—Original Article—

Effects of insulin-like growth factor-1 on the *in vitro* maturation of canine oocytes

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Abstract. The maturation rate of canine oocytes during *in vitro* maturation (IVM) needs to be improved. The present study was designed to evaluate the effects of insulin-like growth factor-1 (IGF-1) on the IVM of canine oocytes. Ovaries were obtained by ovariectomy and were sliced to release cumulus-oocyte complexes (COCs). In Experiment 1, the effects of different concentrations of IGF-1 on the nuclear maturation of oocytes was investigated. The COCs were cultured in a modified medium (mTCM199) with IGF-1 (0, 0.5, 5, 10, and 50 µg/ml). At the end of the 48 h culture, oocytes were fixed and stained to evaluate their nuclear stage. Supplementation with 50 µg/ml IGF-1 induced a significantly higher metaphase II (MII) rate ($P < 0.05$) compared to the 0 and 0.5 µg/ml IGF-1 groups. In Experiment 2, the expression levels of *insulin receptor* (*INSR*), *IGF-1 receptor* (*IGF-1R*), and *IGF-2 receptor* (*IGF-2R*) genes, localized to canine oocytes and cumulus cells, were investigated before and after IVM. The expression level of *IGF-1R* in cumulus cells after IVM was higher than that before IVM ($P < 0.05$). In Experiment 3, it was investigated whether an inhibitor of PTEN (phosphatase and tensin homolog), bpV, affects the nuclear maturation of oocytes. Regardless of bpV supplementation at a concentration of 0.2 to 200 µmol/l, there was no significant difference in the proportion of oocytes that reached the MII stage. These results indicated that IGF-1 has a favorable effect on the IVM of canine oocytes, possibly through the stimulation of the Ras/MAPK pathway via *IGF-1R* expressed in cumulus cells.

Key words: bpV, Canine oocyte, Insulin-like growth factor-1 (IGF-1), Insulin-like growth factor-1 receptor (IGF-1R), *In vitro* maturation

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The *in vitro* maturation (IVM) technique can be applied to various assisted reproductive technologies (ART), such as *in vitro* fertilization, the microinjection of sperm or DNA into an oocyte, or the cryopreservation of embryos and the generation of cloned animals [1]. ART procedures are also used in canine species, to preserve endangered species [2] and to create efficient breeding systems for working dogs [3]. In many mammalian species, the maturation rate of oocytes during IVM (80% in humans [4]; 80% in mice [5]; 90% in bovine species [6]) is much higher than that in canine species (0 to 39.5% [7–11]). In bovine oocytes, nuclear maturation has been shown to complete after 21 to 24 h in culture

medium containing follicle-stimulating hormones and 17β-estradiol in 5% CO₂ in air [12]. In mouse and human oocytes, the proportion of oocytes that reach metaphase II (MII) after IVM is around 80 to 90% [13, 14]. Since the maturation rate of canine oocytes *in vitro* remains extremely low, many studies have already begun to address this problem by investigating the influence of various IVM conditions, such as co-culture with oviductal epithelial cells [7], the type of culture medium [8], and supplementation of the maturation media with serum, pyruvate, glutamine, and hormones [9–11].

Some growth factors have been shown to promote oocyte maturation in many mammalian species [15, 16]. It has been demonstrated that insulin-like growth factor-1 (IGF-1) promotes the maturation of bovine oocytes and swelling of cumulus cells [17], and can also inhibit apoptosis [18]. It has also been shown that IGF-1 improves *in vitro* oocyte maturation, fertilization, and embryo development to the blastocyst stage in mice [19]. The addition of IGF-1 to IVM medium also increased the maturation rate, and improved IVF results in a porcine model [20]. Activation of the *IGF-1 receptor* (*IGF-1R*) can result in cell signaling via two pathways: the Ras/mitogen activated

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protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K)/Akt pathway [21]. The *IGF-1R* activates the insulin receptor substrate (IRS) protein, and Src and collagen homologues protein, stimulates Ras through a GTPase, and triggers a kinase cascade, eventually resulting in the activation of MAPK. Otherwise, *IGF-1R* is activated through phosphorylation, and subsequently phosphorylates insulin receptor substrate 1 (IRS-1). Activated PI3K then leads to increased levels of phosphatidylinositol 3, 4, 5-trisphosphate (PIP3), which results in the activation of the critical protein Akt/PKB through phosphorylation [22]. PI3K is thought to participate in the meiotic maturation of oocytes [23]. The phosphatase and tensin homolog (PTEN) inhibits the action of PI3K by dephosphorylating the signaling lipid PIP3 [24]. A bisperoxovanadium inhibitor of the PTEN [25], bpV, has been shown to regulate cell survival signaling through the PI3K/Akt pathway.

