Effect of β-mercaptoethanol or epidermal growth factor supplementation on in vitro maturation of canine oocytes collected from dogs with different stages of the estrus cycle

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Supplementation of β-mercaptoethanol (β-ME) in in vitro maturation (IVM) medium was shown to improve embryo development and quality in several species. Epidermal growth factor (EGF) was also shown to improve IVM of human oocyte and embryo development after in vitro fertilization (IVF). The effect of these two compounds were suggested to be mediated through the synthesis of glutathione (GSH) which is known to play an important role in protecting the cell or embryos from oxidative damage. Thus, it is suggested that supplementation of canine IVM medium with β-ME or EGF may be of benefit due to its positive role in IVM of various mammalian oocytes and embryo development, including cattle, pigs, rodents and humans. This study investigates the effect of ovarian estrus stage on canine oocyte quality and supplementation of medium with β-ME or EGF on IVM of canine oocytes. As results, a significantly higher percentage of oocytes progressed to metaphase II (MII) stage in 50 or 100 µM of β-ME supplemented oocytes collected from the follicular stage. The maturation rate to metaphase I (MI) stage was also significantly higher in oocytes collected from follicular stage and cultured with 25 or 100 µM compared to other experimental groups. After IVM culture, oocytes recovered from dogs with the follicular stage and matured in TCM-199 supplemented with 20 ng/ml EGF yielded better oocyte maturation to MII phase compared to other groups. Taken together, supplementation of β-ME (50 or 100 µM) or EGF (20 ng/ml) improved IVM of canine oocytes to MII stage.

Key words: β-mercaptoethanol (β-ME), epidermal growth factor (EGF), canine oocytes, in vitro maturation (IVM).

Introduction

The efficiency of in vitro maturation (IVM) of canine oocytes is still very low compared to that found in other mammalian species. Small proportions of canine oocytes completed nuclear maturation after being cultured for 48 to 72 h in vitro [20,27,30]. Low maturation rates could be due to either suboptimal culture conditions or low meiotic competence of the oocytes [7]. Studies are in progress to determine both the culture requirements for canine oocytes and some parameters indicative of maturation competence. The morphological appearance of cumulus-oocyte complexes (COCs) and oocyte diameter have been identified as parameters indicative of maturation competence [14]. In contrast to most mammals, bitches ovulate immature oocytes that require 2-5 days for the completion of meiosis within the oviduct [16] and remain surrounded by a tight and multilayered cumulus cell mass [28]. The immature stage of oocytes at ovulation and the persistence of cumulus cells during transport and maturation within the oviduct, indicating that investigation of the relationship between cumulus cells and oocytes should help to clarify the reasons for the low IVM rates of canine oocytes. This results also suggests the importance of selecting good quality of COCs by morphological appearance for IVM of oocytes.

Increased oxidative stress is one of the causes of impaired IVM of oocyte. The GSH is the major nonprotein sulphynyl compound in mammalian cells and is known to play an important role in protecting the cell from oxidative damage. The β-mercaptoethanol (β-ME) is low molecular weight thiol and supplementation of β-ME in IVM medium has shown to increase intracellular glutathione (GSH) content in bovine oocytes and to improve embryo development and quality in several species [24,25,29]. Moreover, the presence of thiol compound in a maturation medium increased the GSH level and improved developmental competence of pig
oocytes [1]. GSH is also known to be important for sperm chromatin decondensation and male pronucleus formation after sperm penetration, supporting its possible role in encouraging oocyte cytoplasmic maturation [8]. A mechanism by which β-ME promotes intracellular GSH synthesis was proposed by Takahasi et al. [32]. In lymphocytes, β-ME promotes the uptake of cysteine, enhancing GSH synthesis [18]. It has been suggested that GSH protects cells from oxidative damage [9,26,34]. Thus, increased intracellular GSH induced by β-ME may provide embryos with a better intracellular condition, preventing oxidative damaged possibly promoting late embryonic development.

Several hypotheses suggest that epidermal growth factor (EGF) acts on cells as both a paracrine and autocrine regulator of ovarian function [3,12]. The EGF is one of many growth factors found throughout the body, including growing antral follicles within the ovary, and has shown to stimulate DNA synthesis and cell proliferation in porcine granulosa cells. The EGF also improves IVM of human germinal vesicle breakdown (GVBD), and to cause cumulus expansion in bovine and mouse COCs.

