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(Original Article)

Effect of Season on Oocytes and in Vitro Fertilization by Fresh and Frozen Semen of Cattle

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Abstract

The aim of the research is to study the effect of season on the quality of bovine oocytes and semen (fresh and frozen) used for in-vitro maturation and fertilization. Fresh ovaries were collected from slaughtered animals by aspiration with 18- gauge needle attached to a 5 ml syringe filled with (flushing fluid). The oocytes were classified to Grades A and B after washing 3 times by the fluid, the oocytes were left in the incubator for 18-22 h to mature at 38°C and CO_2 5%. After 18-22 h, the cumulus cells were removed and washed 1-2 times to remove all the cumulus cells around the oocytes. The matured oocytes and the capacitated sperms (fresh and frozen) were incubated in the fertilizing medium and checked daily for seven days. The results showed that the aspiration technique significantly ($p < 0.01$) gave a high percent of good quality oocytes with homogenous cytoplasm in comparison to fair and denuded oocytes. Moreover, the maturation (the first polar body appeared) also was significantly increased during summer, autumn and winter compared to spring ($p <0.05$). The rate of embryo morula stage increased significantly ($p < 0.05$) with using the fresh semen compared to frozen ones (54.52% vs. 38.02 %). In conclusion, season and fresh semen play an important role in the rate of success of in-vitro fertilization in cattle.

Keywords: *Bovine oocyte, in-vitro fertilization, season, fresh and frozen semen.*

Introduction

In vitro fertilization technique began with the recovery of oocytes from the ovaries of slaughtered animals in (2019-2022), which results in a large number of oocytes (Nandi *et al.,* 2002). The method of the recovery of oocytes from the ovary is important to obtain oocytes. with high quality The aspiration is an easy and fast method for oocyte recovery, so it is widely used since long decades (Rakshitha *et al.,* 2019). Slicing is another method in which ovaries put are placed in a petri dish with normal saline solution and cut into small parts by a blade (Das *et al.,* 1996; Kumar *et al.,* 1997). Slashing method, in which the surface of the follicles was cut

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several times to remove all the follicular fluid (Saleh, 2017). In comparison to the previous methods, it was found that the aspiration process increased number and quality of oocytes (Wani *et al.,* 2000; Saleh, 2017).

The effect of season studded by (wang *et al.,* 2009) who found that oocytes collected in summer showed a low in vitro fertilization (Wang *et al.,* 2009). Similarly, It was found that spring-summer season decreased the number of follicles and cumulus cells compared to the spring-winter season (Acar *et al.,* 2013). Blastocyst production declined in mid and late summer while, increased in winter and autumn seasons (Rutledge *et al.,* 1999).

There are many factors play a role to complete the fertilization process in the laboratory, such as flushing and fertilizing media components used in maturation and fertilization, the temperature degree and percentage of carbon dioxide in the incubator, and the type of semen that has been evaluated in order for the fertilization process to be successful in the laboratory (Majeed *et al.*, 2019).

Most of the time, fresh semen is used in vitro fertilization because of the higher rate of motility than frozen semen, but the use of frozen semen is useful in the case of bull death with good traits. Frozen semen can be used on the in vitro production of embryos to minimize inbreeding and the subsequent risk of expression of lethal genetic traits (Saha *et al.*, 2014).

The aim of the research is to study the effect of season on the quality of bovine oocytes and type of semen (frozen or fresh) used for in-vitro maturation and fertilization.

Material and methods

Experiment 1. Oocytes Classification

Ovaries were obtained from animals slaughtered in the Dachlout abattoir, Dayrout, Assiut Governorate. Ovaries were kept in a storage box until reaching the laboratory, there, the ovaries were washed several times with distilled water to remove any external sediments. The follicular fluid was aspirated through sterilized 18- gauge needle attached to a 5 ml syringe containing a collection medium (TL HEPES Stock) (Tríbulo *et al*. 2019). The oocytes were collected and washed 3 to 5 times with the same media to remove any other cells. They are classified to: Grade A (Good): an oocyte surrounded by 5 layers of compact cumulus cells or more with a homogenous cytoplasm. Grade B (Fair): an oocyte surrounded by a 1-4 compact cumulus cell layers with a homogenous cytoplasm. Grade C (Poor and denuded): partial or complete denuded oocyte with heterogeneous cytoplasm (Das *et al*. 1996; Saleh, 2017).

