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Effect of Leptin Supplementation in Maturation Medium on *in vitro* Nuclear Maturation and Fertilization of Camel Oocytes (*Camelus dromedarius*)

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ABSTRACT

The present study aimed to evaluate the effect of leptin addition (0, 10, 20 and 30 ng mL^{-1}) to maturation media on *in vitro* maturation (IVM) and fertilization (IVF) of dromedary camel oocytes. Ovaries were collected from slaughtered animals, oocytes were collected by slicing technique and matured in TCM-199 medium with different leptin levels in CO_2 incubator (5% CO_2) at 38.5°C and high humidity for 42 h. After maturation, oocytes were categorized as Germinal Vesicle (GV), Germinal Vesicle Breakdown (GVBD), metaphase-I (MI), metaphase-II (MII) and degenerated oocytes. Epididymal spermatozoa were recovered from camel testes immediately after slaughter. *In vitro* fertilization was carried out for oocytes matured in TCM-199 with 10% FDCS plus 20 μg mL^{-1} of leptin. Spermatozoa and oocytes were co-incubated at 38.5°C in a moisture atmosphere of 5% CO_2 in air for 20-24 h. The cleaved oocytes were examined after five days of culture for cleavage stages (2, 4 and 8-16 cell, morula and blastocyst stages). Results revealed that supplementation of leptin (20 mg mL^{-1}) showed ($p<0.05$) the highest percentage of oocytes at MII (58.8%) and the lowest percentages of those at GV (7.8%), GVBD (9.8%), MI (10.8%) and degenerated oocytes (12.7%) as compared to control medium (40.0, 10.9, 11.8, 14.5 and 22.7%, respectively) and other levels of leptin. Supplementation of leptin, matured with 20 ng mL^{-1} leptin increased fertilization rate (27 vs. 25.3%) and blastocyst rate (3.6 vs. 1.4%) as compared to the control medium. In conclusion, supplementation of leptin to maturation medium (TCM-199) at a level 20 ng mL^{-1} increased *in vitro* nuclear maturation rate of immature oocytes and their fertilization rate and blastocyst production.

Key words: Camel, oocytes, *in vitro* maturation, fertilization

INTRODUCTION

Camels are induced ovulations and exhibit follicular cycles with follicles developing and regressing successively and ovulation will occur only when mating takes place (Elias *et al*., 1984; Ismail, 1987). The dromedary camels are regarded as seasonal breeders with a relatively short breeding season, based on the seasonal, distribution of births and the status of ovarian activity (Shalash, 1987). Low reproduction efficiency of camels under their natural postural conditions is due to many factors such as age of puberty (4-5 years), breeding seasonality, long gestation period (13 months), long inter-calving interval (2 years), high incidence of early embryonic losses and the continuous use of traditional breeding systems.

In vitro Embryo Production (IVEP) may proved to be suitable for the low reproductive efficiency of camels. However, as camels are unique, concerning their physiological and reproductive characteristic, the usage of assisted reproductive techniques such as Artificial Insemination (AI), Embryo Transfer (ET) and IVEP are often difficult to implement. The IVEP has been successfully applied in a number of domestic species with transferred embryos resulting in live offspring (Gordon, 2003). However, this is not true in case of camel because there is a relatively limited information about *in vitro* Maturation (IVM) and *in vitro* Fertilization (IVF) of camel oocyte as well as about early embryonic development *in vitro* (Khatir *et al*., 2004). Consequently the study on IVEP in camels will open gates to more accurate understanding of factors leading to better reproductive efficiency and less early embryonic death.

Leptin is a metabolic hormone secreted primarily by adipose tissues (Zhang *et al*., 1994). It was believed that, leptin was originally being expressed only in white adipose tissues. But several authors assured that leptin in expressed in areas such as granular layer of cumulus oophorus, theca and interstitial cells of ovaries, testes, uterus (Cioffi *et al*., 1997; Karlsson *et al*., 1997), kidneys, lungs (Sharma and Considine, 1998), skeletal muscles, heart and liver (Bernardis and Bellinger, 1998). Also, leptin has been detected in mice oocytes (Ryan *et al*., 2002), in follicular fluid and in granulosa and cumulus cells (Cioffi *et al*., 1997) and in corpora lutea of rabbits (Zerani *et al*., 2004).

