Oocytes Extraction from Iraqi Local Goat Ovaries by Using Aspiration and *in vitro* Fertilization Techniques

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Abstract. This study was conducted on 72 local slaughtered female goats, 144 ovary were collected from the different places of the city of Basrah, a local slaughterhouse, Basrah markets and agriculture station for scientific research at the university of Basrah. Oocytes was obtaining from the ovaries by using aspiration technique and the number of it was 1044 after the division of ovarian follicular depending on the size of its diameter into two group, one < 2 mm and the second > 2.5 mm and the rate of oocytes obtained from follicles with a diameter < 2 mm (22.7%), while the rate of oocytes obtained of follicles with a diameter > 2.5 mm (77.2%) .these oocytes is divided depending on the number of layers of granulosa cells that surround it to: -**1**-Typical oocytes that contain all layers of the granulosa cells, and its rate was (10.44%). **2** – Oocytes that invested with more than 3 layers of granulosa cells, and its rate was (48.84%). **3** – Oocytes are partially surrounded by granulosa cells and its rate was (25.67%). **4** – denuded oocytes from granulosa cells was (15.80%). The highest rate of oocytes that reached to the stage of maturation were from oocytes that surrounded by more than 3 layers of granulosa cells (82.44%). These oocytes incubated for maturation in TCM-199 medium in 39 °C, 5% CO2 and 96% humidity for different periods (24,25,26 and 27) hours in order to determine the optimal time for maturation was obtained at the highest rate of maturity (18.29%) within 27 hours of incubation, mostly from the follicles with diameter> 2.5 mm where the rate (19.10%).Fertilized with sperm capacitated *in vitro* and the rate of fertilized oocytes (53.43%). Fertilized oocytes were cultured for 168 hours after fertilization at a temperature 39 °C, 5% CO2 and 96% humidity to cleavage up blastocyst stage, which was represented (40%) and stage of morula which was represented (31.42%).

Key words: Oocytes, Iraqi local goat, ovaries, *in vitro* fertilization

1.Introduction

Biotechnology is one of the most fast growing scientific disciplines of the twenty-first century, and the *in vitro* fertilization technique (IVF) consider one of new techniques which used for solving and treating many problems, most important of which infertility in both human and animals which may result neither due to genital reproductive disease (Adhesion or blocked in fallopian tube) or when the infertility result from low in capacity of sperm to penetrate the ovum, Also IVF process used for other purposes or aims as improvement of the economic state by using Oocytes and sperms from viable females and males respectively and doing fertilization for its in laboratory this enable keeping of good characteristic even after died of these animals. also IVF process increase production (twinning) and enable import and export animals as fertilized egg (4).

The goat consider one of small farm animals which can be used their meat and milk daily in our food, It is well known that the use of human oocytes for research purposes is severely limited. Therefore, due to their convenient size

and management, goat can be considered as unique laboratory animal models to study the reproductive processes in humans, necessitating a better understanding of the mechanisms underlying the biology of the reproductive process. Development of new and appropriate ARTs would also improve the management of infertile/ sub-fertile buck and will eliminate reproductive diseases (3). Breeding of goats in some regions of the world (e.g., cold and temperate regions) are limited to a specific period of the year and therefore, development of appropriate ARTs would enable greater flexibility to produce offspring that would be economically viable to produce high quality milk, meat, skin and wool all year round (1). This study involved collect of ovaries from slaughterhouse and doing fertilization in laboratory with fresh semen that have been collected from buck goat using artificial vagina (AV). Theres critical point of the maturation culture is the selection of the oocytes. Immature oocytes are generally recovered from ovaries of slaughtered animals, which results in a mixing of oocytes at different growth phases. Results obtained by (13) show that oocytes from large follicles (more than 5 mm in diameter) have more ability to develop into embryos than oocytes from small follicles (<3 mm in diameter). However, in terms of efficiency, the diameter of follicles is difficult to control. Furthermore, after aspiration of the follicles, the oocytes are commonly selected using various criteria such as their morphology, including the numbers of cumulus cell layers and evaluation of the granulation of the cytoplasm. Those morphological evaluations are subjective, and categorization standards vary among investigators.

In this study the IVF process have done in local Iraqi goat because there is less studies for this kind of animals as comparison with cow and sheep in Iraq. *In vitro* embryo culture is an important procedure for improving the developmental competence of in *vitro*embryos produced by IVM–IVF Furthermore (21 and 8)

2.Material and Methods

2.1. Ovaries collection: The ovaries in this study were collected from three location local slaughterhouse in Basrah city, Iraq, Abattoir in Basrah markets, Iraq and from agricultural research station, agriculture college, university of Basrah. Transported to the laboratory in 0.9 % saline which was supplemented with antibiotic (100 µg/mL) in thermo flask at 37°C and transported to the laboratory within 2 hr (7) to 4 hr of slaughter (19). The ovaries in the laboratory were handled aseptically. Ovaries were taken from the normal saline and lightly rinsed with 70 % alcohol (Ethanol) to remove any contaminants present. The ovaries were washed 3 times in fresh warm saline before processing. All ovaries were freed of the surrounding tissues, using sterile scalpel blades and forceps (20).

2.2.Categories of follicles: This study involved determine the optimal follicular size for maturation and fertilization. This done by measurement the size of follicles per each ovary using vernier as in figure (1-B) then categories in to 2 groups according to (5). Large size group recognized by its diameter more than 2.5

mm and small size group which have diameter less than 2 mm. This enable measurement the competence oocytes for IVM and IVF depending on the size of follicle from which extracted.

