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# ASSESSMENT OF EMBRYO PRODUCTION OF DROMEDARY (*Camelus dromedarius)* USING TWO SEMEN SOURCES AND TWO *IN VITRO* FERTILIZATION TECHNIQUES

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## SUMMARY

*The current study was conducted to evaluate the effects of maturation time (30 or 40 hours) on maturation rate (%) and two sources of spermatozoa; epididymal spermatozoa (G1), frozen semen (G2) and two insemination methods IVF (G1 and G2) and intra-cytoplasmic sperm injection (ICSI) (G3) on the cleavage rate and development competence of in vitro produced embryos as a trial to improve the reproductive efficiency of the dromedary camel. Cumulus oocytes complexes (COCs) were recovered from ovaries by slicing technique. Based on morphological characteristics (number of cumulus cells enclosed the oocytes and the clearance of the cytoplasm) only grade A and B oocytes were selected to be cultured in TCM-199 medium for maturation at 5% CO2 and 38.5 $^{\circ}$C for 30 or 40 hours. According to maturation results, the mature oocytes were subjected to two sources of spermatozoa. For G1, a number of 205 oocytes were inseminated with fresh epididymal spermatozoa ($1x10^6$ spermatozoa/ml). In G2, a number of 290 oocytes were inseminated with frozen thawed semen ($3x10^6$ spermatozoa/ml). Whereas in G3, 28 oocytes were denuded and inseminated (individually injected) by ICSI technique. The results showed a significantly ($P \leq 0.05$) higher maturation rate (83%) in oocytes subjected to 30 h compared to 40 h group (64%). There was no significant ($P > 0.05$) difference in cleavage rate between the three groups being 21, 20 and 23 % for G1, G2 and G3, respectively. Blastocyst rate calculated to fertilized oocytes was higher in G1 (8.27%) and G2 (8.33%) compared to G3 (5.5%). In conclusion, this study validates the first application of the ICSI technique as a successful method for embryo production in dromedary camel. Moreover, there is no difference between frozen and epididymal spermatozoa on blastocyst rate when applying in vitro fertilization.*

***Keywords: Dromedary, Fertilization protocols, ICSI, Oocytes, Embryo production***

## INTRODUCTION

Dromedary camel is considered a multi-purpose animal. It is used for milk, meat productions, transport and sports (Skidmore, 2005). In Egypt, despite of its major important and high potential value, camel participate a small part in agriculture system for meat production with 47250 ton/year (FAO, 2013).

The reproductive efficiency of camels under natural conditions is approximately 40%, this is probably due to the relatively short breeding season, long pre-pubertal period, long gestation period, prolonged lactation period and the lack of use of assisted reproductive techniques (Skidmore, 2005).

*In vitro* embryo production technology (IVP) has been successfully applied to number of animal species with different success rate (Gordon, 2003). The purposes of produced embryos vary from commercial to research such as studies the pre-implantation development and application of new technologies. However, there is still a lack of application of such technique in camels (Skidmore, 2005).

Many trails were conducted to study the possibility of success of *in vitro* oocyte maturation in camelids and dromedary (Abdoon, 2001; Torner *et al*., 2003; Khatir *et al*., 2004; Nowshari and Wani, 2005; Khatir and Anouassi, 2006 and El-Sayed *et al*., 2012) indicating that preparation of semen and culture media are restricting the success on *in vitro* embryo production.

Previous works aimed at reaching an appropriate protocol for *in vitro* camels' embryo production. The results indicated that the success rate of reaching to blastocyst stage ranged from 14 to 23% (Khatir and Anouassi, 2006; Abdoon *et al*., 2007 and Wani, 2009).

Semen preparation is one of the corner stone's on *in vitro* embryo production in camels. Different methods were used to separate good motile sperm to be used in *in vitro* fertilization step (Suthar and Shah, 2009). Fresh ejaculated spermatozoa have been used for IVF of dromedary oocytes with blastocyst rate up to 23% (Khatir *et al.*, 2004 and 2005), reduced to 17% when epididymal spermatozoa were used after storing in tris extender (Nowshari and Wani, 2005). Up to the knowledge of the authors

rare data are available to describe the success rate of camel's embryo production using frozen semen and little has done applying intracytoplasmic sperm injection (ICSI). This one is a powerful technique in the field of assisted reproduction technologies (ART). It implies the injection of the spermatozoa inside an oocyte, bypassing the natural process of sperm-oocyte interaction. It could be used in livestock species to solve the fertilization problems in IVF systems (García-Roselló *et al.*, 2009). Moreover, it can be very useful to use the sex-sorted semen in which spermatozoa are low in numbers and motility (Probst and Rath, 2003).