The present study was designed to evaluate the influence of IGF-1 supplementation during IVM on canine oocyte maturation. In Experiment 1, different concentrations of IGF-1 were supplemented to an IVM medium, and the nuclear configuration of the oocytes was evaluated after 48 h of IVM culture. In Experiment 2, the expression levels of *insulin receptor (INSR)*, *IGF-1 receptor (IGF-1R)*, and *IGF-2 receptor (IGF-2R)* were investigated in cumulus cells and oocytes before and after IVM by quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay. In Experiment 3, it was investigated whether an inhibitor of PTEN (bpV) supplemented to the IVM medium affected the nuclear maturation of the oocytes.

Materials and Methods

Collection of ovaries and immature oocytes

This study was conducted in accordance with the Utsunomiya University Guide for Experimental Animals, under an experimental design approved by the Animal Care and Use Committee of Utsunomiya University. Ovaries were obtained from female dogs (bitches) following routine contraceptive operations at a local veterinary hospital, and were stocked in hygienic plastic bags and transported to the laboratory at room temperature within 6 h after the operation. The dogs involved in this study were either pure or crossbred, ranged from 6 months to 9 years in age, and were at different stages of estrus.

Cumulus oocyte complexes (COCs) were collected from the ovaries by repeatedly slicing the ovarian cortex with a scalpel blade in Medium 199 (No. 12340, Thermo Fisher Scientific, Waltham, MA, USA) containing 0.1% (w/v) polyvinyl alcohol (No. 341584, MilliporeSigma, St Louis, MO, USA), 0.5 mM sodium pyruvate (No. 29806-54, Nacalai Tesque, Kyoto, Japan) and 1% (v/v) antibiotic/antimycotic agent solution (No. 15240062, Thermo Fisher Scientific, Waltham, MA, USA). The medium described above was defined as mTCM199, and was used for the subsequent IVM. Perfectly spherical oocytes that exhibited an even, smooth, and dark pigmented cytoplasm, measured more than 110 μ m in diameter, and were surrounded by more than three layers of cumulus cells, were selected for IVM.

IVM of canine COCs

COCs were washed in the mTCM199. In each culture, 10 COCs were introduced into 50 μ l drops of the mTCM199 covered with

mineral oil in culture dishes (Falcon 351007, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and were cultured for 48 h under 5% CO₂ in humidified air at 38.8°C. In Experiment 1, the mTCM199 supplemented with 0, 0.5, 5, 10, or 50 μ g/ml of IGF-1 (human, I-3769, MilliporeSigma) was used as the IVM medium. In Experiment 2, the mTCM199 without IGF-1 supplementation was used as the IVM medium. In Experiment 3, the mTCM199 containing 10 μ g/ml of IGF-1, to enhance the IGF-1 cascade, was supplemented with 0, 0.2, 1, 5, 20, 100, or 200 μ mol/l of bpV (HOPic, Calbiochem, 203701, Merck Millipore, Billerica, MA, USA).

Assessment of the meiotic stage

At the end of the IVM culture, the expansion of cumulus cells was observed under a stereomicroscope (100 $\times$; Eclipse TE300, Nikon, Tokyo, Japan). The cumulus cells were removed from COCs by gentle pipetting in each 50 μ l drop of culture medium covered with mineral oil. Denuded oocytes were fixed with Carnoy's solution (99.9% ethanol: 99.7% acetic acid in a ratio of 3:1) for 10 to 30 min. Then, fixed oocytes were washed three times in mTCM199 and stained with phosphate-buffered saline (No. 70011044, Thermo Fisher Scientific) supplemented with 90% (v/v) glycerol and 1.9 μ M Hoechst 33342, on glass slides (SO317, Matsunami, Osaka, Japan). The stained nuclear configuration and chromatin morphology of oocytes were then evaluated under confocal laser scanning microscopy (60 $\times$; FV10i, Olympus, Tokyo, Japan). The meiotic stage of each oocyte was classified as belonging to one of the following meiotic stages: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII), as shown in Fig. 1.