In this experiment, whether β-ME promotes completion of nuclear maturation in canine oocytes was evaluated. The concentrations of β-ME tested were 0.0 (control), 25, 50 and 100 μM. After 72 h of in vitro culture, oocytes were evaluated for nuclear meiotic stage as previously defined in Table 1 and Fig. 1.

**Experimental design**

**Supplementation of β-ME in TCM-199 (Experiment 1).**

In this experiment, the effect of supplementation of EGF to basic maturation medium on IVM of canine oocytes was evaluated as previously defined in Table 1 and Fig. 1.

**EGF supplementation in TCM-199 (Experiment 2).**

In this experiment, the effect of supplementation of EGF to basic maturation medium on IVM of canine oocytes was evaluated. The concentrations of EGF tested were 0.0 (control), 10 and 20 ng/ml.

**Statistical analysis**

Multiple comparisons (LSD) were implemented using Generalized Linear Models in the SAS 8.12 program. When the significant effect in each experimental parameter was detected, subsequent comparison was made by the least
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square method. Difference among the treatment were considered statistically significant when the p-values were less than 0.05.

Result

Supplementation of β-ME in TCM-199

The result of Experiment 1 showed that a significantly higher percentage of oocytes progressed to metaphase II (MII) (20 or 13%, respectively) in 50 or 100 µM β-ME supplemented oocytes collected from the follicular stage (Table 2). The maturation rate to metaphase I (MI) stage was also significantly higher in oocytes collected from follicular stage and treated with 25 or 100 µM (29 or 32%, respectively) compared to that observed in other experimental groups (p < 0.05).

EGF supplementation in TCM-199

In Experimental 2, after 72 h culture, oocytes recovered from the follicular stage and matured in TCM-199 supplemented with EGF 20 ng/ml yielded better oocyte maturation to MII phase compared to other groups (13 % vs. 3 to 6 %, respectively) (Table 3).

Discussion

Unlike other mammalian oocytes, ovulated canine oocytes complete meiotic maturation within 2-5 days in the oviduct after ovulation [4,16]. At present, IVM of canine oocytes is characterized by low and greatly variable success rates [7]. Studies have been performed to improve the rate of IVM in canine oocytes by supplementing the culture medium with various serum [25,29], gonadotrophin [15] or steroid hormone [15]. The present study investigated the effect of β-ME or EGF supplementation on the base of the stages of the estrus cycle of the ovaries, and demonstrated that β-ME or EGF supplementation significantly increased maturation of canine oocyte to MII stage.

A number of studies have demonstrated an effect of stage of the estrous cycle on the meiotic competence of canine oocytes matured in vitro. Yamada et al. [35] reported that 32% of preovulatory oocytes collected from superovulated bitches reached MII after 72 h of culture, whereas oocytes from anestrous bitches showed no tendency to resume meiosis even after a culture period of up to 144 h. In a similar investigation by Luvoni et al. [23], a high percentage of the oocytes collected during anestrous were in the germinal

Fig. 1. Canine oocytes stained with Hoechst 33342 and visualized under UV light. Germinal vesicle (A), germinal vesicle breakdown (B), metaphase I (C) and metaphase II (D) (x200).
vesicle breakdown (GVBD) stage at the time of collection. Such oocytes could be derived from atretic follicles, which are usually ruptured by the slicing procedure used for collection. It is generally accepted that GVBD can occur spontaneously in human atretic follicles [11]. In contrast, Hewitt and England [15] reported that no significant differences in maturation rates were observed between oocytes collected at proestrus and estrus stages. In the present study, higher proportion of oocytes collected from the follicular stage ovaries reached MI and MII stage in the absence or presence of β-ME or EGF compared to those collected from ovaries of anestrus and luteal stages. Our results indicate the importance of oocytes recovery phase to select potentially meiotically competent canine oocytes for use of in vitro experiment.

Glutathione (GSH) is the major non-protein sulfydryl compound present in mammalian cells. Multiple actions have been described for GSH, including increasing amino acid transport, stimulating DNA and protein synthesis, reduction of disulfides and protection against toxic effects of oxidative damage [21]. Takahashi et al. [33] demonstrated that the supplementation of low molecular weight thiol compound, to culture medium can increase the cystein-redox of disulfides and protection against toxic effects of oxidative damage [21]. Similarly, addition of β-ME increased intracellular glutathione synthesis [34]. Therefore, addition of these compounds to culture medium can increase the cystein-redox state in bovine embryo produced in vitro. Similarly, the supplementation of low molecular weight thiol compound, to culture medium can increase the cystein-redox state in bovine embryo produced in vitro. Similarly, the supplementation of low molecular weight thiol compound, to culture medium can increase the cystein-redox state in bovine embryo produced in vitro. Similarly, the supplementation of low molecular weight thiol compound, to culture medium can increase the cystein-redox state in bovine embryo produced in vitro. Similarly, the supplementation of low molecular weight thiol compound, to culture medium can increase the cystein-redox state in bovine embryo produced in vitro.