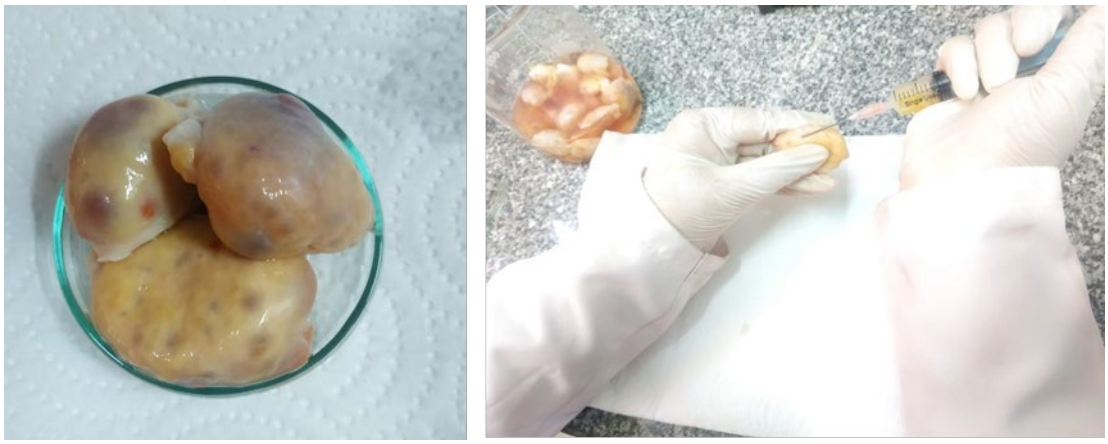

Fig.1. Fresh ovaries and the aspiration method.

Experiment 2. Oocytes Maturation

A total of 452 ovary, were used for maturation (Tríbulo *et al*., 2019). Drops of 50 µl maturation media were placed in a sterilized Petri dish covered with paraffin oil. Ten oocytes were placed in each drop. The oocyte incubated at 38°C, 5% of CO_2 for 18-20 hours to complete the maturing process. The oocytes were considered as mature when the first polar body was detected and cumulus cells expanded (Lodde *et al.,* 2021).

Experiment 3. In Vitro Fertilization

Preparation of Oocytes for In Vito Fertilization:

The oocytes were checked daily for observing the maturity stage, where the oocytes collected in a tube were exposed to the vortex device for 10 minutes for removing the cumulus cells embraced around them. After that the oocytes were (Denuded Oocyte) washed 3 times by flushing solution (Lonergan *et al.,* 2003).

Preparation of Sperm for In Vitro Fertilization

Fresh semen Semen was collected on the day of fertilization by artificial vagina, evaluated for sperm motility and concentration, and then diluted by what type of extender (1:1). the number of sperms used in the fertilization dish was 1.0 x 10^6 sperm/ ml (Majeed *et al.,* 2019).

Frozen semen The straw of frozen semen contained 1 × 10^6 sperm/ml was placed in a water bath at 38°C for 45-60 s to thaw the sample in the fertilization dish (Majeed *et al.,* 2019).

Sperm capacitation

The semen was mixed with capacitation medium (TL-HEPES Stock, BSA, Na Pyruvate, Gentamicin sulfate, Caffeine) (Tríbulo *et al.,* 2019). The ratio between semen and medium a was 1:1, then the sample incubated with 5 % CO_2 for 45 minutes (Majeed *et al.,* 2019).

In vitro fertilization

The fertilization media (Tríbulo *et al.,* 2019) were placed in sterilized dishes in form of 50µl drop/dish and completely covered with paraffin oil Ten oocytes were placed in each drop.

Capacitated sperms were placed and incubated at 39°C, 5% CO_2 (Soliman *et al.,* 2018). Daily examination and change the media for 7 days to detect the blastocyst stage of embryo (64 cells).

Statistical analysis

The data were analyzed by T-test. The obtained data were analyzed by one way analysis of variance.

