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EFFECT OF EPIDERMAL GROWTH FACTOR ON IN VITRO PRODUCTION OF CAMEL (*CAMELUS DROMEDARIUS*) EMBRYOS BY USING FROZEN SEMEN

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SUMMARY

The present study was conducted on 1640 oocytes of camels to investigate the effect of supplementing culture medium (TCM-199) with epidermal growth factor (EGF) on the competence of oocytes to mature and develop in vitro using frozen semen and to study the effect of season on recovery rate and quality of recovered oocytes. Oocytes were collected during breeding (n=883) and non-breeding (n=757) seasons from she camels' ovaries (n=191) delivered in normal saline solution at 37^{o}C from a local slaughterhouse. Cumulus oocytes complexes (COCs) were recovered from ovaries by slicing technique. Good-quality oocytes were selected for in vitro maturation (IVM) in TCM-199 medium. Number of 206 COCs was inseminated with thawed semen (3x10^{6} spermatozoa/ml). Culture process was conducted at 38.5^{o}C in humidified air containing 5% CO_2.

The oocytes' maturation rate in medium contained EGF (81.19 %) was higher (P< 0.05) compared to control group (68.04 %). Cleavage rate, and percentages of obtained morula and early blastocyst were slightly higher when EGF was added to the maturation medium compared to control group.

In conclusion, camel frozen semen could be used successfully for in vitro production of camels embryos. In addition, EGF could improve camel oocytes maturation and cleavage rates.

Keywords: camels, IVM, EGF, season, cleavage rate

INTRODUCTION

Camels play a considerable role in agricultural matrix particularly in desert areas, providing milk, meat and transport (Skidmore, 2005). In Egypt, camels have a growth role particularly in meat production sector (Farah and Fischer, 2004). Reproductive efficiency of camels under natural pastoral conditions was reported to be low due to the short breeding season, late age of sexual maturity and long calving interval (El-Wishy, 1987 and Marai *et al.*, 2009).

In Egypt, camels are displaying its maximum reproductive efficiency during the period from December to April (Basiouni, 2007), which affects with no doubt the capacity of embryo production (Amer and Moosa, 2009).

Applying *in vitro* embryo production technique may help in maximizing the gained embryos. 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) indicating that preparation of semen and culture media are restricting the success on in vitro embryo production.

Addition of epidermal growth factor (EGF) to maturation media of camel oocytes was found to improve maturation rate (Khatir *et al*., 2004; Kafi *et. al.*, 2005 and Wani and Wernery, 2010), which agrees with the obtained results on bovine (Lonergan *et al*., 1996; Park *et al*., 1997 and Lee *et al*., 2005).

Previous works aimed at reaching an appropriate protocol for *in vitro* camels' embryo production. Results indicated success rate of reaching to blastocyst stage 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.

The present study aimed at investigating the effect of epidermal growth factor on *in vitro* production of dromedary camels' embryos using frozen semen. In addition to

study the season effect on recovery rate and quality of recovered oocytes.

MATERIALS AND METHODS

Chemicals and media:

Unless otherwise mentioned, all the chemicals, reagents, media, biologics and media constituents were purchased from Sigma-Aldrich Chemicals, Germany. Media and reagents were prepared under standard protocol following aseptic technique (Parrish et *al.*, 1988). All incubation steps were conducted in CO_2 incubator (5% CO_2) at 38.5%.

In vitro maturation of oocytes:

A total number of 191 she camels' ovaries (*Camelus dromedarius*) were collected from slaughterhouse at El-Warak- Giza, Egypt and transported to the laboratory (Faculty of Agriculture Research Park, Cairo University, Giza) in a thermo flask containing warm physiological saline (35 to 37^{o}C, 0.9% sodium chloride solution, w/v), supplemented with 50 µg/ ml gentamicin within three to four h. post slaughter. Ovaries were rinsed three times in saline at 35°C and attached tissues were removed using a sterile scissors. Thereafter, ovaries were washed with ethanol (70 %) to remove any contamination on the surface of the ovaries before placing in glass gars containing warm physiological saline (37 to 38 ^{o}C) and kept in a water bath adjusted to 37 ^{o}C according (Khatir and Anouassi, 2006).