In this context, the current study was conducted to evaluate the effect of two sources of spermatozoa (epididymal spermatozoa and frozen semen) and two techniques of fertilization (intra-cytoplasmic sperm injection and *in vitro* fertilization) on the cleavage rate and development competence of *in vitro* produced embryos of dromedary camel.

## MATERIALS AND METHODS

### *Chemicals and media:*

Unless otherwise mentioned, all the chemicals and media constituents were purchased from Sigma-Aldrich Chemicals, Germany.

Dromedary camel frozen semen was obtained from artificial insemination and embryo transfer lab - Marriott Research Station - Desert Research Center, Alexandria, Egypt.

### *In vitro maturation of oocytes:*

A number of 376 dromedary camel ovaries were collected from two abattoirs (El-Bassatein and El-warrak).The collected ovaries were placed immediately after slaughtering into thermo flask contains normal saline solution (0.9% NaCl) supplemented with antibiotic (100 IU penicillin and 100 µg streptomycin/ml) at 25-30 ˚C and transported to the laboratory (Cairo University Research Park, Cairo University, Giza, Egypt) within two to four hours from slaughtering. Then, ovaries were washed once with ethanol (70%) to remove any contamination. Followed by final washing with pre-warmed (30 ˚C) phosphate buffered saline (PBS) supplemented with antibiotics (100 IU penicillin and 100 µg streptomycin/ml). All visible follicles on the ovarian surface (2–8 mm diameter) were sliced using normal sharp razor in a glass petri dish contains pre-warmed (30 ˚C) PBS. The harvesting medium containing the COCs transferred to glass tubes for 10 min for precipitation. Searching for COCs and grading has been done under stereo microscope (MZ6, Leica Microsystems, Wetzlar, Germany). Only good quality oocytes were selected and washed three times in washing medium (TCM-199 with HEPES - Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) heat-treated (56 ˚C for 30 min) fetal bovine serum(FBS) and antibiotic(100 IU penicillin and 100 µg streptomycin/ml). Then, once in IVM medium (TCM-199 HEPES free - Gibco, Grand Island, NY, USA) supplemented with 0.3% Cysteine, 10% FBS, 10 µg/ml FSH, 10 µg/ml LH, 20 ng/ml EGF, 0.25 mg/ml $Na^+$ pyruvate and 1 mg/ml estradiol and antibiotic (100 IU penicillin and 100 µg streptomycin/ml) as final washing. Maturation medium was sterilized using 0.22 µm millipore syringe filters and incubated in $CO_2$ incubator two hours before using (Amer and Moosa, 2008). The oocytes were placed in four well plate each well contains 25 – 30 oocytes in 400µl of IVM medium and covered with mineral oil then incubated in $CO_2$ incubator at 38.5 ˚C in 5% $CO_2$ and humidity 95% in the air for 30 or 40 hours depending on the experiment.

### *Semen preparation:*

For epididymal spermatozoa, testicles were collected within their scrotum under hygienic conditions and transported to the laboratory within one hour in an ice box at temperature between 25–30 °C using water bags. Each testicle was dissected away from its tunica vaginalis and other extraneous tissues, washed 3 times by tab water and once by alcohol 70%. Various incisions in the tail of epididymis were performed with a scalpel and then, the spermatozoa were released and allowed to swim out by gently pressing that region manually in 6 cm petri dish containing 5 ml sperm TALP (Parrish *et al*., 1986), for five to 10 min. The recovered spermatozoa were placed in 15 ml falcon tube in water bath at 37 ºC. For IVF, the sperm suspension was washed twice with sperm TALP by centrifugation at 250×g for 10 min each. The pellet was overlaid with fertilization medium (TALP supplemented with 4mg/ml BSA, 50 IU/ml penicillin and 10µg/ml heparin) and allowed to swim up for 1 hour in an incubator (5% $CO_2$ and 38.5 ºC) with angle of 45 degree. The motile spermatozoa were added to the oocytes at the concentration of approximately $1\times10^6$/ml (Wani, 2009).