Results

Experiment 1. Oocyte Classification

Table 1. Classification of Oocytes which collected by aspiration method

Trial	Total no of oocytes	Oocyte quality			A%	B%	C%
		A	B	C			
1	30	12	7	11	40	23.3	36.7
2	16	14	0	2	87.5	0.0	12.5
3	59	24	12	23	40.7	20.3	39
4	50	28	12	15	50.9	21.8	27.3
5	65	29	14	22	44.61	21.54	33.85
Total	225	107	45	73	52.74	17.39	29.87

A. Good, B. Fair, C. Poor and denuded

The results (Fig. 2 and 3) showed that the aspiration technique significantly ($P< 0.01$) gave a high percent of good quality oocyte in comparison to fair and denuded oocyte.

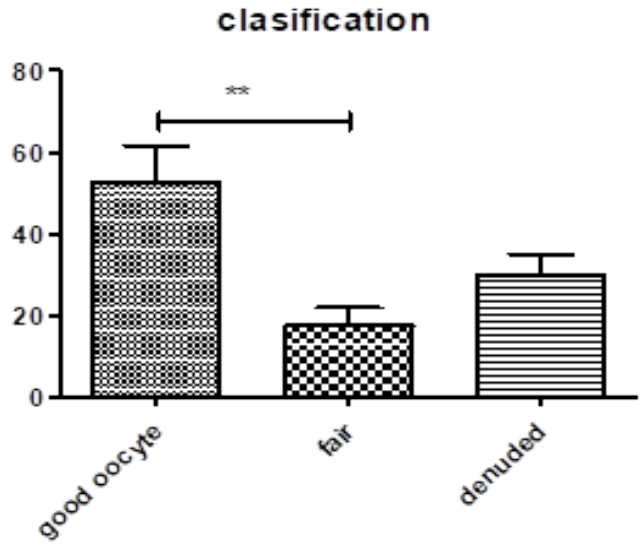

Fig.2. The percent different quality of aspirated oocytes

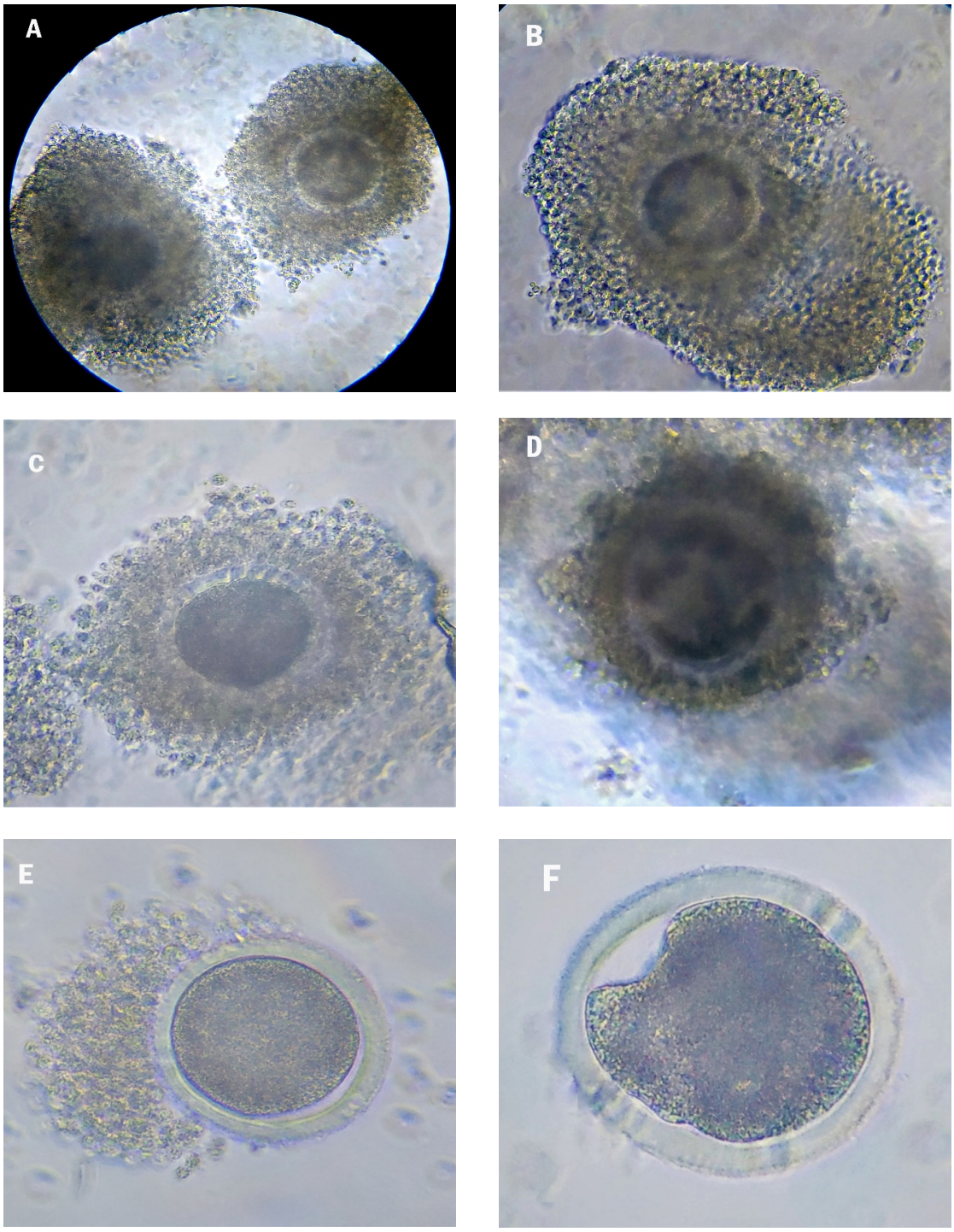