Many authors reported an important role of leptin to improve IVM in bovine (Stojakovic *et al*., 2003), pigs (Zhang *et al*., 2007) and rabbits (Arias-Alvarez *et al*., 2007). Also leptin play role in embryo development and pre-implantation in mice (Kawamura *et al*., 2003; Craig *et al*., 2005; Udagawa *et al*., 2006) and porcine (Kim *et al*., 1992). Previous experiment have demonstrated that leptin addition during IVM in bovine oocytes exerted long-term positive effects, increased blastocyst cell number and the reduced proportion of apoptotic cells in the blastocysts (Boelhauve *et al*., 2005). Moreover, leptin enhanced meiotic progression of pig oocytes and subsequent development during mouse oocytes maturation. While, leptin treatment of mouse cumulus-enclosed oocytes or denuded oocytes does not enhance meiotic progression (Swain *et al*., 2004). The addition of leptin during culture of mouse follicles increased germinal vesicle breakdown (Ryan *et al*., 2002). Therefore the present study was conducted to explore the effect of leptin addition at different levels to maturation media on nuclear maturation (IVM) and fertilization (IVF) of dromedary camel oocytes.

MATERIALS AND METHODS

This study and experimental work was carried out at International Livestock Management Training Center, Sakha, Kafr-El Shiekh Governorate, belonging to Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture, Egypt, in cooperation with Department of Animal Production, Faculty of Agriculture, Tanta University, during the period January to April 2014 (breeding season).

All chemicals used were Sigma Production Chemi. Co. (St Louis, Mo, USA).

Ovaries collection: Ovaries were collected from El-Bassatein slaughterhouse, Cairo, Giza, located at distance approximately 225 km from the laboratory. Ovaries were collected within 15-30 min post slaughter and placed immediately after slaughter into thermos in saline solution (0.9% NaCl) supplemented with antibiotics (100 IU penicillin and 100 μg streptomycin/mL) at 25-30°C. Thereafter, all collected ovaries were transported to the laboratory within 3-3.5 h.

In the laboratory, the excess tissue from the ovarian stalk was cut from the ovaries and then ovaries were washed two times with warmed (28°C) Phosphate Buffer Saline (PBS) supplemented with antibiotics (100 IU penicillin and 100 μg streptomycin/mL) to remove adhering clotted blood. Thereafter, all ovaries were quickly washed one time with ethanol (70%) to remove any contamination on the surface of the ovaries.

Oocyte collection: Phosphate Buffer Saline (PBS) was prepared as harvesting medium by dissolving one PBS tablet (Sigma-Aldrich Chemie GmbH, P4417) in 200 mL sterile distilled water supplemented with antibiotics (100 IU penicillin and 100 μg streptomycin/mL). About 2 mg mL^{-1} of Bovine Serum Albumin (BSA) was added to PBS. The pH value of the solution was adjusted to 7.2-7.4 using pH-meter and to osmolarity of 280-300 mOsmol kg^{-1}. Then, the solution was filtered by 0.22 μm millipore filter (Milieux GV, Millipore, Cooperation Bedford, MOA).

Cumulus oocyte complexes were collected by slicing of ovary in petri dish containing 5 mL of PBS. Each ovary was held by a forceps and by a scalpel blade was made incisions along the whole ovarian surface. Ovary was washed three times with harvesting medium. Petri dishes were kept undisturbed for 5 min, allowing the oocytes to settle down. The excess medium was taken by a pasture pipette without disturbing the oocytes at the bottom of the petri dish.

