three heparin levels out of breeding season in which the highest heparin level gave the best capacitation index than the other two (Table,4).

Table,1: Compare between with and without season in parameters study.

Parameters	Mean ± SE		LSD value
	Within season	Out of season	
Tests weight (gm)	174.94 ± 3.36	105.47 ± 1.83	16.704 *
Volume of testis (mm^3)	5.47 ± 0.09	4.50 ± 0.06	0.581 *
Weight of Epididymis (gm)	8.92 ± 0.18	6.08 ± 0.12	1.269 *
Volume of Epididymis (gm)	1.96 ± 0.02	1.76 ± 0.02	0.386 NS
Length of Epididymis (cm)	14.36 ± 0.12	13.36 ± 0.09	0.633 *
The data displayed mean ± SE and n=?, the stars denoted significance ((P<0.05)			

Table, 2: Effect of testicular orientation on testicular and epididymal parameters under season influences.

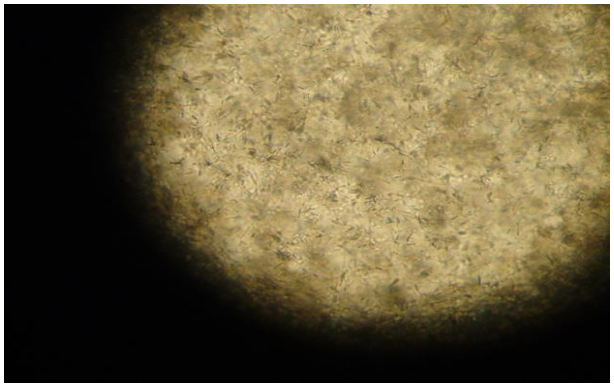
Parameters	Mean ± SE				LSD value
	With season		Without season		
	Left	Right	Left	Right	
Tests weight (gm)	176.25 ± 3.48	173.63 ± 3.46	107.14 1.85 ±	103.80 ± 1.91	15.823 *
Size of the testis (mm^3)	5.57 ± 0.09	5.37 ± 0.09	4.57 ± 0.06	4.42 ± 0.07	0.439 *
Weight of Epididymis (gm)	9.07 ± 0.19	8.77 ± 0.18	6.10 ± 0.14	6.06 ± 0.15	1.062 *
Size of Epididymis (mm)	1.90 ± 0.02	2.03 ± 0.02	1.71 ± 0.02	1.81 ± 0.02	0.388 NS
Length of Epididymis (cm)	14.47 ± 0.11	14.25 ± 0.11	13.50 ± 0.10	13.22 ± 0.09	0.625 *
* (P<0.05).					

Table,3: Effect of breeding season and testicular orientation on spermatozoa mass motility %, dead spermatozoa % and spermatozoa abnormalities %.

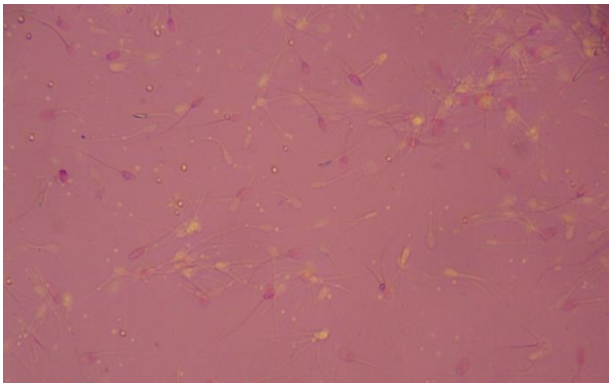
Parameters	Mean ± SE				LSD value
	With season		Without season		
	Left	Right	Left	Right	
Mass motility (%)	77.71 ± 0.48	77.71 ± 0.48	58.85 ± 0.61	54.28 ± 0.54	8.504 *
Dead sperms (%)	22.29 ± 0.48	22.29 ± 0.48	41.15 ± 0.6	45.71 ± 0.54	8.504 *
Abnormality sperr (%)	2.47 ± 0.06	1.97 ± 0.06	5.33 ± 0.08	6.17 ± 0.08	3.601 *
*** (*P*<0.05).**					

Table, 4: Effect of heparin level under season influences on capacitation index.