Fig. 3. A-B: Good quality oocyte with homogenous cytoplasm, C-D: fair, E: Partially denuded, F: Denuded oocyte.

Experiment 2. Oocyte Maturation in relation to season

The results (Fig. 4 and 5) showed that the maturation was significantly increase during summer, autumn and winter ($p<0.05$) and the best one was in summer. On the other hand, the maturation rate was significantly decreased during the spring ($p<0.05$).

Table 2. The total Oocytes and their maturation rates in different seasons

	Total oocytes	Mature oocytes	Maturation rate
Winter	573	452	78.8%
Spring	111	71	63.9%
Summer	463	388	83.8%
Autumn	251	196	78%

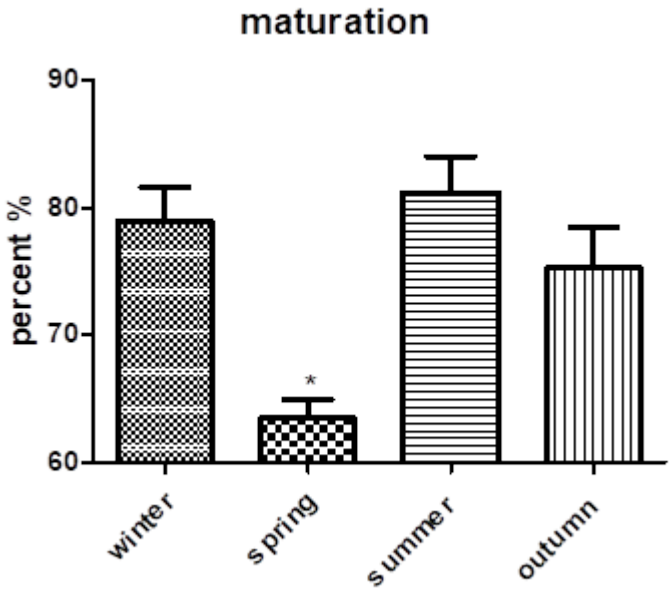

Fig. 4. The effect of season on the quality of bovine oocytes.