2.3. Oocytes collection method: The oocyte is collected by using aspirated method in which visible follicles (<2 to 8 mm) in diameter measured using vernier and aspirated using an 18 gauge hypodermic needle attached with a sterile 5 ml disposable syringe containing 2 ml harvesting medium (Ham's F-10**)** as in figure (1-A). The media along with the collected COCs was then transferred to Petri dish (the oocytes which recovered from the same size follicles were place in the same Petri dish. The Petri dishes were kept undisturbed for 5 min, allowing the COCs to settle down. Excess media were taken out by a syringe without disturbing the oocytes at the bottom of the Petri dish. The Petri dishes were examined under an inverted microscope and the total number of COCs harvested was counted according to (20).

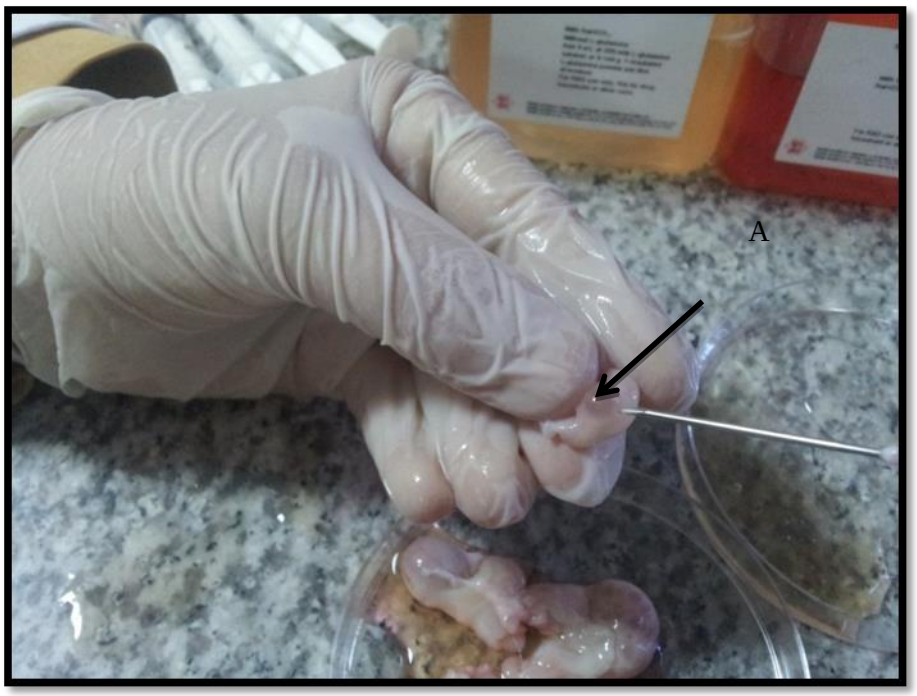


Figure (1): Showing harvesting of oocytes from follicle using aspiration method and the pointer indicated on follicle (A) and measurement the diameter of follicles using vernier and the pointer indicated on follicle (B).

2.4. Grading the COCs depending on the number of cells layers surrounding the oocyte: After examined the Petri dish under inverted microscope and counted the COCs. The COCs which collected from follicles less than 2 mm and more than 2.5 mm in diameter are graded into 4 groups depending on the number of cells layers surrounding it (typical oocytes contain complete layers of granulosa cells, oocytes contain more than 3 layers of granulosa cells, oocytes surrounded partially with granulosa cells and denuded oocytes (poor for granulosa cells)**.** This done according to **(9, 11, 15, 12 and 16)** with little modification.

2.5. *In vitro* maturation (IVM): Incubate the oocytes were for 24 hr and when there is no maturation incubate for 25 hr and also still monitor the time of maturation until 27 hr and counted the number of oocytes which reach the maturation during each of these time to confirm the optimum time for maturation and the procedure of maturation is explained as following: after aspirated method, the oocytes that selected for *in vitro* maturation prefer to be morphologically (having compact, multilayered cumulus granulosa cell complex and evenly granulated cytoplasm) was separated under microscope.

The COCs were washed three time, first in Ham's F-10 medium and subsequently in maturation medium (TCM-199). Each ten COCs were placed in 50 µl droplets of maturation medium in Petri dishes as in figure (2-B) using micropipette. Each Petri dish contain 4-5 droplets of maturation medium and added 10% from fetal bovine serum to the droplets contained COCs then covered with sterile paraffin oil to help prevent evaporation of the maturation medium then incubated the COCs at 39°C at 5% CO2 in 96 % humidity according to (6) (figure 2-B).