A total number of 1640 oocytes were collected all over the year as 883 during the breeding season (BS, from January to April) and 757 during non-breeding season (NBS, May to September). Cumuls oocytes complex (COCs) were harvested by slicing ovaries in warm (37^{o}C) phosphate buffered saline (PBS) supplemented with 50 µg/ ml gentamicin (Torner *et al.*, 2003). The retrieved containing COCs fluid was left for five minutes; the sediment was then transferred to a 60 mm petri dish (Greiner bio-one, Frickenhausen, Germany) and diluted with PBS and examined under a sterio-microscope (Leica Microsystems, MZ6, Wetzlar, Germany) to pick out COCs. Collected oocytes were evaluated and classified as good-quality and degenerated oocytes according to the criteria described by Wani (2009) and Khatir *et al.* (2007). Good oocytes, which characterized with more than one layer of homogeneous cumulus cells and dark cytoplasm were selected for *in vitro* maturation process. Oocytes were matured in tissue culture medium-199(TCM- 199) supplemented with 10% (v/v) heat-treated (56^{o}C for 30 min) fetal bovine serum (FBS), 1 µg/ ml FSH, 1 µg/ml estradiol 17 β (E_2) and 50 µg/ml gentamicin (control medium). The medium was sterilized using 0.22 µm millipore syringe filters and before incubation for at least two hours (Amer and Moosa, 2008). COCs were washed twice in warm PBS supplemented with 50 µg/ml Gentamicin and once in maturation medium; before culturing for maturation.

Two media were tested: the first was the control medium and the second was the control medium supplemented with 10 ng/ml EGF (treated medium). Oocytes were cultured in group (10 – 15 oocytes) in a 100 µl drop of maturation medium in a 35 mm petri dish, covered with mineral oil and cultured in incubator for 30 h. Cumulus expansion (the main criterion of oocyte maturation) was examined under a stereomicroscope after the incubation period. Percentage of oocytes with expanded cumulus was recorded and based on the criteria of Amer and Moosa (2009).

In vitro fertilization:

Frozen semen was obtained from Reproduction Center, Desert Research Center, King-Marriott, Alexandria, Egypt to be used in this study. Two straws (0.5 ml each) were thawed in a water bath, 38.5^{o}C for 30 to 40 sec (Skidmore, 2005). The straw was wiped with 70% ethyl alcohol before being opened. The contents of the straw were layered under five ml washing sperm medium, (TCM-199 medium supplemented with four mM caffeine sodium benzoate; pH 7.5, filtered using 0.22 µm syringe filter) and centrifuged twice for five min at 1500 rpm at 32 to 35^{o}C. Sperm pellet was mixed with appropriate volume (two ml) of pre-warmed and pre-filtered fertilization medium as described by Park *et al.* (1997). The final sperm concentration was adjusted to $3x10^{6}$/ml. Four drops of a 100 µl sperm suspension were placed in a 35 mm petri dish and covered with a pre-warmed mineral oil. Matured oocytes were washed three times in fertilization medium. Ten to fifteen oocytes were placed into each of the sperm drops and cultured in CO_2 incubator for 18 h (Khatir *et al.*, 2007). Presumptive zygotes were washed twice in a developmental medium (TCM-199 supplemented with 1% FBS, 12.5 µl/ml sodium pyruvate and L-glutamine 25 µl/ml, pH 7.2). About 10 to 15 of presumptive zygotes were cultured in 100 µl drop of developmental medium covered with mineral oil and incubated for eight days. Medium was renewed every two days during the incubation process.

Statistical analysis:

The obtained data of recovery rate were statistically analyzed by T- test using SPSS 19

(2001) and that of IVM and IVF were analyzed by Cochran's Q test using XLSTAT (2012). This method compares several related samples and can be used as a non-parametric alternative to the two way ANOVA.

RESULTS

Effect of season on oocytes' recovery rate and quality:

Recovery rate:

Number of oocytes recovered per ovary during breeding and non-breeding seasons did not differed significantly, however was higher in non- breeding season compared to breeding one by about 20%. Moreover, percentage of accepted oocytes per ovary was higher by 8.8% in breeding season relative to the non-breeding one (Table 1).

Effect of Medium:

In vitro maturation and fertilization rates:

Adding EGF to maturation medium (G2) improved ($P<0.05$) the maturation rate by approximately 13.5 % relative to the control group (G1). Fertilization rate also was higher by about 2.7% in G2 relative to G1 (Table 2).