For frozen semen, two 0.5 ml straws were thawed in water bath at 37˚C for 40 seconds (El-Bhrawi, 2005). Straws were wiped with 70% ethyl alcohol before being opened. The content over layered on 5 ml of sperm washing medium (Sperm TALP medium) in a 15 ml falcon tube. Then, centrifuged twice at 300xg for 5 min each. The final pellet was re-suspended in appropriate volume (depending on the sperms concentration after washing) of pre-warmed fertilization medium (Fert. TALP medium) then the final sperm concentration was adjusted to $3x10^6$/ml (El-Sayed *et al*., 2012).

***Conventional IVF:***

Sperm suspension and matured oocytes were co-incubated in groups of 20 – 25 oocytes/ 400 µl fertilization medium at 38.5 ºC, 5% $CO_2$ and 95% humidity in air for 18 h.

***ICSI:***

For injection procedure an inverted microscope (DMI 3000B, Leica Microsystems, Wetzlar, Germany) has been used with micro-manipulator system (NARISHIGI, Japan).

Amount of 1 µl of sperm suspension was added to a 10 µl drop of polyvinyl-pyrrolidone (PVP; 10%, FrtiPro, Belgium) shortly before microinjection. Thereafter, injection was done using a pipette with inner diameter of eight µm, and the holding pipette with 25 µm. Sperms were individually immobilized by targeting the mid piece, aspirated tail-first and injected into the ooplasm through the zona pellucida. The first polar body was adjusted to either six or 12 o'clock position, and the injection pipette adjusted to three o'clock position. During the injection, cytoplasm was aspirated to approve that the oolema was broken. The spermatozoon was injected into the ooplasm with a minimum volume of medium at the 9 o'clock position (Shirazi *et al*., 2009) as shown in Plate 2.

***In vitro culture system:***

After IVF or ICSI, the presumptive zygotes were transferred to a washing medium (TCM-199 with HEPES supplemented with 10% (v/v) heat-treated (56 ˚C for 30 min) FBS and AA (100 IU penicillin and 100 µg streptomycin/ml), then cultured in a modified synthetic oviductal fluid (mSOF) medium supplemented with 5% FBS and AA (100 IU penicillin and 100 µg streptomycin/ml) in groups of 5 embryos per 100 µl culture medium. Thereafter, the embryos were incubated at 38.5 ˚C in sterile filtered human lung air containing around 4% carbon dioxide and 16 to 17% oxygen using submarine incubation system (SIS) as stated by (Vajta *et al.*, 1997).

***Statistical analysis:***

Data was statistically analyzed using IBM SPSS 22.0 Software Package (IBM corp., NY, USA, 2013). Independent-Samples T Test was used to analyze the effect of sperm source (epididymal and frozen), insemination methods (IVF and ICSI) on cleavage and blastocyst rates and to compare the maturation rates due to expansion and polar body for oocytes exposed to 30h or 40 h maturation time. Results are expressed as means ± S.E.M. and the significance level was set at $P<0.05$.

## RESULTS AND DISCUSSION

***Effect of maturation time***

The maturation rate (83%) according to expansion of cumulus cells for oocytes incubated for 30 h was significantly higher ($P <0.05$) than that for oocytes incubated for 40 h (64%). In addition, nuclear maturation rate that indicated by extrusion of the first polar body was higher in 30h group but not significantly different ($P <0.05$) than 40h group as shown in Table 1. The results are in agreement with those obtained by Khatir *et al*. (2007) who found that incubation for 30 h has resulted in 84% maturation rate. The lower maturation rate resulted from 40 h maturation, maybe due to the tendency towards a higher percentage of degenerated oocytes as the culture period increased more than 30 h as reported before by Kafi *et al*. (2005). On the other side, this result is lower than that obtained by El-Nahla *et al*. (2014) who recorded maturation rate 92% for oocytes incubated for 40 h and average of 80% extrusion of the first polar body. This discrepancy maybe due to the few numbers of trials which have been done on oocyte maturation testing the incubation time in dromedary especially when different media were used or the experiments were performed in different seasons.

**Table 1. Effect of different incubation periods on maturation rate**

| Trait | 40h | 30h |
|---|---|---|
| No. of oocytes | 157 | 218 |
| Expansion (%) | 64.3±4.0 [a] | 83±2.0 [b] |
| Polar body (%) | 15.7±3.0 [a] | 22.7±2.0 [a] |

Means within the same row having different superscript letters differ significantly at ($P <0.05$).