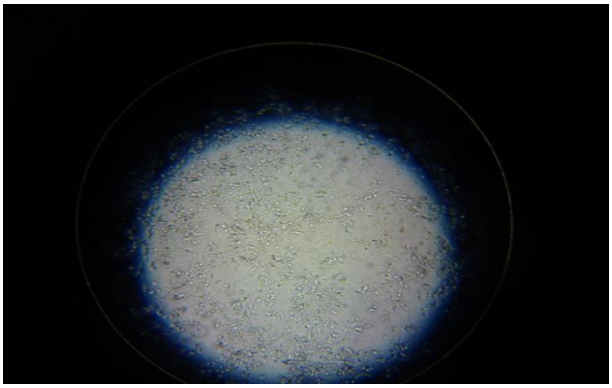
Season	Heparin IU	Capacitation index
With season	50	82.23 ± 0.58
	100	82.23 ± 0.58
	150	82.23 ± 0.58
Without season	50	56.85 ± 0.61
	100	56.85 ± 0.61
	150	62.07 ± 0.62
LSD value	---	9.317 *
* (*P*<0.05).		



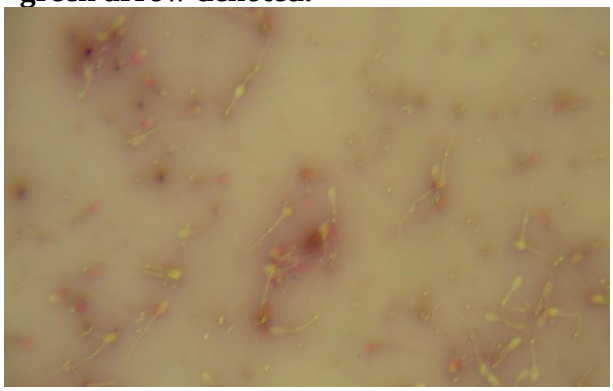
Figure, 3: Capacitated spermatozoa.



Figure, 5: Eosin-Nigrosin stained sample green arrow denoted.



Figure, 4: Progress viability and increased fertilizable activity of sperm after capacitation.



Figure, 6: Out of season sperm sample after capacitation with limited sperms number.

Results of oocytes collection in relation to seasonal influences revealed that; total oocytes number was 2035 oocytes on both season in which 1039 oocytes were obtained within season while a number of 1006 oocytes was collected out of season (Table, 5). The result showed that; the oocytes maturity index within season time is about 40% for the oocytes grade v.good and a good grade, but for the fair and poor grade it is not more than 25%. This percentage decreased for those oocytes collected out of breeding time in which it range between 25% for the v. good and good grade to 10% for the other as shown in (Table,6).

While the fertilizable index for embryos development after capacitated spermatozoa adding to the medium containing matured oocytes is range from 35% for the v.good and good graded oocytes within season to 10% out of season. No embryo found for fair or poor graded oocytes in both season (Table, 7).

Table, 5: Oocytes account and quality grading within and out of season.

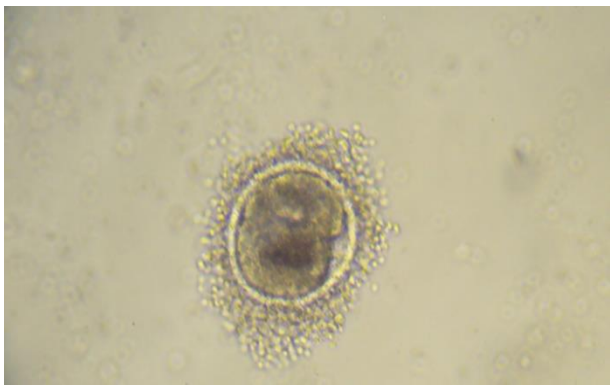
Season	Oocyte grade				Total number
	V. good	Good	Fair	Poor	
Within season	388	331	198	122	1039
Out of season	137	200	305	364	1006
Total number	525	531	503	486	2035

Table, 6: Oocytes maturity index within and out of season.