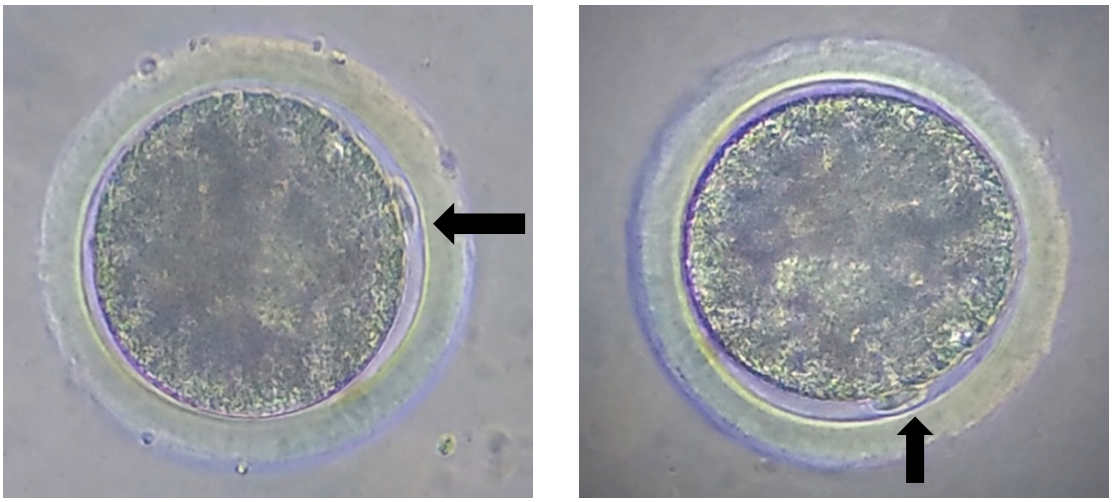

Fig. 5. Matured bovine oocytes after 20 h in 5 % CO_2 incubation, where the first polar body appeared (black arrows).

Experiment 3. In vitro Fertilization

The results (Table 3 and Fig. 6) showed that the rate of morula stage increased significantly ($p< 0.05$) when used fresh semen in comparison to frozen semen (54.52% vs. 38.02 %).

Table 3. Developmental competence of cattle oocytes fertilized by fresh and frozen semen

	Semen	
	Frozen	**Fresh**
Oocytes number	187	386
Morula rate %	38.02%	54.52%

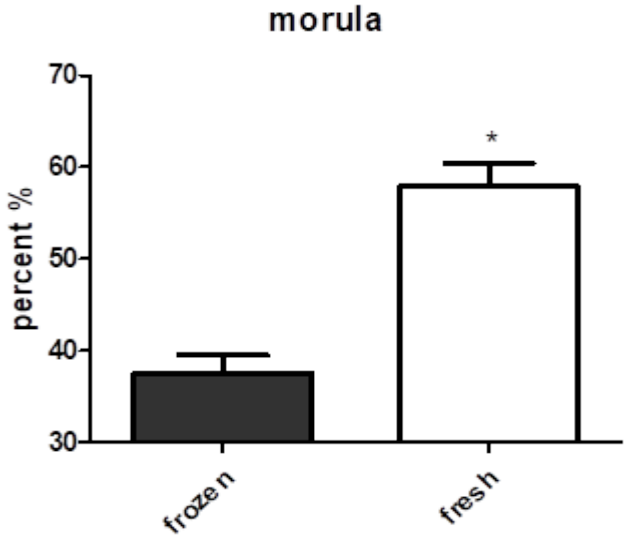

Fig. 6. The effect of type of semen (frozen or fresh) used for in-vitro fertilization.

Discussion

The results showed that aspiration technique was an appropriate method to obtain the largest number of high-quality oocytes. In cow, it was reported that the aspiration method produces high quality oocytes compared to the other methods used to collection oocytes (Saleh, 2017). In goat, it was explained that although the number of oocytes produced by the slicing method is greater than the aspiration method, it was found that the percentage of mature oocytes from the aspiration method is higher than the mature oocytes from the slicing method (Wang *et al.,,* 2007). Similarly, in sheep, it was reported that the total number of oocytes produced by the aspiration method is less than the puncture and slicing methods, while the percentage of good oocytes was higher in the aspiration method (Wani *et al.*, 2000). This results explained why the maturation rate of aspirated follicles was higher than the puncture and slicing methods (Wani *et al.*, 2000). In contrast, in bovine, it was found that high-quality oocytes were obtained from the surface dissection method rather than the aspiration method (Carolan *et al*., 1994). The reason for the decrease in the number of aspirated may be related to lost during aspiration or the difficulty of obtained follicles which embedded in the ovarian cortex (Wani *et al*., 2000).