***Effect of sperms source and fertilization techniques on cleavage and blastocyst rates:***

The results of this study (Table 2 and plate 2) show that the cleavage rate of the *in vitro* dromedary embryos resulted from fertilization by using epididymal spermatozoa, frozen semen or ICSI were not significant ($P> 0.05$) where it has recorded 21%, 20% and 23% respectively. There was no significant difference ($P > 0.05$) in the proportion of blastocyst resulted from oocytes fertilized with epididymal spermatozoa, frozen semen or ICSI (8.27%, 8.33% and 5.5% respectively). The result of epididymal spermatozoa regarding cleavage rate is higher than that obtained (17%) by Fathi *et al*. (2014) and it is related to that obtained by Moawad *et al*. (2012) who reported that the cleavage rate was 26.8% in *in vitro* camel embryos. On the

other side this result using epididymal spermatozoa tended to be low compared to the results (37% - 43%) reported by Wani (2009) and Badr and Abdel-Malak (2010). The blastocyst rate resulted from oocytes fertilized by epididymal spermatozoa is higher than the rate (1.2%) obtained by Badr and Abdel-Malak (2010) and that (3%) obtained by Fathi *et al*. (2014) but comparable to the rate (10%) obtained by Wani (2009). The cleavage and blastocyst rates resulted from fertilization by frozen semen is considered to be high compared to the few studies of using frozen semen for *in vitro* production of camel embryos done by Abdoon *et al*. (2007) who reported 17% and 0% for cleavage and blastocyst rates respectively, and previous study reported by, El-Sayed *et al*. (2012) who reported 19% and 3.8% for cleavage and blastocyst rates respectively. The ICSI results even though the low percentage of blastocyst rate (5.5%) but according to the author's knowledge it is considered to be novel in this species.

**Table 2. Effect of sperm sources and fertilization techniques on cleavage and blastocyst rates (mean ± S.E)**

| Sperm source/technique | No. of fertilized oocytes | Cleavage rate | Blastocyst rate |
|---|---|---|---|
| Epididymal (G1) | 290 | 21.2±1.7 [a] | 8.27±2.3 [a] |
| Frozen (G2) | 205 | 20.7±2.0 [a] | 8.33±2.5 [a] |
| ICSI (G3) | 28 | 23±9.3 [a] | 5.5±5.0 [a] |

Means within the same column with different superscript letters differ significantly at ($P$ <0.05).

In conclusion, the present results validate for the first time ICSI technique as a successful method for *in vitro* production of camel embryos with further investigations to enhance the success rate. Moreover, frozen semen could be used for *in vitro* production of camel embryos with comparable results as epididymal spermatozoa.