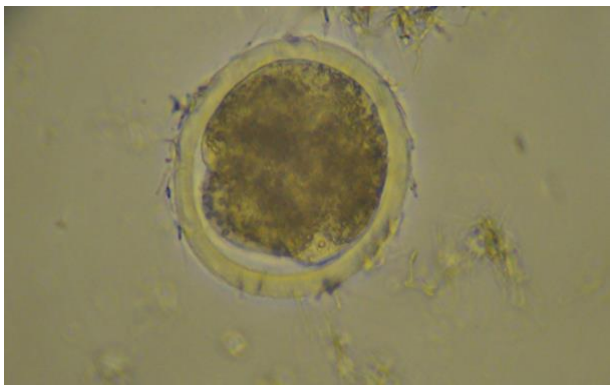
Season	Oocyte maturity index				Total number
	V. good	Good	Fair	Poor	
Within season	155 (40%)	132 (40%)	40 (25%)	30 (25%)	357
Out of season	35 (25%)	50(25%)	30 (10%)	36 (10%)	151
Total number	190	182	70	66	508

Table, 7: Embryo development within and out of season.

Season	Embryo development				Total number
	V. good	Good	Fair	Poor	
Within season	54 (35%)	53 (35%)	3 (10%)	0	110
Out of season	4 (10%)	5 (10%)	0	0	9
Total number	58	58	3	0	119



Figure, 7: two cells embryo 24-36 hrs. after IVF.



Figure, 8: Four cells embryo 36-48 hrs. after IVF.

Results and Discussion

This study is designed to evaluate the suitable way for spermatozoa capacitation *in vitro* by using abattoir samples (testes and ovaries) under seasonal influences in sheep. Both testicular and epididymal parameters are significantly influenced by season, those parameters showed variations in relation to the breeding season which affect the spermatozoa production, viability then capacitation and IVF index. This seasonal influences is agreed with (34) in which; seasonality is more obvious in some breeds of sheep, influenced the reproductive capability which regulated by photoperiod and the correlation between melatonin and testosterone (35) found same results, in which, season influenced testicular parameters (weight and size) and altered the parameters when applied out (–) ve or within (+) ve season. (36) found the same results when investigates about the testicular parameters (weight, size, spermatozoa characters) and demonstrates that season is directly affected those parameters and involve spermatozoa production.

The effect of testicular orientation influenced by season factor on testicular and epididymal parameters; Testicular and epididymal parameters showed humble and not significant variations as in the season does, mainly seasonal factor significantly modulates those parameters. (30) agreed with this result as there is a significant ($P<0.05$) effect of season on testicular weight and size; these effects altered epididymal parameters, spermatozoa concentration and abnormalities which affect the final capacitation process to consequences powerful affect the final step (IVF) and embryo production (IMP). Other Atheros (37) described the same result that testicular parameters exhibit seasonal variations; those variations are in turn influenced other parameters as testicular size, sperm concentration which affect other processes as capacitation. 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 these results are influenced by seasonal factors mainly out of breeding season these 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. (38) agreed with these results and showed that sperm capacitation with different heparin levels mainly showed variable index when seasonal factor influenced the final result. Also (39) revealed to; 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. Authors (40) 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 (41) approved that; even the capacitated medium may 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.