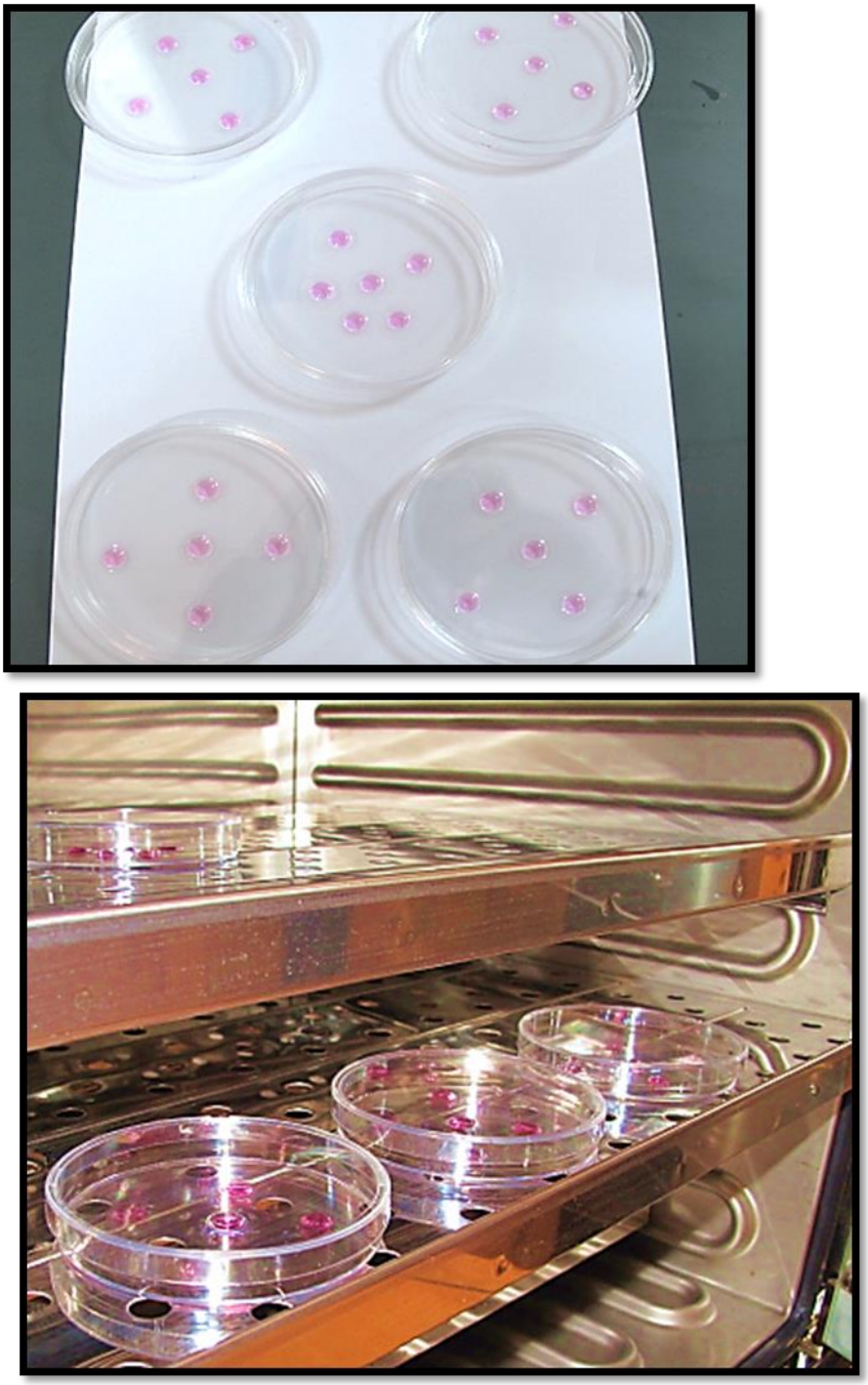


Figure (2): Explained droplets of media contained immature oocytes in Petri dishes after aspirated from ovaries and ready for incubation.(A) and Petri dishes contained oocytes inside CO2 incubator for maturation occurrence (B)

2.6. Preparation of semen and *in vitro* capacitation method: Fresh semen was collected from buck goat that sure previously from their fertility by using artificial vagina at agricultural research station in agriculture college of Basrah university. The selected buck are known by genital efficiency and have been trained on artificial vagina and must be healthy .The semen is preparation according (17) which briefly included: Examined the semen microscopically to

ensure from motility of sperm and that is done on a short time from the semen collection. Make dilution of semen (0.5) ml with 5 ml of TCM-199 medium and then make centrifugation (1500 rpm/minute for 3 minute. Discarded the supernatant and take sperm pellet which contain high concentration of viable sperms and also diluted other once with TCM-199 then incubated in CO2 incubator at 39°C for 1 hr to permit the sperm to capacitated. At this time the semen become ready for fertilization.

2.7. *In vitro* fertilization method (IVF): After 27 hr from maturation, select the oocyte with first polar body and expanded cumulus cells because it is consider mature. The expanded cumulus cells which surrounded the matured oocyte removed by repeated pipetting using micropipette, after that add 300U/ml of hyluronidase enzyme to the matured oocytes for about 3-5 minutes to remove (digest) the granulosa layers which surrounded the oocytes and facilitated fertilization process then groups of 5-10 oocytes picked up by micropipette and transferred into 50 µL of TCM-199 and then add 0.5 ml of capacitated sperm (1×10^6 sperm per mL) in sterilized Petri dishes under paraffin oil. Then IVF drops incubated at 39°C at 5% CO2 in 96 % humidity for 24-27 hr.

2.8. IVC technique: After *in vitro* fertilization step remove the Petri dishes from incubator and examined it under inverted microscope to ensure from fertilization occurrence which characterize by appearing the second polar body, after ensure from presence of second polar body pick up the fertilized oocytes by using micropipette and transferred to sterilized petri dishes contained 200 µL of TCM-199 with 10% of fetal bovine serum then incubate at 39°C at 5% CO2 in 96% humidity for 168 hr (7 days) to permit it to cleavage and reach it to the morulla or blastocyst stage, after 168 hr stop the incubation and examined the Petri dishes under inverted microscope to observe and counted the cells which formed during this period of incubation. After that transferred the cleavaged oocytes (embryos) to sterilized Eppendorf tube contained drops of distilled water and stored in freezer.