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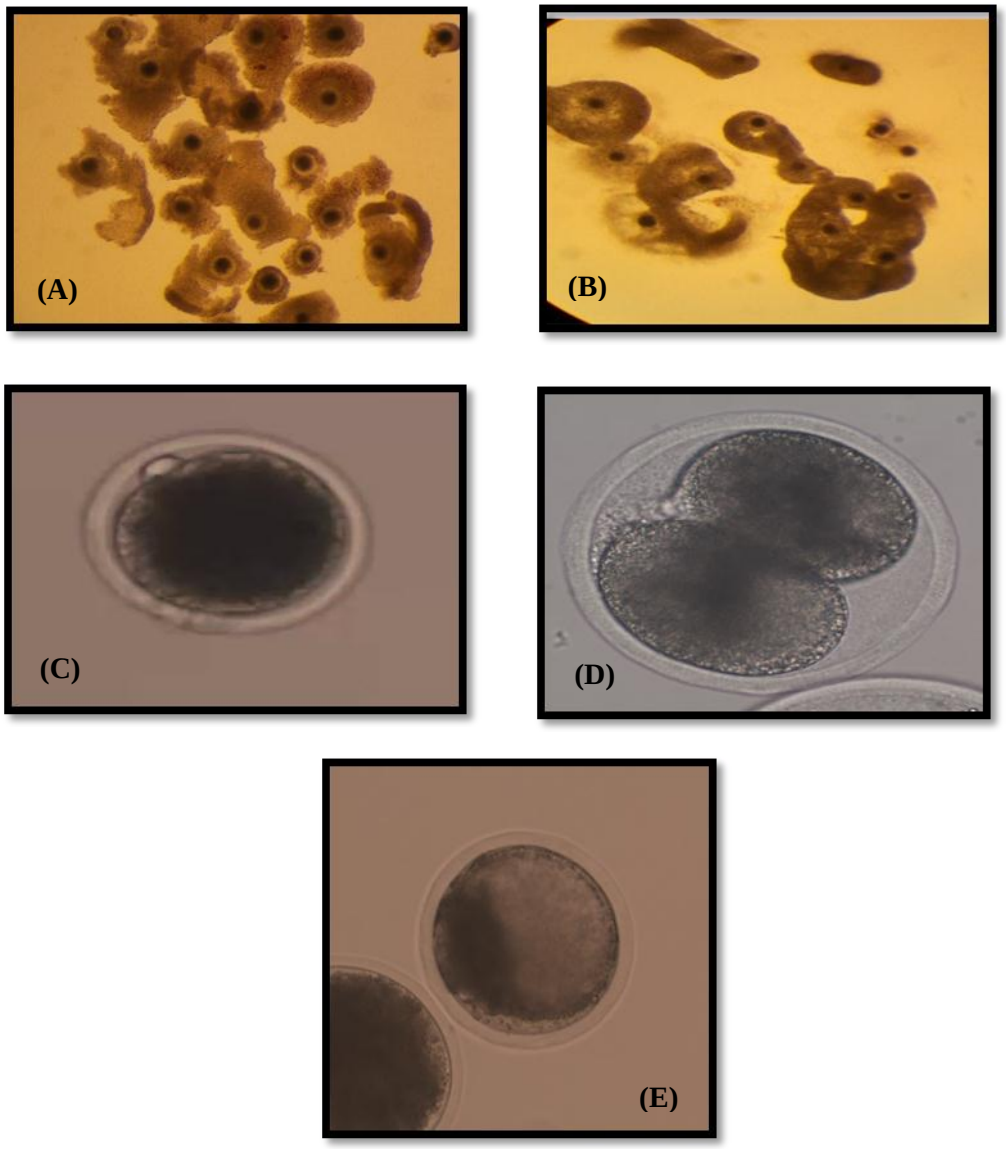


**Plate.1. Developmental stages of *in vitro* produced camel embryos showing: A- immature oocytes, B- mature oocytes with expanded cumulus cells, C- denuded mature oocyte with 1st polar body, D- 2 cell stage embryo and E- blastocyst.**