Table 2. Meiotic status of canine oocytes recovered from different ovarian estrus stages and cultured in TCM-199 supplemented with various concentrations of β-mercaptoethanol (β-ME)

<table>
<thead>
<tr>
<th>Estrus stages</th>
<th>β-ME (µM)</th>
<th>No. of oocytes examined</th>
<th>% nuclear status of oocytes (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GV</td>
</tr>
<tr>
<td>Anestrus</td>
<td>0</td>
<td>51</td>
<td>31.3 ± 6.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>27</td>
<td>37.0 ± 8.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td>50</td>
<td>65</td>
<td>29.2 ± 5.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>100</td>
<td>46</td>
<td>30.4 ± 6.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Follicular</td>
<td>0</td>
<td>36</td>
<td>22.2 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>21</td>
<td>19.0 ± 9.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>13.3 ± 8.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>100</td>
<td>31</td>
<td>25.8 ± 8.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Luteal</td>
<td>0</td>
<td>84</td>
<td>36.9 ± 4.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>69</td>
<td>18.8 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>84</td>
<td>47.6 ± 4.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>100</td>
<td>65</td>
<td>21.5 ± 5.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values with different superscripts are significantly different (p < 0.05)

Table 3. Meiotic status of canine oocytes recovered from different ovarian estrus stages and cultured in TCM-199 supplemented with various concentrations of epidermal growth factor (EGF)

<table>
<thead>
<tr>
<th>Estrus stages</th>
<th>EGF (ng/ml)</th>
<th>No. of oocytes examined</th>
<th>% nuclear status of oocytes (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GV</td>
</tr>
<tr>
<td>Anestrus</td>
<td>0</td>
<td>80</td>
<td>27.5 ± 4.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>126</td>
<td>28.5 ± 5.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>20</td>
<td>122</td>
<td>31.9 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Follicular</td>
<td>0</td>
<td>36</td>
<td>16.6 ± 7.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>34</td>
<td>8.8 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>20</td>
<td>45</td>
<td>8.8 ± 6.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Luteal</td>
<td>0</td>
<td>24</td>
<td>12.5 ± 8.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>31</td>
<td>25.8 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>36</td>
<td>16.6 ± 7.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values with different superscripts are significantly different (p < 0.05)
folicular stage, consistent with the result from Takahashi et al. [32] in bovine IVM. As described above, it is important to mention that the outcome of our experiments was most likely influenced by the stage of the estrus cycle of the dogs from which the oocytes were collected; the positive effect of β-ME on IVM of canine oocytes was only observed in the oocytes collected from the follicular stage.

It is well established that the addition of EGF promote in vitro oocyte maturation, and also improve fertilization and cleavage rates in many species, including the pig, rat, mice [5], fox [31], cow [19], and humans [17]. In addition to accelerating nuclear maturation in the oocyte, literature also suggests that the addition of EGF improves cytoplasmic maturation [20]. Furthermore, studies indicate that EGF induces oscillations in calcium efflux in COCs of mice, and also promotes the synthesis of GSH within the oocyte [8]. The role of calcium oscillations in immature oocytes is not as widely studied as it is in fertilization events, but it is suggested to have a role in resumption of meiosis, as described in research on amphibian and invertebrate oocytes [15]. The results of the experiments presented in this study demonstrated the benefit of supplementing canine IVM medium with EGF. Supplementation of 20 ng/ml EGF in TCM-199 showed significantly improved rate of meiotic competence of oocytes collected from follicular stage. In support of our results, a study reports a significant increase in the blastocyst development from oocytes that were cultured in serum-free medium supplemented with 10 ng/ml EGF, and this was attributed to an increase in GSH synthesis influenced by EGF [2]. It is also important to mention that the outcome of our experiments was most likely influenced by the stage of the estrus cycle of the dogs; the positive effect of EGF supplementation on IVM of canine oocytes was only observed in the oocytes collected from the follicular stage.

In conclusion, supplementation of canine IVM medium with GSH-synthesis stimulator, β-ME or EGF improved IVM of canine oocytes collected from dogs with the follicular stage of the estrus cycle.

References