3. Results and Discussion

3.1. Samples collection: This study used 72 slaughtered does and 144 ovaries. (Table 1).

Table (1): Site of samples collection and numbers of samples

Sites of samples collection	No. of slaughters females	No. of ovaries
Local slaughterhouses	30	60
Abattoir in Basra markets	7	14
Agricultural research station, agriculture college	35	70
Total	72	144

3.2. Oocytes collection technique: In this study the oocytes are harvested from the ovaries by using aspirated technique to extracted the oocyte from the ovaries because the number of normal COCs per ovary was observed in aspiration than those of puncture and slicing this reported by (14). According to the results of harvesting technique the total number of oocytes that consider typical for IVM and IVF containing complete layers of granulosa cells were 109 (10.44%). whereas the number of oocytes that contain approximately more than 3 layers were 502 (48.08%) (figure 3-A). whereas the number of oocytes that surrounded partially with granulosa cells were 268 (25.67%)(figure 3-B) and the number of denuded oocytes (poor for granulosa cells layers) were 165 (15.80%) (figure 4-B). Table (2 and 3) showed this results and classification grading of oocytes.

The results showed that the oocytes surrounded by more than 3 layers of granulosa cells have higher rate of maturation (82.44%). These agreed with (2). These cells consider source for nutrition and contain necessary protein for maturation and fertilization process and protect the oocytes therefore, cumulus cells are considered to play an important role in oocyte maturation by regulating meiotic progression and by supporting cytoplasmic maturation (18)**.**

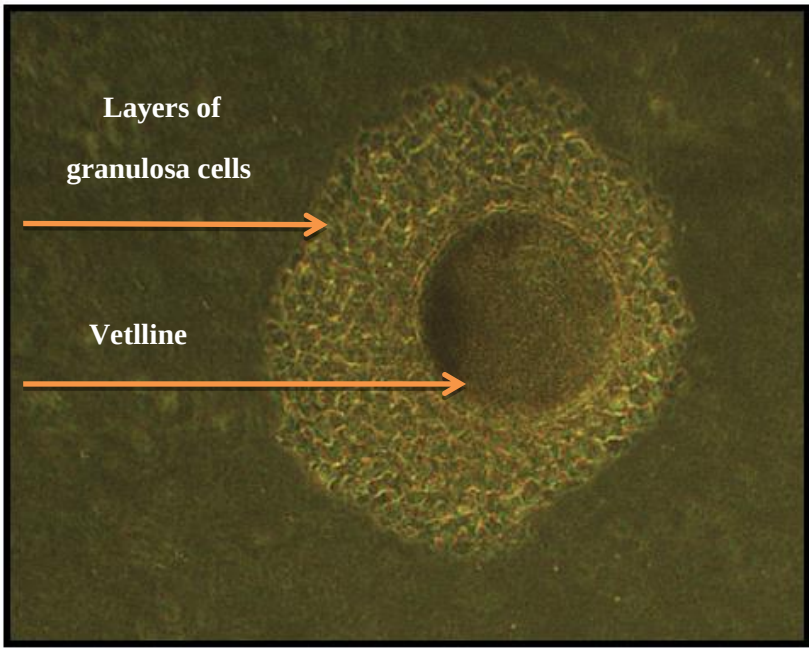
3.3. Determine the optimal follicular size for maturation and IVF: According to the results of measurement the size of follicles from which the oocytes extracted, the number of oocytes that harvested from follicles less than 2 mm in diameter were 238 and the number of oocytes that harvested from follicles more than 2.5 mm in diameter were 806. (figure 4-A and table 4).

Table (2): Grading of oocytes based on presence of granulosa cells around oocytes (9,11,15,12 and 16).

Grade	No. of oocytes (%)
Typical oocytes contain complete layers of granulosa cells	109 (10.44)
Oocytes contain more than 3 layers of granulosa cells	502 (48.08)
Oocytes surrounded partially with granulosa cells	268 (25.67)
Denuded oocytes (poor for granulosa cells)	165 (15.80)
Total	1044

Table (3): Results of classification grading of oocytes.

No of cumulus layers present / Follicles diameter (mm)	No. of matured typical oocytes (%)	No. of matured oocytes with more than 3 layers (%)	No. of matured oocytes which surrounded partially with layers (%)	No. of. matured denued oocytes (poor for layers) (%)	Total maturation oocytes (%)
<2 (238)	12 (15.18)	67 (84.81)	0	0	79 (33.19)
<2.5 (806)	72 (16.17)	365 (82.02)	8 (1.79)	0	445 (55.21)
Total (1044)	84 (16.03)	432 (82.44)	8 (1.52)	0	524 (50.19)