Embryonic development:

Developmental stages of embryo are shown in plate (1) (a, b, c, d, e, f and g). The cleavage rate, morula and early blastocyst was higher ($P<0.05$) in embryos of G2 than of G1. In G2 percentages of the cleaved embryos increased by about 10 % than in G1 for 2-32 cell stage, 11.4 % for morulla stage and 2.86 % for early blastocyst stage (Table 3).

DISCUSSION

The non-significant increase in recovery rate during non-breeding season in the dromedary camel may be due to cessation of ovulation during this season. This result is in contrary with the results of Abdoon (2001) which reported the yield and quality of oocytes in camel were higher during the breeding season. On the other hand, high percentage (90.8%) of recovered good oocytes (Table 1) during breeding season is in accordance with the results of Amer and Moosa (2008).

Maturation rate of embryos cultured in TCM-199 medium (68 %,Table 1) is higher than to the results of Khatir *et al.* (2004) and less than that of Kafi *et al.* (2005) and Wani and Wernery (2010) (67-71%). This difference may be due to the short time of incubation period applied in the present work (30 h) compared to the longer period applied in the previous works (36- 42 h).

The obtained higher maturation rate in TCM-199 medium supplemented with EGF during culture process after fertilization (G2) compared to G1 is in accordance with that stated in buffaloes (Dinesh and Govind, 2010), Boer goat (Wang *et al.*, 2007), bovine (Sirisathien and Brackett, 2003; Bastan *et al.*, 2010) and sheep (Shabankareh and Zandi, 2010). These results may be due to the positive effect of EGF on nuclear and cytoplasmic maturation of cumulus enclosed oocytes *in vitro* (Lonergan *et al.*, 1996 and Park *et al.*, 1997). EGF in cultural medium enhances cleavage (Conti *et al*., 2005).

Comparable maturation rate obtained in the present results after 30 h. incubation may conclude that the maturation time for dromedary camel oocytes could be 30 h in TCM 199 media supplemented with EGF, which is in consistence with studies of Khatir *et al.* (2004 & 2005) on camel. The importance of determining the optimal *in vitro* oocyte maturation time is to avoid oocyte aging if *in vivo* insemination is delayed (Hunter, 1989) and subsequent poor development (Amer and Moosa, 2008).

In conclusion, the present results suggest that EGF might be one of the factors improved camels oocytes maturation, cleavage rate and proportion of blastocyst stage. Further detailed studies are required to compare between fresh and frozen semen on embryo development, in addition to test the effect of EGF on culture media.

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REFERENCES

Abdoon, A.S.S., 2001. Factors affecting follicular population, oocyte yield and quality in camels (*Camelus dromedarius*) ovary with special reference to maturation time *in vitro*. Anim. Reprod. Sci., 66: 71-79.

Abdoon, A.S., O.M. Kandil, B. Berisha, H. Kliem, and D. Schams, 2007. Morphology of dromedary camel oocytes and their ability to spontaneous and chemical parthenogenetic activation. Reprod. Domest. Anim., 42: 88−93.

Amer, H. and A. Moosa, 2009. Effect of season and culture media on the competence of dromedary camel oocyte to mature *in vitro*. Global Veterinaria, 3(1): 01-08.

Amer, H. and A. Moosa, 2008. Relationship between season of the year, culture medium and *in vitro* oocyte competence in dromedary camels. Bulg J. Vet. Med., 11(3): 195- 204.

Basiouni, G.F., 2007. Follicular wave pattern, folliculogenesis and assisted reproductive techniques in the non-pregnant female dromedary camel (*Camelus dromedarius*). J. Biol. Sci., 7(6): 1038- 1045.

Bastan, A., B. Polat, D. Bakiacar, Ö. Korkmaz, and A. Colak, 2010. Determination of optimal dose of EGF for bovine oocyte maturation and subsequent in vitro ertilization and culture in two media. Turk. J. Vet. Anim. Sci., 34(1): 33-38.

Conti, M., M. Hsieh, J.Y. Park, and Y.Q. Su, 2005. Role of the epidermal growth factor Network in ovarian follicles. Mol. Endocrinol., 20(4): 715–723.

Dinesh, K. and N.P. Govind, 2010. Effect of epidermal and insulin-like growth factor-1 on cumulus expansion, nuclear maturation and fertilization of buffalo cumulus oocyte complexes in simple serum free media DMEM and Ham's F-10. Eterinarski arhiv, 74 (1): 13-25.

El Wishy, A.B., 1987. Reproduction in the female dromedary (*Camelus dromedarius*): Anim. Reprod. Sci., 82: 587-593.

