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Improvement of the developmental competence of canine oocyte using caffeine supplementation during IVM at different maturation time

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Summary

The aim of the current study was to investigate the effect of caffeine supplementation during *in vitro* maturation (IVM) for different maturation times on the developmental potential of canine oocytes recovered from ovariohysterectomized bitches. The recovered cumulus–oocytes complexes were *in vitro* matured for 72 h. Here, 10 mM caffeine was added to the maturation medium for different incubation times (caffeine from 0–72 h maturation, caffeine for the first 24 h of maturation only, caffeine addition from 24 to 48 h maturation time, caffeine addition from 48 to 72 h maturation or in caffeine-free medium, control group). The matured oocytes were *in vitro* fertilized using frozen–thawed spermatozoa. The presumptive zygotes were *in vitro* cultured in synthetic oviductal fluid medium for 5 days. The results showed that both maturation and fertilization rates were significantly higher ($P < 0.05$) using caffeine-treated medium for the first 24 h of maturation compared with the control and other two groups of caffeine treatment (from 24 to 48 h and from 48 to 72 h), whereas use of caffeine-treated medium for a 0–72 h incubation time did not affect these rates ($P > 0.05$). Interestingly, the matured oocytes in caffeine-supplemented medium for the first 24 h or from 0–72 h showed a significant ($P < 0.05$) increase in the total number of cleaved embryos compared with the control group. In conclusion, supplementation of the maturation medium with 10 mM caffeine for the first 24 h of maturation or during the whole maturation time (0–72 h) improved nuclear maturation and subsequent embryo development preimplantation following *in vitro* fertilization.

Keywords: Caffeine, Canine oocytes, IVC, IVF, IVM

Introduction

There is an increasing interest for canine management and reproduction from dog breeders, pet owners and in institutions such as forensic medicine, police academies, fire brigades and blind associations. However, the application of assisted reproductive technologies in dogs is still limited compared with other mammalian species.

Interestingly, the maturation process comprises two main steps: nuclear maturation in which germinal vesicle (GV) oocytes resume meiosis up to metaphase II (MII), and cytoplasmic maturation in which the redistribution of different organelles followed by functional and structural changes have occurred (Lane & Lofstedt, 1996).

The application of a successful protocol for *in vitro* maturation, fertilization and culture (IVMFC) has not been well established in *Canis familiaris* (Songsasen & Wildt, 2007; Rodrigues & Rodrigues, 2010). Canine oocytes appear refractory to various approaches that have been used for their *in vitro* maturation and fertilization processes (Farstad, 2000). This refractoriness is mainly attributed to the complexity of nuclear maturation from prophase I to MII, high lipid content and their fragile status during immature

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oocyte recovery (Salavati *et al.*, 2012). Moreover, arrested status immature oocytes were recovered from preantral follicles at the GV stage when not recruited by gonadotropic hormones (Vannucchi *et al.*, 2009). The meiotic arrest of nuclear maturation was mainly due to the high concentration of cyclic adenosine monophosphate (cAMP) as described by Guixue *et al.* (2001) and Takeda *et al.* (2006).

The initial changes in cAMP concentration inside the oocytes are mediated by luteinizing hormone (LH) during the preovulatory period leading to continuation of meiosis (Zhang *et al.*, 2009). Different pathways are thought to act on changes in cAMP concentration within the oocytes through LH receptors, intracellular calcium and phosphodiesterases pathways (Mehlmann, 2005). So, use of phosphodiesterase inhibitors such as methylxanthines (caffeine or theobromine) has been applied during oocyte maturation to enhance their *in vitro* nuclear maturation (Barretto *et al.*, 2007). Changes in cAMP levels accompanied by caffeine supplementation have been studied and trigger the activity of maturation-promoting factor (MPF) and phosphorylating mitogen activated protein kinase (MAPK) in oocytes leading to progression of meiotic resumption (Tripathi *et al.*, 2010).

Maturation-promoting factor appears before germinal vesicle breakdown (GVBD) and increases until MI, while its greatest intensity is reached at MII (Tripathi *et al.*, 2010).

Generally, the biphasic effect of caffeine supplementation during *in vitro* maturation of canine oocytes had been studied before, via its effect on cAMP concentration, moreover its effect on MPF and MAPK helps in oocyte maturation (Salavati *et al.*, 2012).