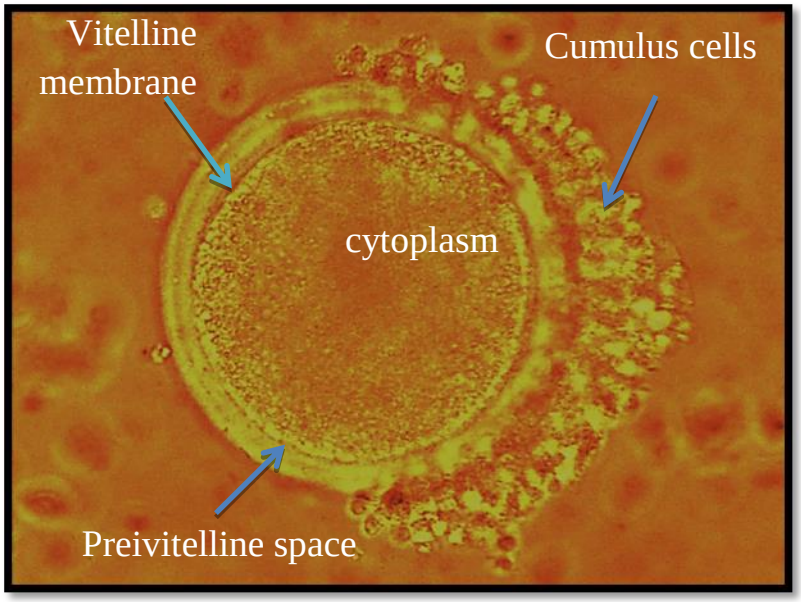
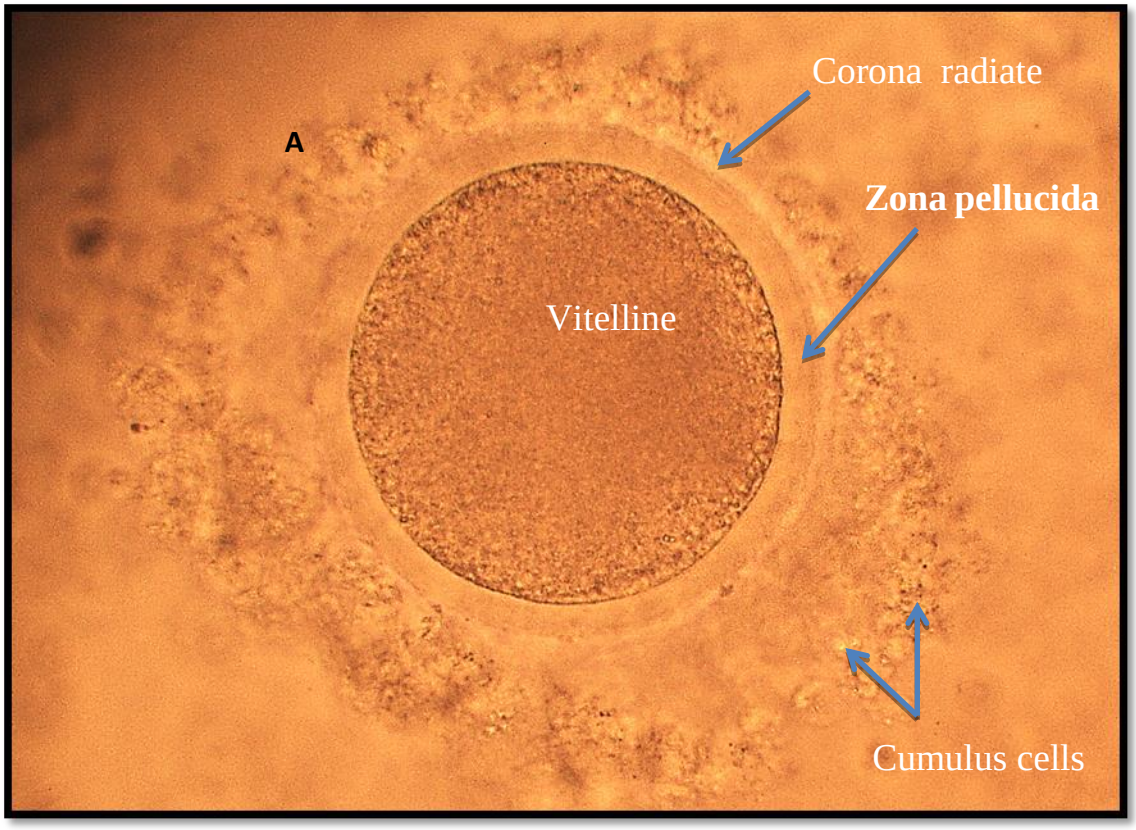


Figure (3): The immature COCS containing more than 3 layers of granulosa cell after transfer from collection medium to TCM-199 medium under inverted microscope (40 X) (A) and the immature COCS that surrounded partially with granulosa cells under inverted microscope (40 X) (B)