***In vitro* maturation**

Preparation of maturation medium: The base maturation medium (TCM-199, Egyptian Orgnaization for Biological Product and vaccine, Agoza) containing 10% FDCS, PMSG (20 IU mL^{-1}), hCG (10 IU mL^{-1}), E-17β (1 μg mL^{-1}), Na-Pyruvate (20 mMol), Na-Penicillin G (100 IU mL^{-1}) and Streptomycin (100 μg mL^{-1}), supplemented with 0 (M1), 10 (M2), 20 (M3) or 30 (M4) ng mL^{-1} of leptin (195807; leptin Human Recombinant, Mp) were used in this study.

The medium was adjusted to pH of 7.2-7.4 and osmolarity of 280-300 mOsmol kg^{-1} and filtered by 0.22 μm-millipore filter. About 100 μL from prepared maturation medium was placed into sterile petri dish (30×60 mm) and covered by sterile mineral oil. Before placing oocytes in culture dish, petri dishes were incubated in CO_2 incubator (5% CO_2) at 38.5°C and high humidity for at least 1 h for equilibration.

Oocytes maturation: Oocytes were washed three times in PBS plus 2% BSA and two times in maturation medium to remove substances in follicular fluid, which may prevent maturation. Thereafter, about 10-20 oocytes per droplet 100 μL were allocated by pasture pipette and cultured petri dish was incubated for about 42 h in CO_2 incubator (5%CO_2) at 38.5°C and high humidity.

At the end of maturation period, COC_s were removed from the maturation medium and the cumulus cells were removed from the oocytes by repeated pipetting. About 10-20 oocytes in a drop of the appropriate medium were pippeted and added into glass tube 5 mL, well-closed and containing 3 mL of fixative solution (fresh mixture of acetic acid ethanol (1:3, v/v)) for 24 h. After that oocyte mounted into glass slide, a cover slip with inert paraffin wax spots at each of its four corners was placed directly over the center of drop of the fixative containing oocyte.

The slide was stained with a 1% aceto-orcein stain (1% orcein stain in 40% glacial acetic acid, w/v) for few minutes and washed by (aceto-glycerol 3:1, v/v). Thereafter, oocytes were examined under microscope by high magnification. Oocytes were categorized as Germinal Vesicle (GV), Germinal Vesicle Breakdown (GVBD), metaphase-I (MI), metaphase-II (MII) and degenerated oocytes.

***In vitro* fertilization:** Oocytes matured in TCM-199 with 10% FDCS plus 20 μg mL^{-1} of leptin as well as spermatozoa capacitated with IVF-TALP medium supplemented with 10 μg mL^{-1} heparine for 2 h were used for *in vitro* fertlilzation.

Preparation of spermatozoa: Epididymal spermatozoa were recovered from testes of mature camel bulls (Kom-Hamada slaughterhouse) immediately after slaughter and transported to laboratory on normal saline in ice (0-1°C) within 2-3 h. Spermatozoa were collected from the epididymal regions by dissection and spermatozoa were released and collected by aspiration with sterile disposable (5 mL) syringe containing 2 mL TALP media. The recovered spermatozoa were placed in a 5 mL tube. IVF-TALP medium was prepared according to Leibfried and Bavister (1982).

***In vitro* fertilization (IVF):** On the day of fertilization, 50 μL of epididymal spermatozoa (≥70% motility) were allowed to swim up for 1 h in TALP medium in an incubator (5% CO_2 and 38.5°C) at an angle of 45°. The sperm suspension was washed twice with sperm TALP (Parrish *et al*., 1986) by centrifugation at 1500 rpm for 10 min. The pellet was overlaid with fertilization medium (IVF-TALP supplemented with 4 mg mL^{-1} BSA, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} Streptomycin).

The matured oocytes were washed twice in fertilization medium and randomly distributed in 4-well culture plates (20-25 COCs/well) containing 500 μL of the fertilization medium. The motile spermatozoa were added to the oocytes at the concentration of approximately 1×10^6 mL^{-1}. Sperm and oocytes were then co-incubated at 38.5°C in a moisture atmosphere of 5% CO_2 in air for 24 h. The cleaved oocytes were examined after five days of culture by inverted microscope for kinetic cleavage stages (2, 4 and 8-16 cell, morula and blastocyst stages).