Also, caffeine had been proven to enhance *in vitro* sperm capacitation prior to fertilization by increasing cAMP and by its effect on the concentration of calcium ions resulting in sperm capacitation, acrosome reaction and enhancing sperm motility (Choi *et al.*, 2010). So, the main objective of the current study was to investigate the effect of caffeine supplementation during *in vitro* maturation of canine oocytes at different maturation times on the rates of maturation, fertilization and subsequent preimplantation embryo development *in vitro*.

Materials and methods

All reagents and chemicals were purchased from Sigma Co. (UK), unless otherwise mentioned.

Ovaries collection and oocytes recovery

Ovaries were collected after routine ovariohysterectomy of mixed breed bitches (No. 30) belonging to the Egyptian Society for Mercy to Animals (ESMA) under supervision and approval of the shelter's owners with application of the ethical standards. The bitches were of different ages ranging from 1–5 years. Ovaries were placed in a clean thermos flask containing prewarmed (37°C) sterile phosphate-buffered saline immediately following ovariohysterectomy and transferred to the laboratory within 1–2 h post operation. At the laboratory, the ovaries were trimmed of the ovarian bursa and sliced using a surgical scalpel blade in a clean Petri dishes and rinsed using a washing medium (TCM-199 with HEPES modification) as described by Alhaider & Watson (2009). Cumulus–oocytes complexes (COCs) with a dense and homogenous ooplasm surrounded with multiple layers of cumulus cells were obtained, washed and selected for *in vitro* maturation as mentioned by Evecen *et al.* (2011).

In vitro maturation (IVM) of COC

The selected COCs were washed twice in washing medium then twice in the maturation medium [TCM-199 supplemented with 10% fetal calf serum (FCS), 5 µg/ml follicle stimulating hormone (FSH), 5 µg/ml LH, 50 µg/ml pyruvate and 50 µg/ml gentamycin]. Groups of 5–10 oocytes were transferred into four-well culture dishes (NUNC, Milan, Italy) containing 100 µl droplets of prewarmed maturation medium covered with mineral oil for 72 h (De los Reyes *et al.*, 2006) at 39°C in 5% CO_2 in an air humidified atmosphere (control group). For our approaches with caffeine treatment, the oocytes were matured either in 10 mM caffeine-supplemented maturation medium for 72 h (caffeine-treated group from 0 to 72 h IVM) or caffeine-supplemented medium at first 24 h maturation only then transferred to pre-equilibrated caffeine-free medium (caffeine at first 24 h maturation; from 0 to 24 h only) or matured in caffeine-free medium for 24 h then in pre-equilibrated caffeine-supplemented medium from 24 to 48 h then in caffeine-free medium until 72 h maturation (caffeine from 24 to 48 h) or matured in caffeine-treated medium for the last 24 h of maturation (caffeine from 48 to 72 h maturation) under the same maturation conditions.

Assessment of cumulus expansion and the nuclear maturation

At the end of the maturation time, the partial or complete expansion and loosening of cumulus cells was assessed under a stereomicroscope (Silva *et al.*, 2009). For nuclear maturation, the COCs were exposed to repeated gentle pipetting in TCM-199 supplemented with 10% FCS medium until denuded and then loaded on a glass slide beneath a glass cover slip supported with four Vaseline drops on its four corners and stained with aceto-orcein stain (2% orcein dissolved in 45%

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acetic acid) as described by Evecen *et al.* (2011). Oocytes at MII that were visualized under phase contrast microscope were considered as mature (El-Shalofy *et al.*, 2017).