Gene expression analysis

Oocytes and cumulus cells, obtained pre-IVM and post-IVM, were pooled and frozen at -196°C and then stored at -80°C . Subsequently, total RNA was isolated from 20 to 100 denuded oocytes or from 4,000 to 20,000 cumulus cells (approximately 200 cumulus cells per COC) [26] using the PicoPure RNA Isolation Kit (KIT0204, Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The concentration and quality of each RNA sample was determined using a NanoVue system (GE Healthcare, Little Chalfont, UK). Purified RNA samples were incubated in gDNA Wipeout Buffer at 42°C for 2 min to remove contaminating genomic DNA, and then the messenger RNA (mRNA) was used for reverse transcription using the QuantiTect Reverse Transcription Kit (No. 205311, QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's instructions. Real-time Polymerase Chain Reaction (PCR) amplifications were performed in a LightCycler 96 (Roche, Basel, Switzerland) using a QuantiTect SYBR Green PCR kit (No. 204143, QIAGEN). Real-time PCR was then performed to determine the relative expression levels of *INSR* [27], *IGF-1R* [28], and *IGF-2R* [29] by three steps under the following conditions: 95°C for 15 min, followed by 45 cycles of 15 sec at 95°C for denaturing, 25 sec at 55°C for annealing, and 20 sec at 72°C for extension. The annealing temperature was optimized for our experimental conditions. To confirm specificity of the product amplification, melting curves were examined. The results of the mRNA expression were normalized to the mRNA expression of *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* [30] as an internal control. Primer sequences are shown in Table 1.

Fig. 1. Chromatin configuration in canine oocytes stained with Hoechst 33342. (A) Oocyte at the germinal vesicle (GV) stage; (B) Oocyte at the germinal vesicle breakdown (GVBD) stage; (C) Oocyte at the metaphase I (MI) stage; (D) Oocyte at the metaphase II (MII) stage. (A), (B) and (C) were single optical sections selected from Z stacks. (D) was a merged image of Z stacks.

Statistical analysis

The proportions of oocytes in each meiotic stage were analyzed using a one-way analysis of variance and the Tukey-Kramer test. In addition, the pre-IVM and post-IVM expression levels of each receptor were tested for normality using the F-test and, if normally distributed, the data were analyzed using a paired *t*-test. Differences at $P < 0.05$ were considered to be statistically significant.

Results

In Experiment 1, the proportion of MII oocytes in the 50 $\mu\text{g/ml}$ IGF-1 group (12.2%) was significantly higher than those in the control 0 $\mu\text{g/ml}$ IGF-1 group (2.7%) and the 0.5 $\mu\text{g/ml}$ IGF-1 group (5.3%; Table 2). The average diameter of COCs before IVM was 126.7 ± 5.1 , and those after IVM in the 0, 0.5, 5, and 50 $\mu\text{g/ml}$ IGF-1 groups were 138.5 ± 7.4 , 142.6 ± 10.5 , 145.5 ± 6.2 , and 176.6 ± 7.3 μm , respectively (Fig. 2).

In Experiment 2, the expression levels of each receptor in the oocytes were not significantly different when compared between pre- and post-IVM oocytes ($P > 0.05$; Fig. 3A). However, the expression level of *IGF-1R* in the cumulus cells from post-IVM COCs (1.19 ± 0.20) was significantly higher (3.27 ± 0.64 -fold) than that from pre-IVM COCs (0.45 ± 0.09) ($P < 0.05$; Fig. 3B). No significant increases were detected in the expression of *INSR* and *IGF-2R* during the IVM.

In Experiment 3, an inhibitor of PTEN (bpV) did not affect the nuclear maturation of oocytes regardless of the bpV supplementation

Table 1. Primers used for the quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Gene	Primer Sequence (5'→3')	Amplicon (bp)	Reference
<i>INSR</i> †	(F) CACCAGTACGTCATCCACAA	136	[27]
	(R) GTCTTCTCGCCTTCCAGAAT		
<i>IGF-1R</i> ‡	(F) CCGACGAGTGAGAAATCTGT	99	[28]
	(R) GGAGGTAGCCCTCGATCACT		
<i>IGF-2R</i> §	(F) GCTCAGCCTGCTGCTGGT	196	[29]
	(R) GGATCTCTTCCATCAGCCACTC		
<i>GAPDH</i> *	(F) TGTCCCCACCCCAATGTATC	100	[30]
	(R) CTCCGATGCCTGCTTCACTACCTT		

† *INSR*, Insulin receptor; ‡ *IGF-1R*, Insulin-like growth factor-1 receptor; § *IGF-2R*, Insulin-like growth factor-2 receptor; * *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase.