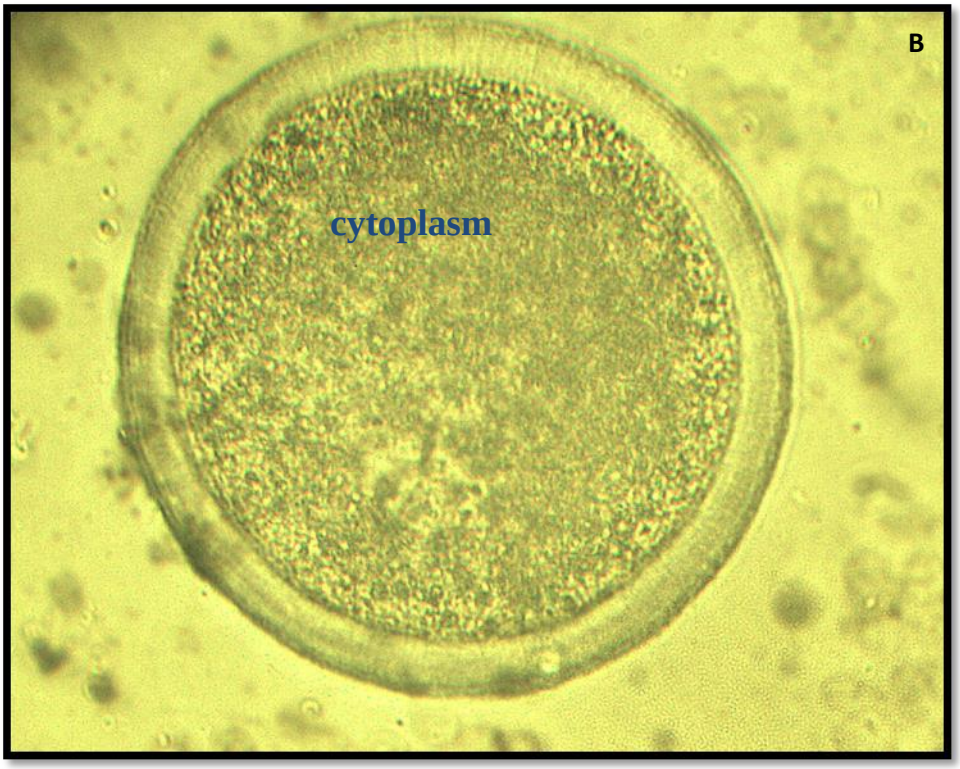


Figure (4): Showing immature oocyte after aspirated from follicle > 2.5 mm in diameter as observed under inverted microscope (40X) (A) **and the unfertilized denuded oocyte (poor for granulosa cells layers) under inverted microscope (40 X).(B)**.

Table (4): Number of oocytes collected from follicles <2 mm and from follicles >2.5 mm diameter.

Diameter of oocytes	No. of oocytes (%)
<2	238 (22.7)
>2.5	806 (77.2)
Total	1044

According to the results that showed in table (3) which explained that the high rate of maturation is obtained from follicles with diameter > 2.5 mm and were 445 (55.21%) whereas the rate of matured oocytes that which extracted from follicles with diameter < 2 mm were 79 (33.19%). These results is proved the effect of follicles size on maturation these agreed with (10) Who established that large follicles contain more oocytes capable of maturation and developing into blastocysts than do smaller

follicles. these agreed with (13) show that oocytes from large follicles (more than 5 mm in diameter) have more ability to develop into embryos than oocytes from small follicles (<3 mm in diameter).

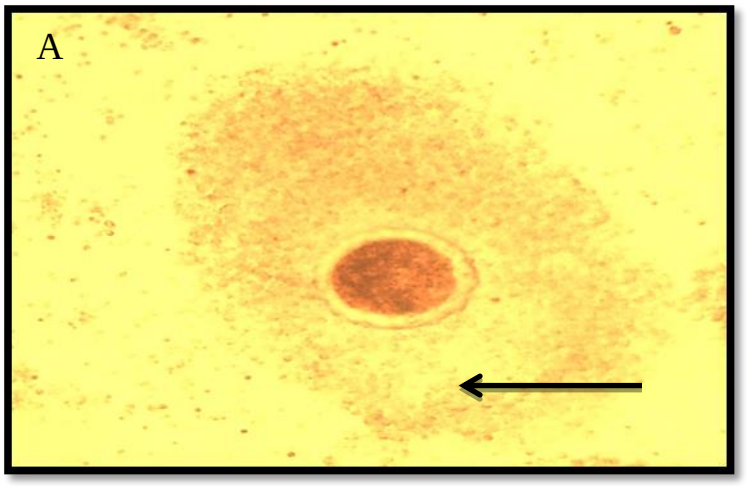
4.3. IVM of oocytes: According to the results of maturation, the signs of maturation showed in (figure 9) and (figure 10), the total number of oocytes that submitted to maturation were 1044 from both follicles size (less than 2 and more than 2.5 mm in diameter). The number of oocytes taken for IVM from follicles less than 2 mm diameter were 238 and according to results of different incubation times about 7 (2.94%) of it reach maturation during 24 hr, 11 (4.62%) were matured during 25 hr from the time of incubation. 24 (10.08%) were matured during 26 hr, 37 (15.54%) were maturation during 27 hr. and 159 (66.80%) were failed to mature throughout the periods of incubation, but the number of oocytes that subjected for IVM from follicles more than 2.5 mm diameter were 806. 66 (8.18 %) of it were matured during 24 hr, 105 (13.02 %) reach to maturation during 25 hr, 120 (14.88%) were matured during 26 hr and 154 (19.10%) matured during 27 hr. While 361 (44.78%) of it do not reach maturation throughout all periods of incubation. (table 5). According to the results in table (5) showed the best time for maturation was during incubation for 27 hr during this period obtained on highest rate of matured oocytes 191 (18.29%) from total number of oocytes whereas when incubating for (24, 25 and 26 hr) obtained on these results respectively 73 (6.99%), 116 (11.11%) and 144 (13.79%) this indicate that the time of incubation have impacted on the rate of maturation

3.5. IVF technique: According to the results of IVF as showing in (table 6) and the rate of graded oocytes which are reaching to fertilization shows in (table 7) the total number of matured oocytes that submitted to IVF were 524, 79 of it were from follicles has < 2 mm in diameter, only 17 (21.51 %) were fertilized *in vitro*. While the number of matured oocytes which from follicles > 2.5 mm in diameter were 445 oocyte, only 263 (59.1 %) were fertilized *in vitro* the results showed that the high rate of fertilization were from oocytes with more than 3 layers of granulosa cells 213 (76.07%) (figure 6, A) shows the moment of fertilization occurrence.