Farah, Z. and A. Fischer, 2004. The camel (*C. dromedarius*) as a meat and milk animal: Handbook and product development. Vdf. Hochschulverlag. http://www.camelgate.com/.

Hunter, R.H., 1989. Aging of the unfertilised cow egg *in vivo*: How soon is fertility compromised? Vet. Record., 124, 489−490.

Kafi, M., F. Mesbah, H. Nili, and A. Khalili, 2005. Chronological and ultrastructural changes in camel (*Camelus dromedarius*) oocytes during *in vitro* maturation. Theriogenology, 63: 2458-2470.

Khatir, H., and A. Anouassi, 2006. The first dromedary (*Camelus dromedarius*) offspring obtained from in vitro matured, in vitro fertilized and in vitro cultured abattoir-derived oocytes. Theriogenology, 65: 1727- 1736.

Khatir, H., A. Anouassi, and A. Tibary, 2007. Effect of follicular size on *in vitro* developmental competence of oocytes and viability of embryos after transfer in the dromedary (*Camelus dromedarius*). Anim. Reprod. Sci., 99: 413- 420.

Khatir, H., A. Anouassi, and A. Tibary, 2005. *In vitro* and *in vivo* developmental competence of dromedary (*Camelus dromedarius*) embryos produced *in vitro* using two culture systems (mKSOMaa and oviductal cells). Reproduction in Domestic Animals , 40(3):245-249.

Khatir, H., A. Anouassi, and A. Tibary, 2004. Production of dromedary (*Camelus dromedarius*) embryos by IVM and IVF and co-culture with oviductal or granulosa cells. Theriogenology , 62: 1175–1185.

Lee, G.S., H.S. Kim , S.H. Hyun, H.Y. Jeon , D.H. Nam , Y.W. Jeong , S. Kim , J.H. Kim, S.K. Kang , B.C. Lee and W.S. Hwang, 2005. Effect of epidermal growth factor in preimplantation development of porcine cloned embryos. Molecular Reproduction and Development, 71(1):45-51.

Lonergan, P., C. Carolan, A.V. Langendonckt, and I. Donnay, 1996. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. Biol. Reprod., 54: 1420-1429.

Marai, I.F.M., A.E.B. Zeidan, A.M. Abdel-Samee, A. Abizaid, and A. Fadiel, 2009. Camels reproductive and physiological performance traits as affected by environmental conditions. Tropical and Subtropical Agroecosystems, 10: 129–149.

Nowshari, M.A., and N.A. Wani, 2005. Camelid embryo development *in vitro*: effect of protein supplementation in maturation medium and subsequent culture in two different media on fertilization and development. Reprod. Fertil. Dev., 17: 276 (Abstract).

Park, K.W., K. Iga, and K. Niwa, 1997. Exposure of bovine oocytes to EGF during maturation allows them to develop to blastocysts in a chemically-defined medium. Theriogenology, 48(7): 1127-1135.

Parrish, J.J., J.L. Susko-Parrish, M.A. Winer, and N.L. First, 1988. Capacitation of bovine sperm by heparin. Biol. Reprod., 38: 1171-1180.

Shabankareh, H.K., and M. Zandi, 2010. Developmental potential of sheep oocytes cultured in different maturation media: effects of epidermal growth factor, insulin-like growth factor I, and cysteamine. Fertil. Steril., 94(1):335-340.

Sirisathien, S., and B.G. Brackett, 2003. TUNEL analyses of bovine blastocysts after culture with EGF and IGF-I. Mol. Reprod. Dev., 65(1):51-56.

Skidmore, J.A., 2005. Reproduction in dromedary camels: an update. Anim. Reprod., 2(3): 161-171.

SPSS Inc. (2001) SPSS 11.0 Advanced Models: Englewood Cliffs, NJ: Prentice Hall.

Suthar, V.S., and R.G. Shah, 2009. Bovine *in vitro* embryo production: an overview. Vet. World, 2(12): 478- 479.

Torner, H., B. Heleil, H. Alm, I.M. Ghoneim, V. Srsen, W. Kanitz, A .Tuchscherer, and E.M. Fattouh, 2003. Changes in cumulus-oocyte complexes of pregnant and non-pregnant camels (*Camelus dromedarius*) during maturation *in vitro*. Theriogenology, 60: 977-987.