In vitro fertilization (IVF) of oocytes and evaluation of fertilization potential

Four frozen straws obtained previously from two tested dogs were thawed in a water bath at 37°C for 30 s, frozen–thawed spermatozoa were resuspended in sperm-TALP medium with 5 mM caffeine addition as capacitating mediator (Fathi *et al.*, 2014) and then subjected to centrifugation at 500 *g* for 5 min, the supernatant was discarded and the sperm pellet was resuspended in sperm-TALP and allowed to swim up for 30 min at 39°C in 5% CO_2 humidified air. Spermatozoa were assessed for motility and then adjusted to 2 × 10^6 sperm/ml. Groups of 10–15 matured oocytes were transferred to 100 µl droplets of fertilization medium (fertilization-TALP medium supplemented with 6 mg/ml bovine serum albumin) covered by mineral oil (El-Shalofy *et al.*, 2017). Sperm and oocytes were co-cultured at 39°C in 5% humidified air for 18 h. At the end of the incubation, the co-incubated oocytes with spermatozoa were washed twice in prewarmed TCM-199 supplemented with 10% FCS to remove any adherent spermatozoa followed by fixation and staining with aceto-orcein stain as mentioned before, the oocytes with swollen sperm heads or male and female pronuclei in the ooplasm were considered as fertilized (Fathi *et al.*, 2014).

In vitro preimplantation embryo culture and evaluation

The presumptive zygotes following fertilization were washed twice in TCM-199 with HEPES modification and washed twice using culture medium (synthetic oviductal fluid medium supplemented with 20% BSA). Each five zygotes were cultured per 50 µl droplets of embryo culture medium covered with mineral oil at 39°C 5% O_2, 90% N_2 and 5% CO_2 in an humidified atmosphere until day 5 (day 0 was counted as the insemination day). Different developmental stages were recorded.

Statistical analysis

Three replicates were collected for each experimental group. Data were presented as percentages (the percentage of oocytes maturation, fertilization and culture) and were calculated and compared with the total number using chi-squared test. Significance was considered at a *P*-value < 0.05.

Results

Evaluation of cumulus expansion and oocytes maturation

At the end of maturation time (72 h), the recorded percentages of cumulus cell expansion did not significantly differ between the different caffeine-treated groups and the control (the percentages ranged from 30 to 41%) (Fig. 1). Maturation of canine oocytes in caffeine-treated medium at the first 24 h showed a significantly higher ($P < 0.05$) maturation rate (42.2%) than those matured in caffeine-treated medium from 24 to 48 h (28.6%), caffeine-treated medium from 48 to 72 h (27.0%) and the control (25.5%), this rate was non-significantly higher ($P < 0.05$) than those matured in caffeine-treated medium for 72 h (35.5%) as illustrated in Table 1.

Fertilization rates following IVM in caffeine-treated medium

The current study showed that the fertilization rates obtained from matured oocytes using caffeine-supplemented medium for the first 24 h maturation (28.8%) were significantly higher $P < 0.05$ than those obtained from oocytes matured in caffeine-supplemented medium from 24 to 48 h, 48 to 72 h and the control (16.7, 16.3 and 15.8%, respectively), while this significance was not recorded with the fertilization rate obtained in the oocytes matured in caffeine-treated medium for 72 h (21.6%), as shown in (Table 2).

In vitro culture following IVM and IVF

The developmental rates following insemination of the oocytes that matured in caffeine-supplemented medium were recorded and summarized in Table 3. The total number of preimplantation cleaved embryos per oocytes matured in caffeine-supplemented medium at first 24 h maturation followed by fertilization (19.6%) was significantly higher ($P < 0.05$) than those fertilized and cultured following maturation in caffeine-supplemented medium from 24 to 48 h, 48 to 72 h and the control (6.8, 7.2 and 7.1%, respectively). This rate was also higher than those obtained in oocytes cultured and fertilized following maturation in caffeine-supplemented medium for 72 h (11.3%), but the difference was not significant at $P < 0.05$.

Discussion

Maturation of canine oocytes *in vitro* requires unprecedented and complex conditions that are still under optimization. Meiosis resumption during canine oocytes maturation dissimilar to other mammalian

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Table 1 *In vitro* maturation of canine oocytes for different maturation times using caffeine-treated medium

Treatment	Number of examined oocytes	Maturation rates no. (%)
Caffeine for 72 h	110	39 (35.5%)[a,b]
Caffeine at first 24 h	128	54 (42.2%)[a]
Caffeine from 24 to 48 h	98	28 (28.6%)[b]
Caffeine from 48 to 72 h	104	28 (27.0%)[b]
Control	94	24 (25.5%)[b]

[a,b]Values with different superscripts in the same column are significantly different at $P < 0.05$.