The changing photoperiod acts as a Bioregulatory factor that regulates the reproductive activity and fertility in sheep through the mediation of central nervous system, hypothalamus, adenohypophysis and the pineal gland. Onset of breeding season in sheep which is a typical seasonal breeder is much similar to the onset of puberty. Transition from non-breeding (anestrous) to breeding (estrous) represents a kind of transferring from a sexually quiescent state to active state (42). These physiological changes directly affect the ovarian morphology by increasing follicular number and size then weight, size of ovary will increase, these results agreed with (43) in which; in non-breeding season there is an increase in negative feedback effect of estrogen on GnRH and gonadotrophin secretion, reduced frequency of GnRH pulses, suppressing the gonadotrophin drive to the gonads thereby deprived its activity factor (44).

The result showed; at breeding season there is an oocyte quality improvement appeared as an increased in the number of very good and good quality oocytes in regarding to the other two grades (fair and poor quality), this quality improvement is suppressed due to the effect of a circadian rhythm of day light, this changing in daylight duration will be at optimal limit on middle to the end of May (out of breeding season) then it goes down gradually to be equal at mid-August (breeding season). This is agreed with (45) in which the season affects yield and quality of blastocyst in the way that the autumn period is more favorable for embryo development, and this is mainly initiated from the good quality of oocytes collected through the period of decreased daylight (autumn) .

(46) agreed with the results found in this study, the effect of daylight might influence the cyclicity of ewe which affect ovarian functions, modulate hormones action by increased its level and then more follicular development as this period proceed and changed from transitional to breeding season.

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تقييم معامل الاخصاب الخارجي لحيامن ذيل البربخ بعد زيادة طاقتها للاخصاب بمستويات مختلفة من الهيبارين في الاغنام العراقية

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الخلاصة

أن مصطلح زيادة طاقة اخصاب وتكييف الحيمن يعني تلك التغييرات الفسيولوجية التي تحدث للحيمن لكي يكتسب من خلالها قابليته على الاخصاب. ان هذه الظاهرة مهمة جدا للاخصاب الخارجي ، علم الاجنة و بايولوجيا التكاثر. ان هذه الدراسة اعدت لزيادة طاقة اخصاب وتكييف الحيمن في ثلاثة مستويات من الهيبارين (50 و 100 و 140) وحدة دولية. 140 زوج من خصى الاكباش جمعت من المجزرة، 70 زوج لكل موسم و70 جهاز تناسلي للنعاج (35 لكل موسم). خضعت الحيامن المستخلصة من ذيل البربخ لعملية زيادة طاقة الاخصاب بمادة الهيبارين لثلاث مستويات وولكل موسم حفظت في حاضنة ثاني اوكسيد الكاربون لمدة 45 دقيقة، كانت زيادة الحركة الجماعية وقابلية الحيمن في اخصاب البويضة الناضجة هو المقياس لزيادة طاقة الاخصاب للحيامن.اظهرت النتائج تاثير الموسم المباشر على عملية زيادة طاقة الحيامن،كما اظهرت النتائج ان موقع الخصى كان لصالح الجهة اليسرى اكثرمنها الجهة اليمنى من ناحية زيادة الحجم والوزن كما ظهر تاثير مستويات الهيبارين متساويا خلال موسم التناسل وللثلاث مستويات بينما اشرت المستويات العالية للهيبارين تاثيرا ملحوضا اكثر من المستويات المنخفضة خارج موسم التناسل.كانت اعداد البويضات التي تم الحصول عليها (1034) خلال موسم التناسل اعلى منها (1006) خارج موسم وبتقيم جيد جدا، كما كانت مستوى نضوجها (40%) اكثر منها (25%) خارج موسم التناسل الامر الذي ادئ للحصول على اجنة (35%) ذات تقييم جيد مقارنة بمستوى متدني (10%) خاج موسم التناسل. نستنتج من هذه الدراسة ان الهيبارين باي مستوى خلال موسم التناسل أدى الى التكييف و زيادة طاقة الحيمن لاخصاب البويضات الناضجة بينما يعجز على ذلك خارج موسم التناسل الا بمستويات عالية.

الكلمات المفتاحية: تكييف الحيمن ، البويضه، الهيبارين،الحيامن، البربخ،الاغنام.