Wang, Z.G., Z.R. Xu, and S.D Yu, 2007. Effects of oocytes collection techniques and maturation media on *in vitro* maturation and subsequent embryo development in Boar goat. Czech J. Anim. Sci., 52(1): 21-25.

Wani, N.A., 2009. *In vitro* embryo production in camel (*Camelus dromedarius*) from *in vitro* matured oocytes fertilized with epididymal spermatozoa stored at 4°C. Anim. Reprod. Sci, 111(1): 69- 79.

Wani, N.A, and U. Wernery, 2010. *In vitro* maturation of dromedary (*Camelus dromedarius*) oocytes: effect of different protein supplementations and epidermal growth factor. Reproduction in Domestic Animals, 45(5):e189-193.

XLSTAT, 2012, statistical software for MS Excel- statistics and data analysis with MS Excel Addinsoft 224 centre street, 3rd floor New York, NY 10013 USA.

Table 1. Mean values (Mean±S.E.) of oocytes recovered from ovaries in breeding season (BS) and non-breeding season (NBS)

Trait	BS	NBS	Overall
No. of Ovaries	112	79	191
No. of collected oocytes	883	757	1640
Recovery rate (oocytes/ovary)	8.6 ± 0.8	10.30 ± 1.4	9.4 ± 0.8
No. of accepted oocytes /ovary	7.7 ± 0.7	8.6 ± 1.2	8.1 ± 0.7
Good oocytes (%)	(90.8%)	(82.0%)	(86.4 %)

Table 2. Oocytes maturation (%) in dromedary camel cultured in TCM-199 medium (G1) or in TCM-199 medium supplemented with epidermal growth factor (G2)

Trait	G1	G2
No. of oocytes	654	654
Matured oocytes (%)	68.04	81.19*
Fertilized ova (%)	17.6	20.3

Values differ significantly at (P <0.05).

Table 3. *In vitro* development of camel embryos matured in TCM-199 medium (G1) or TCM-199 supplemented with EGF and fertilized with frozen semen

Experimental Groups	No. of fertilized oocytes	2-32 cells stage	Morulla Stage	Early blastocyst stage
G1	105	9.52	6.67	0.95
G2	105	19.05*	18.10*	3.81

* Values differ significantly at P<0.05.