3.6. IVC technique of zygote: According to the results of IVC technique, the culture of 280 zygotes up to 168 hr (7 days after fertilization) to permit it to reach progressive stages of growing lead to forming 112 compacted morula (figure 6, A) and 88 blastocysts (figure 7).

Table (5): The numbers of oocytes that reach to the maturation during different times of incubation.

Size of follicles	No. of oocytes into maturation	No. of oocytes that matured during:					No. of immature oocytes (%)
		24 hr (%)	25 hr (%)	26 hr (%)	27 hr (%)	Total matured oocyte (%)	
<2	238	7 (2.94)	11 (4.62)	24 (10.08)	37 (15.54)	79 (33.19)	159 (66.80)
>2.5	806	66 (8.18)	105 (13.02)	120 (14.88)	154 (19.10)	445 (55.21)	361 (44.78)
Total	1044	73 (6.99)	116 (11.11)	144 (13.79)	191 (18.29)	524 (50.19)	520 (49.8)



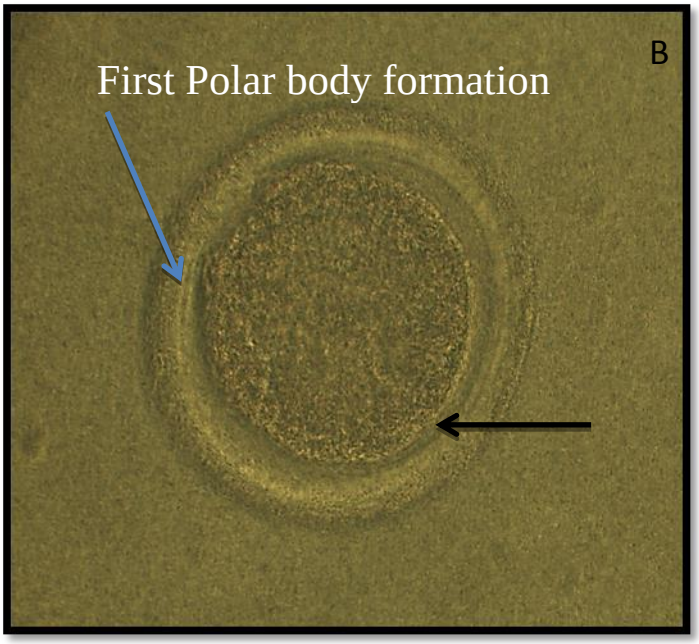


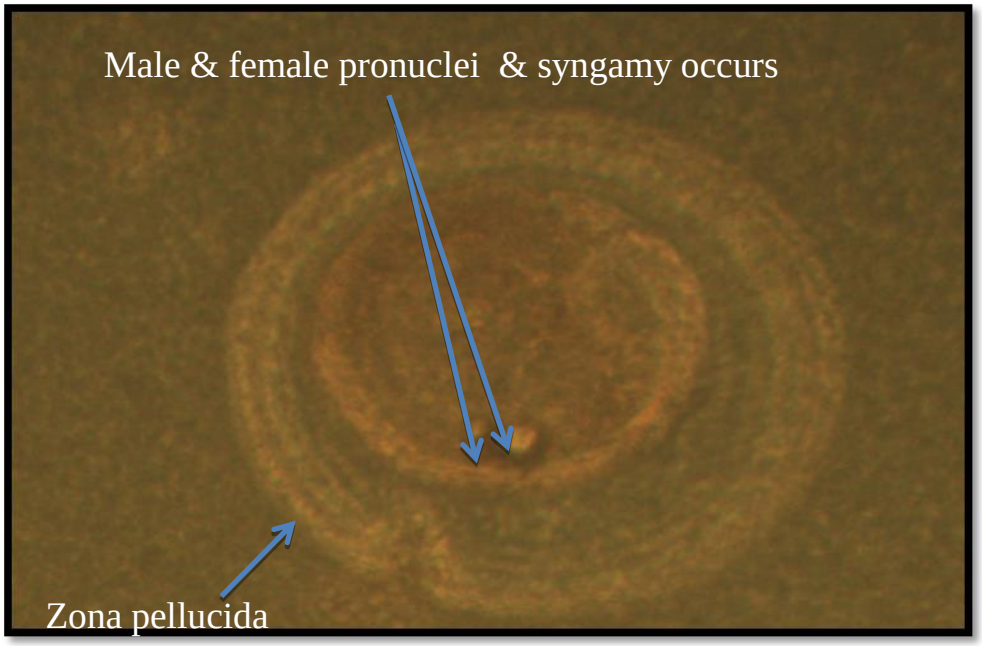
Figure (5): Matured oocyte show the expanded of granulosa cells which surrounding it under inverted microscope (40 X) Pointer A indicated on first polar body and pointer B indicated on expanded of granulosa cells which surrounding oocyte (A) and Matured oocyte under inverted microscope (40 X). Pointer indicated on first polar body (B).

Table (6): Number and percentage (%) of oocytes which fertilized *in vitro*.

Size of follicles (mm)	No. of matured oocytes submitted to IVF	NO. of oocytes that fertilized *in vitro* (%)
< 2	79	17 (21.51)
>2.5	445	263 (59.1)
Total	524	280 (53.43)

Table (7): Rates of fertilization in graded oocytes which extracted from different follicles diameter.