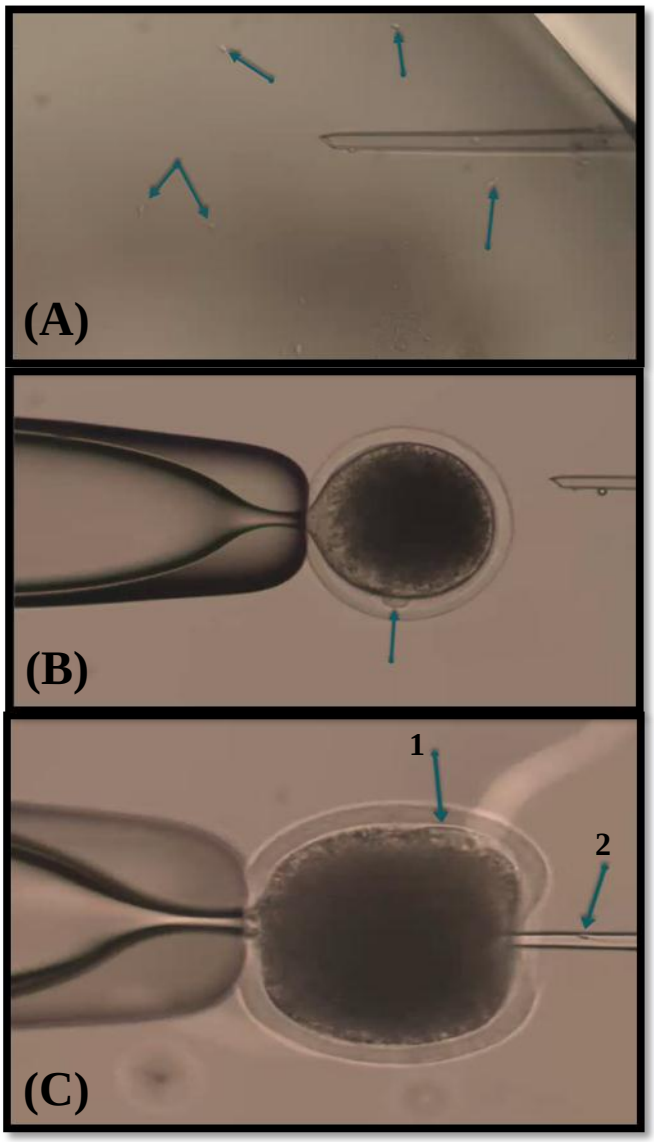


**Plate. 2. ICSI process: A- Sperm immobilization (arrows toward sperms) B- Adjusting the polar body to 6 or 12 o'clock (arrow toward polar body) C- Sperm injected into the ooplasm (arrow 1: the polar body to 12 o'clock, arrow 2: injection pipette penetrated the oolema with sperm inside).**

## تقييم إنتاج أجنة الإبل معمليا بإستخدام مصدرين للحيوانات المنوية وتقنيتين للاخصاب المعملى

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تم إجراء هذه الدراسة لتقدير تأثير فترة التحضين٣٠- ٤٠ ساعة على نسبة الإنضاج وكذلك تأثير استخدام مصدرين للحيوانات المنوية (حيوانات منوية من البربخ اوالسائل المنوي المجمّد)وتقنيتين للإخصاب، (الحقن المجهري للحيوان المنوي داخل السيتوبلازماو الإخصاب المعملى) على نسبة الاخصاب ومراحل التطور ما قبل الإنغراس للأجنة المنتجة معمليا. في محاولة لإنتاج أجنةالجمال وحيدة السنام معمليا. تم جمع البويضات من المبايض بإستخدام طريقة التشريح. بناءا على الخصائص الشكلية (عدد طبقات الخلايا المحيطة بالبويضة، وتجانس السيتوبلازم) تم إختيار البويضات ذات الجودة العالية (A&B) فقط لكي تزرع في بيئة الـ TCM-199 للإنضاج على درجة حرارة ٣٨.٥ مئوي و ٥% ثاني اكسيد الكربون لمدة ٣٠او ٤٠ ساعة.

طبقا لنتائج الإنضاج، البويضات الناتجة من ٣٠ ساعة تحضين خضعت لطرق إخصاب مختلفة. المجموعة الأولى (G1) عدد ٢٠٥ بويضة تم اخصابها بحيوانات منوية من البربخ بتركيز (١ x ١٠$^{٦}$ حيوان منوي/ سم$^{٣}$). في المجموعة الثانية (G2) عدد ٢٩٠ بويضة تم إخصابها باستخدام سائل منوي مجمد مسال (٣ x ١٠$^{٦}$ حيوان منوي/ سم$^{٣}$). وكذلك ٢٨ بويضة تم تعريتها من الخلايا المحيطة بها وحقنها بصورة منفردة كلا على حدى بطريقة الحقن المجهري(G3).

أظهرت النتائج نسبة إنضاج مرتفعة بصورة معنوية (٨٣%) في البويضات التي خضعت للتحضين ٣٠ ساعة مقارنة بالمجموعة المحضنةلمدة ٤٠ ساعة (٦٤%)كذلك كانت هناك فروق معنوية فى نسبة الانضاج المعتمدة على ظهور الجسم القطبى الاول. أيضا لم تكن هناك فروق معنوية في نسبة الاخصاب بين المجموعات الثلاث حيث سجلت (٢١%، ٢٠%، ٢٣%) للمجموعة الأولى والثانية والثالثة على التوالي التى تم إنضاجها لمدة ٣٠ ساعة. مع ملاحظة ان نسبة البلاستوسيست قد تم حسابها بالنسبة للبويضات المخصبة. واظهرت النتائج انها كانت اعلى في المجموعة الأولى والثانية (٨.٢٧% و ٨.٣٣%) على التوالي مقارنة بالمجموعة الثالثة (٥.٥%).

نستخلص من هذه الدراسة انه يمكن استخدام تقنية الحقن المجهري كطريقة لإنتاج أجنة الجمال. بالاضافة الى ان الدراسة قد اثبتت انه لايوجد إختلاف بين الحيوانات المنوية المتحصل عليها من السائل المنوي المجمد او من البربخ في نسبة الإخصاب او البلاستوسيست عند استخدامهم في الإخصاب المعملي لبويضات الجمال.