Statistical analysis: The obtained data was analyzed using general linear model of SAS (2001) while differences among the treatment mean were performed using Duncan Range Test (Duncan, 1955). The percentage values were adjusted to arcsine transformed before performing the analysis of variance. Means were presented after being recalculated from transformed values to percentages.

RESULTS AND DISCUSSION

Effect of leptin addition to maturation medium

***In vitro* maturation of camel oocytes:** Table 1 showed that the effect of leptin supplemented to maturation medium was significant ($p<0.05$) on *in vitro* maturation of camel oocytes in terms of

Table 1: Effect of addition of leptin hormone with different concentration to maturation media on maturation rate of camel oocytes

		GV		GVD		MI		MII		Degeneration	
Medium (ng mL^{-1})	Total	No.	%	No.	%	No.	%	No.	%	No.	%
Control	110	12	10.9±1.38^{ab}	13	11.8±1.28	16	14.5±0.99^{a}	44	40.0±1.61^{c}	25	22.7±0.32^{a}
Leptin 10	80	9	11.3±1.15^{a}	10	12.5±2.40	12	15.0±1.53^{a}	37	46.3±1.40^{b}	12	15.0±0.81^{b}
Leptin 20	102	8	7.8±0.77^{b}	10	9.8±0.85	11	10.8±1.17^{b}	60	58.8±0.87^{a}	13	12.7±0.833^{c}
Leptin 30	91	9	9.9±1.06^{ab}	11	12.1±1.88	10	10.9±0.58^{b}	47	51.6±0.82^{b}	14	15.4±0.84^{b}

a, b and c: Means denoted within the same column with different superscripts are significantly different at $p<0.05$

percentage of oocytes reaching Germinal Vesicle (GV), metaphase-I (MI), metaphase-II (MII) and degenerated oocytes. However, percentage of oocytes reaching Germinal Vesicles Breakdown (GVBD) was not affected by leptin supplementation.

Results revealed that supplementation of leptin to maturation medium at a level of 20 ng mL^{-1} significantly ($p<0.05$) increased the maturation rate in term of significantly ($p<0.05$) the highest percentage of oocytes at MII (58.8%) and the lowest percentages of those at GV (7.8%), GVBD (9.8%), MI (10.8%) from one side and degenerated oocytes (12.7%) on the other side as compared to leptin free-medium (40.0, 10.9, 11.8, 14.5 and 22.7%, respectively) and other levels of leptin. On the same line, supplementation of leptin to maturation medium at levels of 10 and 30 ng mL^{-1} significantly ($p<0.05$) increased percentage of oocytes at MII (46.3 and 51.6%) and decreased percentage of degenerated oocytes (15.0 and 15.4%) as compared to the control medium, respectively.

In accordance with the present results, many authors showed the beneficial effect of leptin supplementation on oocyte maturation *in vitro* which enhanced meiotic progression of oocytes in bovine (Stojakovic *et al*., 2003; Popelkova *et al*., 2006; Guo *et al*., 2008), pig (Craig *et al*., 2004; Zhang *et al*., 2007), rabbit (Arias-Alvarez *et al*., 2007) and horse (Consiglio *et al*., 2009). Similarly, Jia *et al*. (2012) found that addition of leptin to IVM medium enhanced meiotic maturation. Ahmed *et al*. (2008) significantly decreased the degenerated oocytes. In the present study, maturation rate (oocytes at MII) showed significantly ($p<0.05$) the highest increase by increasing level of leptin only up to 20 ng mL^{-1} but leptin at a level of 30 ng mL^{-1} did not exhibit significant increase as compared to 20 ng mL^{-1}. However, increasing leptin level more than 30 ng mL^{-1} (100 and 200 ng mL^{-1}) as reported by Wei *et al*. (2008) had no effect on *in vitro* maturation of pig oocytes.