Table 2 Fertilization rates of canine oocytes matured in caffeine-treated medium for different maturation times

Treatment	Number of inseminating oocytes	Fertilization rates no. (%)
Caffeine for 72 h	88	19 (21.6%)[a,b]
Caffeine at first 24 h	104	30 (28.8%)[a]
Caffeine from 24 to 48 h	78	13 (16.7%)[b]
Caffeine from 48 to 72 h	92	15 (16.3%)[b]
Control	76	12 (15.8%)[b]

[a,b]Values with different superscripts in the same column are significantly different at $P < 0.05$.

Table 3 Developmental potential of canine oocytes matured in caffeine-treated medium for different maturation times

Treatment	Number of inseminating oocytes	2–16-cell stage no. (%)	Morula no. (%)	Total number of cleaved embryos/oocytes no. (%)
Caffeine for 72 h	80	8 (10.0%)[a,b]	1 (1.3%)	9 (11.3%)[a,b]
Caffeine at first 24 h	92	14 (15.2%)[a]	4 (4.3%)	18 (19.6%)[a]
Caffeine from 24 to 48 h	74	5 (6.8%)[b]	0 (0.0%)	5 (6.8%)[b]
Caffeine from 48 to 72 h	83	6 (7.2%)[b]	0 (0.0%)	6 (7.2%)[b]
Control	70	5 (7.1%)[b]	0 (0.0%)	5 (7.1%)[b]

[a,b]Values with different superscripts in the same column are significantly different at $P < 0.05$.

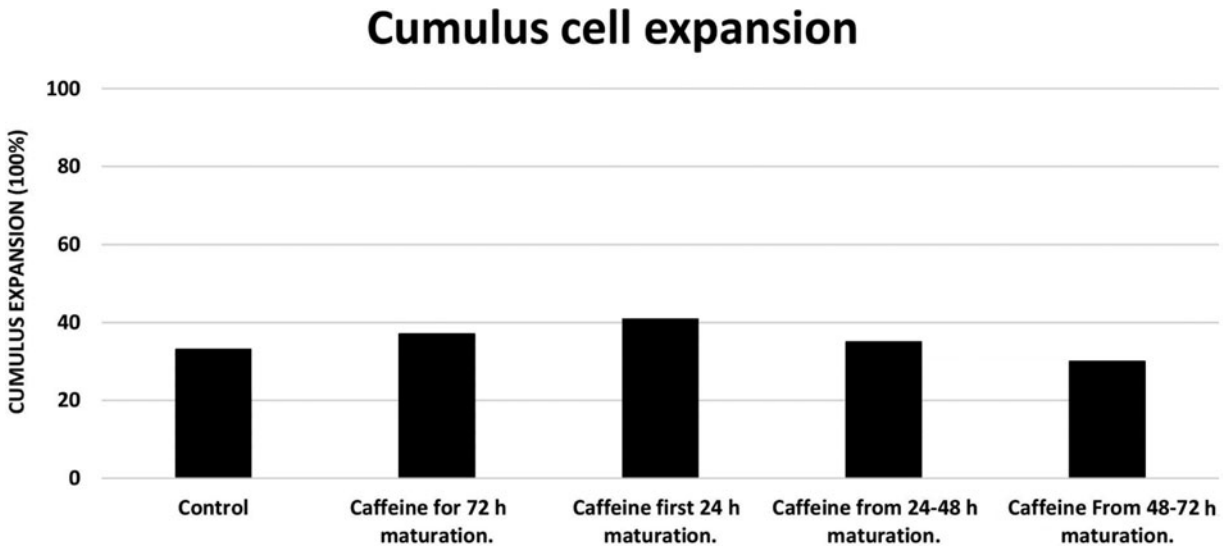

Figure 1 Cumulus cell expansion following (IVM) of canine oocytes using caffeine-treated medium for different maturation times.

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species is not characterized due to the complexity of its delayed progression (Saint-Dizier *et al.*, 2004). Only a few reports are available on *in vitro* maturation and embryo production in canine species despite its importance. So, the current study focused on studying the effect of caffeine addition as a non-selective competitive phosphodiesterase inhibitor during oocyte maturation on the acquisition of developmental competence of canine oocytes *in vitro*.