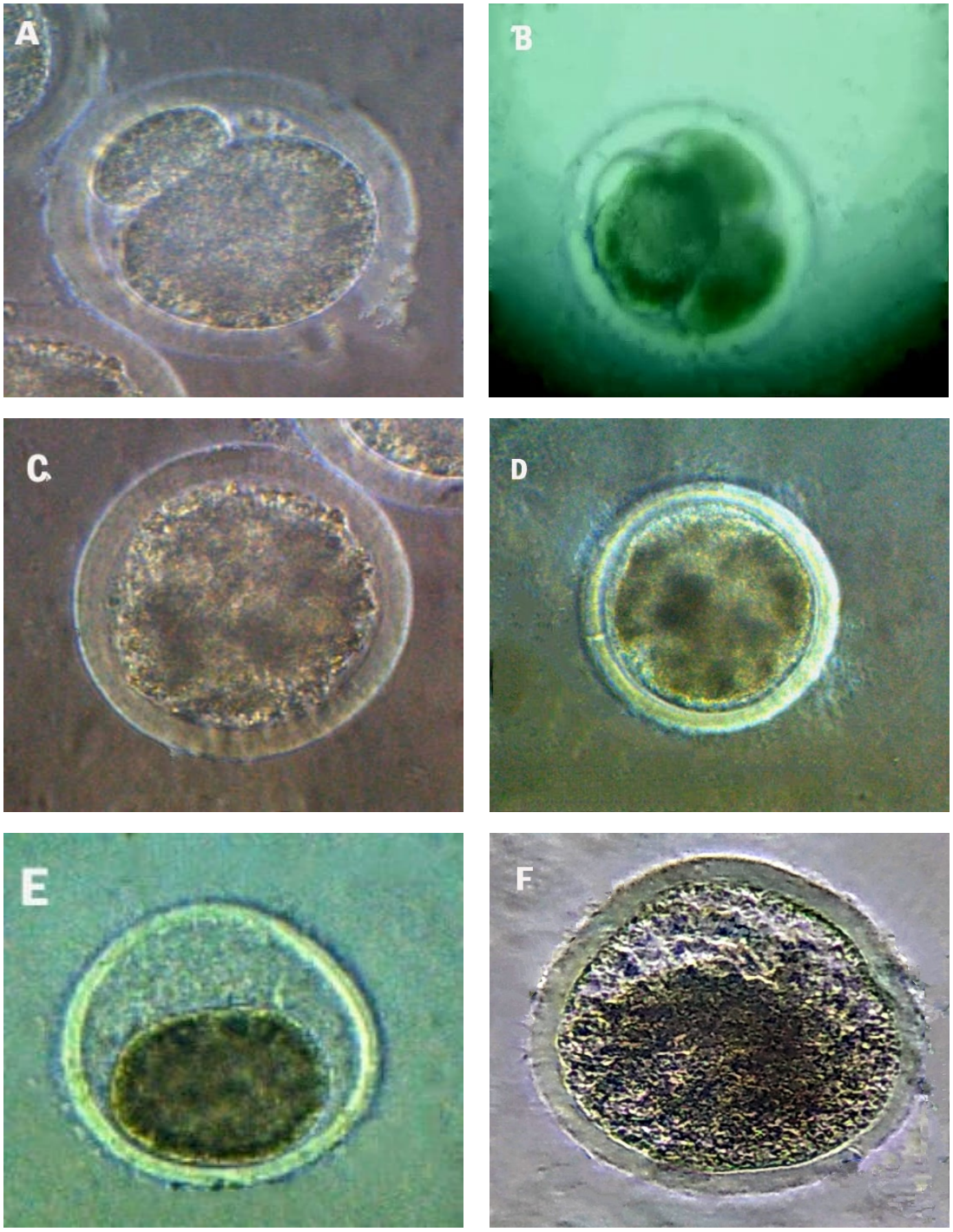

Fig.7. A) 2 cells stage embryo, note clear cytoplasm, B) Normal 8-cells embryo, C) 16 cells, D, E) Morula, compact morula 16-32 cells formed by cleavage of the zygote that precedes the blastocyst and F) Early blastocyst, embryo will have a clearly defined trophoblast layer, blastocoel cavity, along with a normal-thickness zona pellucida. Blastocyst embryo contains 64 cells.