In general, cytoplasmic maturation *in vitro* is generally compromised, leading to low rates of development. Thus, the positive effect of leptin on the developmental potential of oocytes may be related to their cytoplasmic maturation. Potential modes of action include direct or indirect (cumulus cell-mediated) effects of leptin on restructuring oocyte cytoskeleton and reprogramming protein synthesis (Thibault *et al*., 1987), or inhibiting apoptosis (Almog *et al*., 2001). Leptin enhances oocyte nuclear and cytoplasmic maturation of porcine oocytes via mitogen-activated protein kinase pathway (Craig *et al*., 2004).

In the current study, all levels of leptin significantly ($p<0.05$) decreased percentage of degenerated oocytes. Apoptosis plays an important role in mammalian development as a quality control mechanism to eliminate cells (degenerated oocytes) that are damaged, non-functional, abnormal, or misplaced (Byrne *et al*., 1999; Neuber *et al*., 1999). The occurrence of apoptosis has been demonstrated in many cell types, including bovine oocytes and cumulus cells (Kolle *et al*., 2003). The beneficial effect of leptin during oocyte maturation suggests a role for leptin as a survival factor minimizing cellular damage to oocyte and/or cumulus cells. It is possible that leptin may rescue oocytes that would otherwise have generated arrested embryos.

***In vitro* fertilization of camel oocytes:** Table 2 showed that supplementation of leptin to maturation medium of matured had no significant effect on fertilization rate of camel oocytes and morula/blastocyst production rate, although fertilization rate tended to be higher for oocytes matured in medium supplemented with 20 ng mL^{-1} (27 vs. 25.3%). Also, there was tendency of reducing rate of morula production (26 vs 21.7%) and increasing blastocyst rate of oocytes matured in leptin-maturation medium as compared to the control medium (3.6 vs. 1.4%).

In agreement with the present results Jia *et al*. (2012) found that addition of leptin to IVM medium enhanced embryo development from calf oocytes and improves the quality of embryos

Table 2: *In vitro* development of camel oocytes matured in medium supplemented with lipten as compare to control medium

		Embryonic stage							
		Un-cleaved oocytes		Cleaved oocytes		Morula		Blastocyst	
Mediums	Total	No.	%	No.	%	No.	%	No.	%
Control	69	50	72.0	19	27.0	18	26.0	1	1.4
Leptin (20 ng mL^{-1})	83	62	74.7	21	25.3	18	21.7	3	3.6

derived from these oocytes. Cordova *et al.* (2011) asserted that leptin has beneficial effects on blastocyst development, apoptosis and transcription levels of developmentally important genes. In buffaloes, Arias-Alvarez *et al.* (2007) found beneficial effects of leptin supplementation to *in vitro* culture medium (10 ng mL^{-1}) on developmental competence of embryos at blastocyst stage. Also, Boelhauve *et al.* (2005) demonstrated that leptin supplementation during *in vitro* maturation of oocytes exerted long-term positive effects as shown by an increased proportion of development to blastocyst by increasing cell number and reducing proportion of apoptotic cells in blastocyst. The oocyte fertilization *in vitro* increased by leptin supplementation in maturation medium according to Craig *et al.* (2005).

In addition, several authors reported no effect of leptin supplementation during oocyte maturation on cleavage rate after fertilization but an increased proportion of oocytes developed to blastocysts in leptin treatment group as compared to the control (Ryan *et al.*, 2002; Swain *et al.*, 2004; Suzuki *et al.*, 1992). Similarly, Arias-Alvarez *et al.* (2011) showed that addition of leptin during IVM of porcine oocytes had no effect on apoptotic cells in blastocysts. It has been demonstrated that leptin has no effect on expression of apoptotic genes in bovine blastocyst *in vitro*.

CONCLUSION

Increasing quantity and quality of matured oocytes as well as production rate of morula and blastocyst are important considerations in the *in vitro* production of embryos from slaughtered animals. Assisted reproductive technologies such as *in vitro* maturation have been introduced to increase the number of offspring from selected females and to reduce the generation intervals. According to the obtained results from the current study, supplementation of leptin to maturation medium (TCM-199) at a level 20 ng mL^{-1} increased *in vitro* nuclear maturation rate of immature oocytes and their fertilization rate and blastocyst production.

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