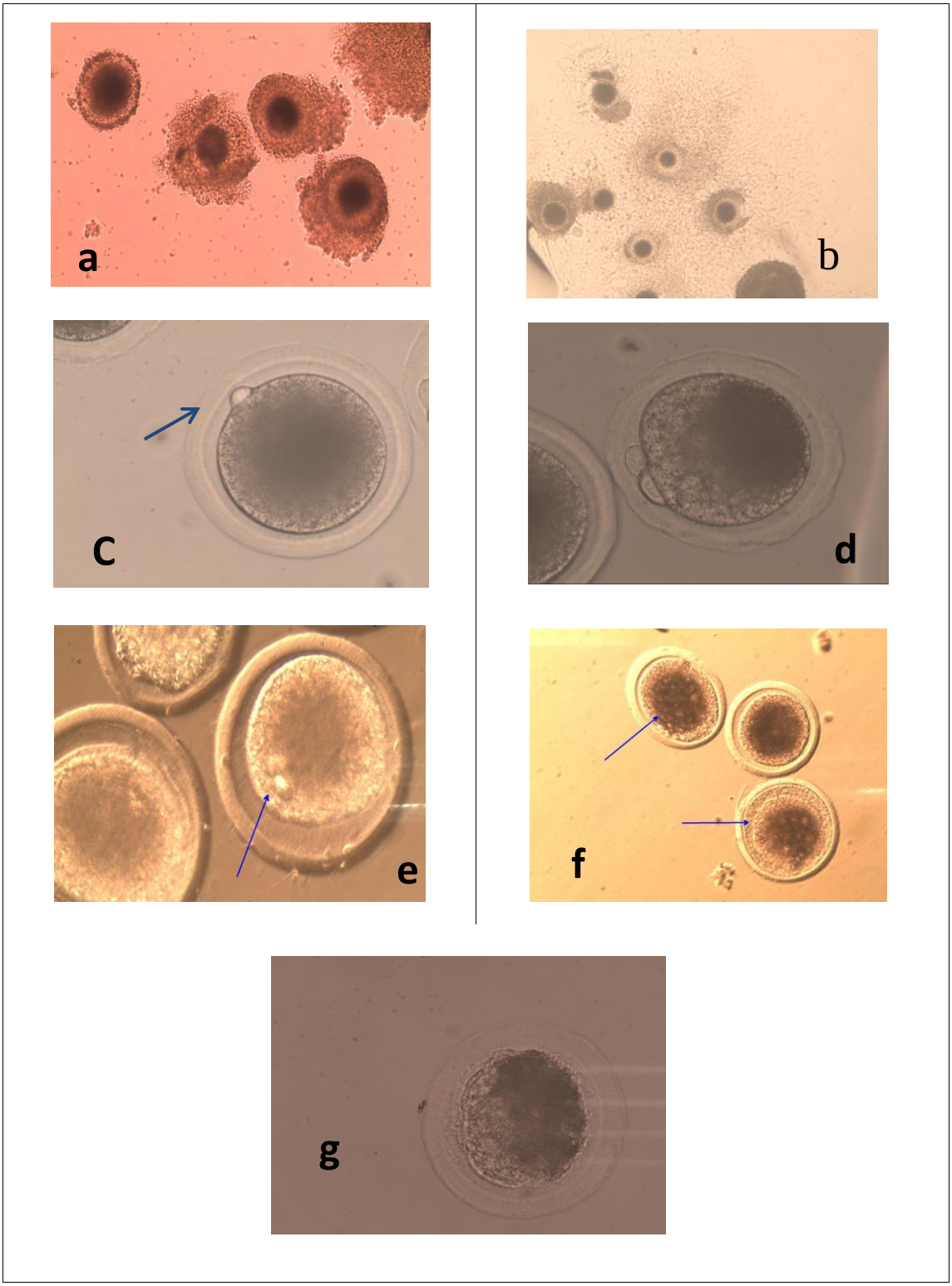

Plate.1. Developmental stages of *in vitro* produced embryos showing: a- immature oocytes with the condensed cumulus cells 100X, b- matured oocytes with expanded cumulus cells 100X, c- denuded matured oocyte with 1st polar body 200 X, d- matured oocyte extruded the 2nd polar body 320X, e-fertilized oocyte with two pronuclei 400X, f- Morula 160X and g- early blastocyst 320X

تأثير إضافة عامل نمو الجلد علي إنتاج أجنة الجمال معمليا بإستخدام السائل المنوي المجمد

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تمت هذه الدراسة على عدد 1640 من بويضات الجمال لدراسة تأثير إضافة نمو الجلد على بيئة النمو في تحسين نضج البويضات معمليا و قدرة الأجنة الناتجة على التطور باستخدام السائل المنوي المجمد بالإضافة إلى تأثير الموسم على عدد البويضات المستخلصة لكل مبيض وكذلك جودتها.

تم جمع عدد 883 بويضة أثناء موسم التناسل و 757 بويضة أثناء موسم الراحة الجنسية من اجمالي عدد مبايض 191، تم إحضار المبايض من مجزر الوراق بالجيزة إلى مجمع المعامل البحثية بكلية الزراعة- جامعة القاهرة في محلول فسيولوجي على درجة حرارة 37 درجة مئوية.

تم الحصول على البويضات باستخدام طريقة تشريط المبايض , و استخدمت البويضات الجيدة فقط للزراعة في بيئة النمو TCM-199 لمدة 30 ساعة.

تم إخصاب عدد 206 بويضة بواسطة السائل المنوي بتركيز (3×10^6 حيوان منوي/ سم^3) و تم تحضينها على درجة حرارة 38.5 درجة مئوية و تركيز 5% ثاني أكسيد الكربون.

أدت إضافة عامل نمو الجلد إلى تحسين معدل النضج حيث وصل الى81,19 % بالمقارنة بالمجموعة الكنترول 68.04 %على درجة معنوية اقل من 0.05 %.

كذلك أدت إضافة عامل نمو الجلد الى تحسين نسبة الانقسام والموريولا و كذلك البلاستوسيت بالمقارنة بمجموعة الكنترول إلا أن هذا الاختلاف لم يكن معنويا في مرحلة البلاستوسيست.

نستخلص من نتائج هذه التجربة أنه يمكن استخدام السائل المنوي المجمد بنجاح في إنتاج أجنة الجمال معمليا, كذلك إضافة عامل نمو الجلد من الممكن أن يؤدى إلى تحسين نضج البويضات و كذلك معدل الإنقسام.