The results showed that the maturation rate was significantly increase during summer, autumn and winter ($p < 0.05$). In cattle, (Rutledge *et al*., 1999) indicated that maturation rate of oocytes increased during winter and autumn which improved the stability of high embryo production. Moreover, it was observed that production of blastocysts decreased significantly in mid and late summer. On the other hand, in buffalo (Zoheir *et al*., 2007) explained that the percentage of high-quality oocytes increased in winter and spring compared to autumn and summer. This is because buffaloes are less active in summer.

In cows, it has been confirmed that heat stress can affect the endocrine glands, reducing the ovarian follicular growth, and oocyte production impairment (Zeron *et al*., 2001). Similarly in the mare, there is a difference in the rate of embryo production in relation to the season., (Brück *et al*., 1996). It was noted that high temperature and humidity have harmful effects on the ability of the oocyte maturation and fertilization (Leibfried-Rutledge *et al.*, 1989). In cows, it was clarified that the oocytes are less efficient in the summer, however, the rate of division did not decrease. But the fertilized oocyte is less developed into a blastocyst. (Al-Katanani *et al.*, 2002). It was shown that cows are able to regulate body temperature during heat stress and that there is a slight change at beginning of each season. And low percentage of blastocyst in subtropical areas (Rivera *et al.*, 2000). It was explained in previous study that the most environmental factors that affected the reproductive performance of cows began after April, which led to the low follicular growth. this occurred after ending the cold season in the tropical regions (Kanwichai *et al.*, 2019).

In deer, it was found that the photoperiod affects the secretion of melatonin, which in turn reflects on the pituitary gland activity to secrete the reproductive hormones (Lincoln, 1998).

The results showed that the morula rate increased significantly ($p< 0.05$) when used fresh semen for in-vitro fertilization in comparison to frozen semen. In buffalo, there were no significant differences in the rate of embryo division between fresh and frozen semen, But using fresh semen improved the rate of fetal growth compared to the frozen semen (Soliman *et al.,* 2018). Similar results were recorded in sheep (Lehloenya *et al.*, 2010). There were no significant differences between the use of frozen semen and fresh semen in goats, Usually, IVF is performed using fresh semen due to the high rate of sperm motility (Saha *et al*., 2014). It was shown that there is a decrease in the rate of cleavage when the freezing time of semen is increased (Lehloenya *et al*., 2011). The fertilization rate using frozen semen may vary for several reasons, such as animal breed, animal age, semen processing, and semen quality.

In conclusion, the season and type of semen used for in-vitro fertilization play a role in the rate of success of in-vitro fertilization in cattle.

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تأثير الموسم على البويضات والاخصاب المعملي باستخدام السائل المنوي الطازج والسائل المنوي المجمد في الأبقار

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الملخص

يهدف البحث إلى دراسة تأثير الموسم على جودة بويضات الابقار ونوع السائل المنوي (المجمد أو الطازج) المستخدم في الاخصاب في المعمل. تم جمع مبايض الأبقار الطازجة من الحيوانات المذبوحة. وتم تجميع البويضات عن طريق الشفط بإبرة قياس 18 ملحقة بحقنة 5 مل تحتوي على وسط التجميع. تم تصنيف البويضات إلى (الدرجة أ، ب) وغسلها 3-5 مرات باستخدام وسط تجميع ثم نضجت لمدة 18-22 ساعة في وسط النضج عند 38 درجة مئوية و5% CO_2 بعد 18-22 ساعة، تمت إزالة خلايا المحيطة بالبويضات وغسلها 1-2 مرات. تم تحضين البويضات الناضجة والحيوانات المنوية المكثفة (طازجة ومجمدة) في وسط الإخصاب ومتابعتها يومياً. أظهرت النتائج أن تقنية الشفط أعطت فرق معنويا جدا بين البويضات ذات جودة عالية مقارنة بالبويضات الاخرى. علاوة على ذلك، زاد النضج معنويا خلال الصيف والخريف والشتاء مقارنة بموسم الربيع. وزادت نسبة الاخصاب معنويا عند استخدام السائل المنوي الطازج للإخصاب المعملي مقارنة بالسائل المنوي المجمد (54.52% .vs 38.02%). في الختام، يلعب الموسم ونوع السائل المنوي المستخدم في الاخصاب المعملي دورًا في معدل نجاح الإخصاب في الابقار.