The current study revealed that use of 10 mm caffeine-supplemented maturation medium for the first 24 h of maturation resulted in a significant increase in the maturation rate up to 42.2%, this increase may be due to the biphasic effect of caffeine on canine oocyte maturation or the enhancement effect of caffeine on both MPF and MAPK as previously mentioned by Salavati *et al.* (2012). In the same way, Salavati *et al.* (2012) concluded that use of 10 mM caffeine pre-treatment for 12 h in the maturation medium of canine oocytes improved MII oocytes and lowered the degeneration rate to 25.9%. The crucial effect of caffeine on oocyte maturation was also studied in different species such as sheep (Lee & Campbell, 2006; Choi *et al.*, 2010) and camels (Fathi *et al.*, 2014). The role of caffeine in oocyte maturation has been illustrated previously by Zhang *et al.* (2009) who described caffeine as a promoter to the extrinsic pathways that initiates MPF activity via MAPK activation. Also, Ono *et al.* (2011) reported that caffeine can maintain a healthy morphology of the meiotic spindle even in aged oocytes. The optimal time of exposure of caffeine with the oocytes was examined in the current study and the best results were obtained during either caffeine supplementation for the first 24 h of maturation time or during the whole maturation time (72 h) suggesting that caffeine had a role in the activity of MPF and MAPK in canine oocytes, further studies are needed on this aspect. This outcome was similar to the results concluded by Salavati *et al.* (2012).

Conversely, Kren *et al.* (2004) reported that using 2.5 mM caffeine concentration resulted in increasing cAMP and maintaining the meiotic arrest in porcine oocytes, this difference with our results may be attributed to species difference or the difference in caffeine concentration used. Also, Miao *et al.* (2007) found a negative effect of caffeine-supplemented medium on the nuclear maturation of mice oocytes.

For fertilization, the present study revealed that supplementation of the maturation medium with caffeine at first 24 h followed by insemination resulted in a significantly higher ($P < 0.05$) fertilization rate than the control and caffeine-treated medium either from 24 to 48 h or from 48 to 72 h, this may be attributed to the higher percentage of maturation rate obtained using this approach. Otoi *et al.* (2004) found that the oocytes matured for 72 h and co-incubated with spermatozoa for 15 h resulted in a 42% penetration rate while the fertilization rate was only 19%, our higher result is attributed to the use of caffeine during maturation, and moreover the capacitating effect of the caffeine as described by Fathi *et al.* (2014). Our results are slightly lower than the values obtained by De los Reyes *et al.* (2006) who reported that the percentages of fertilization rates following IVM were 33.1 and 34.2% using either Tris, citric acid and glucose diluent or Tris, citric acid and fructose diluent, respectively. This difference among the results may be attributed to some factors such as age of animals, sperm quality, fertilization medium, oocytes quality and the concentration of spermatozoa used during IVF. Interestingly, caffeine treatment for the first 24 h maturation resulted in a significant enhancement of the total number of cleaved embryos following IVF compared with the control group. For the same species, the cleavage rate of canine embryos following IVM for only 48 h and IVF was obtained by Songsasen *et al.* (2002). Otoi *et al.* (2000) reported single blastocyst development following IVF of 72 h matured oocytes. England *et al.* (2001) obtained a single pregnancy subsequent to transferring a 2-cell stage embryo following IVM and IVF. Values higher than ours were demonstrated by Otoi *et al.* (2004) who found that the total number of cleaved embryos were 37.2% after IVM and IVF. This higher value may be attributed to the use of a bitch serum containing progesterone (1.6 ng/ml) and estradiol (31.5 pg/ml) during *in vitro* culture (IVC). Similar to our results, Yamada *et al.* (1992) found that 48 h post insemination 14.6% of the oocytes were at the 2–3-cell stage and 72 h post insemination 4.8% of the oocytes were at 5–8-cell stage.

Conclusion

The current study demonstrated the crucial effect of caffeine supplementation during *in vitro* maturation of canine oocytes based on supplementation time. Supplementation of the maturation medium with 10 mM caffeine for the first 24 h of maturation or during the whole maturation time (72 h) improved nuclear maturation and subsequent embryo development preimplantation following IVF. These findings can improve IVF outcomes in *Canis familiaris*.

Statement of interest

The authors have no financial or personal relationship with anyone that could be affected by current work outcomes.

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