Size of follicles (mm)	Total fertilized oocytes	No of fertilized typical oocytes (%)	No of fertilized oocytes with more than 3 layers (%)
< 2	17 (21.51)	6 (35.29)	11 (64.70)
>2.5	263 (59.1)	61 (23.19)	202 (76.80)
Total	280 (53.43)	67 (23.92)	213 (76.07)



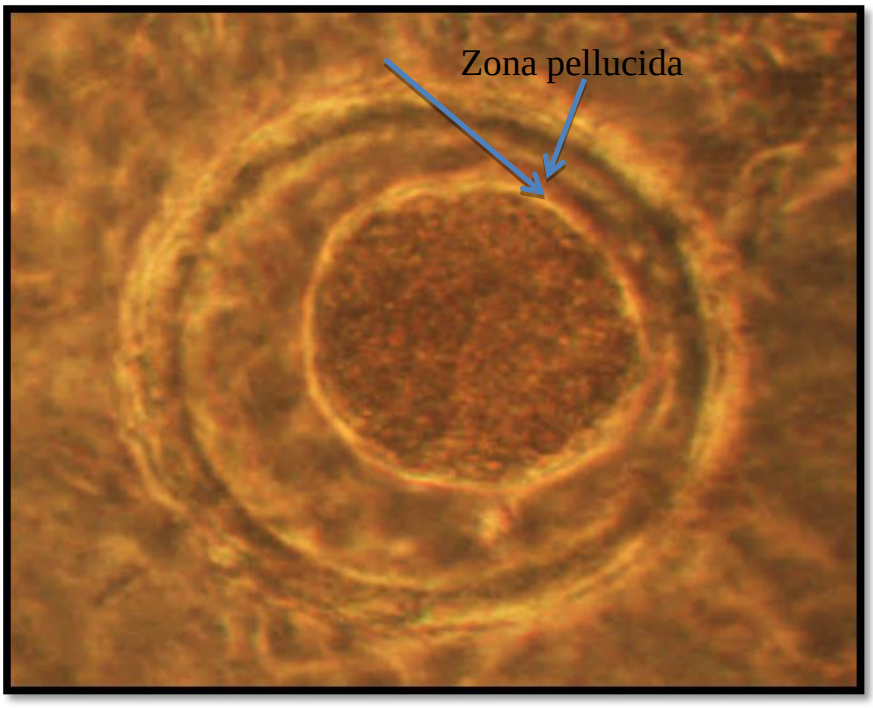


Figure (6): Fertilized oocyte under inverted microscope (40 X) and compacted morula under inverted microscope - 40 X (B).

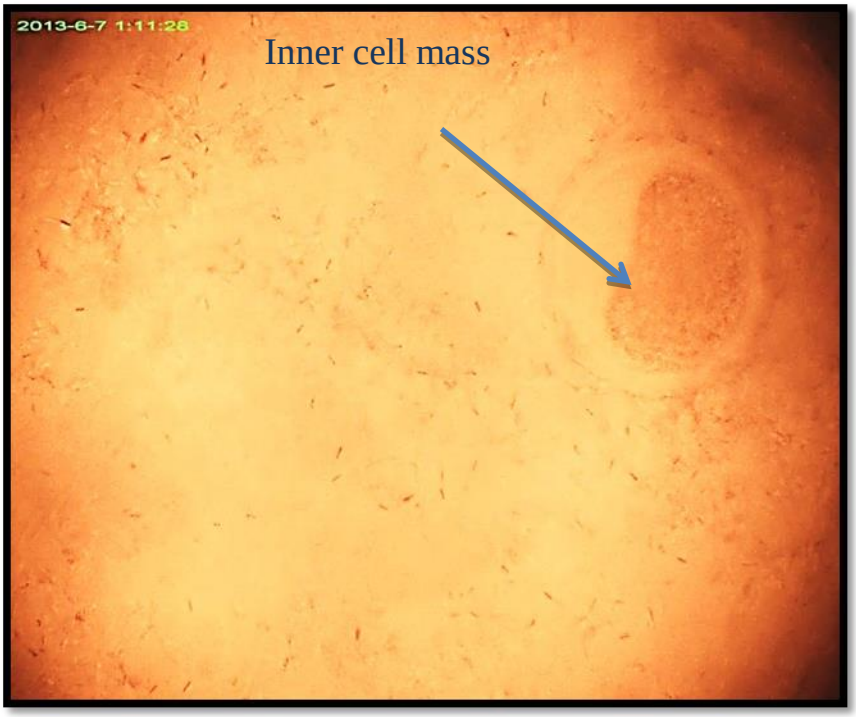


Figure (7): Showing blastocysts stage as seen under inverted microscope (40 X).

Conclusions: Based on the previous findings it may be concluded the following: The optimal time for maturation were 27 hr from incubation because the high rate of maturation occur during 27 hr incubation period, the size of follicles have impact role on IVM and IVF techniques where the high rate of maturation and fertilization were from follicles with > 2.5 mm diameter, the denuded and partially surrounded oocytes grades not prefer in IVM and IVF techniques because the denuded oocytes never mature in this study and there is a low rate of maturation in partially surrounded oocytes grade ,but not fertilized. Therefore not prefer for IVEP and the best grade for IVM and IVF techniques were oocytes that surrounded by more than 3 layers of granulosa cells because the high rate of IVM and IVF were from it.

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