Table 2. The effect of IGF-1 supplementation on the nuclear maturation of canine oocytes

Concentration of IGF-1 ($\mu\text{g/ml}$)	No of oocytes cultured	Meiotic stage of oocytes (%) †				Total	No. of oocytes degenerated
		GV	GVBD	MI	MII		
0	152	10 (7.2)	52 (37.7)	74 (53.6)	3 (2.2) ^a	139	13
0.5	97	15 (16.0)	45 (47.9)	29 (30.9)	5 (5.3) ^a	94	3
5	89	7 (8.5)	43 (52.4)	24 (29.3)	8 (9.8) ^{ab}	82	7
10	96	6 (6.7)	24 (26.7)	48 (53.3)	12 (13.3) ^{ab}	90	6
50	77	11 (14.9)	30 (40.5)	24 (32.4)	9 (12.2) ^b	74	3

† %: No. of oocytes at each meiotic stage / No. of surviving oocytes. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

Fig. 2. Morphology of canine COCs after 48 h of *in vitro* maturation, showing cumulus cell expansion in the presence of IGF-1. (A) No IGF-1; (B) 0.5 µg/ml IGF-1; (C) 5 µg/ml IGF-1; (D) 50 µg/ml IGF-1.

at 0.2 to 200 µmol/l ($P > 0.05$; Table 3). An increased level of cumulus expansion was not observed after IVM in any of the groups.

Discussion

IVM procedures for canine oocytes can be applied to preserve endangered species and to create efficient breeding systems for

working dogs. However, the maturation rate of canine oocytes *in vitro* remains extremely low, with studies reporting rates of 16.7% [7], 33.3% [8], 14.2% [9], 39.5% [10], and 5.5% [11]. Therefore, the aim of the present study was to improve the efficiency of IVM for canine oocytes. In Experiment 1, we confirmed that supplementation with IGF-1 had a positive effect upon the maturation rate of canine oocytes (Table 2) and cumulus expansion (Fig. 2). It has been shown that IGF-1 affects oocyte maturation in several mammalian species, including mice (50 ng/ml) [31], cattle (50 ng/ml) [32], and pigs (25–200 ng/ml) [33]. In addition, IGF-1 (100 ng/ml) has been shown to stimulate cumulus expansion and enhance nuclear maturation in bovine oocytes [17]. Cumulus expansion could be an index of oocyte maturation, as well as their subsequent developmental ability [34]. These previous studies coincide with our present study indicating that IGF-1 can promote the nuclear maturation of canine oocytes and cumulus expansion, although a high level of IGF-1 (50 µg/ml) is required for the promotion. To our best knowledge, this is the first report to demonstrate the positive impact of IGF-1 supplementation during IVM in the canine species.

Two additional experiments were designed to analyze the possible pathway and mechanism of IGF-1 function in canine IVM. In Experiment 2, we investigated the expression levels of *INSR*, *IGF-1R*, and *IGF-2R* localized to canine oocytes and cumulus cells before and after IVM. The expression level of *IGF-1R* in the cumulus cells isolated from post-IVM COCs was significantly higher than that from pre-IVM COCs (Fig. 3B). The IGF system is composed of two ligands (IGF-1 and IGF-2), two types of receptors (*IGF-1R* and *IGF-2R*), and six IGF-binding proteins (IGFBPs) [35]. This IGF system has been shown to be expressed in bovine COCs [36]. It was reported that IGF-1R and IGF-2R proteins were detected on the plasma membrane of cumulus cells in immature and mature porcine COCs [37]. However, there are no reports regarding the possible interaction of *IGF-1R/IGF-2R* expression and the nuclear maturation of canine oocytes. It has been shown that cumulus cells can secrete factors such as angiotensin II [38], basic fibroblast growth

Fig. 3. Relative expression level of mRNA of *IGF-1R*, *IGF-2R*, and *INSR* in canine oocytes (A) and cumulus cells (B), as detected by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and normalized with *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. * *IGF-1R*, Insulin-like growth factor-1 receptor; *IGF-2R*, Insulin-like growth factor-2 receptor; *INSR*, Insulin receptor.

Table 3. The effect of bpV supplementation on the nuclear maturation of canine oocytes

Concentration of bpV ($\mu\text{mol/l}$)	No of oocytes cultured	Meiotic stage of oocytes (%) [†]				Total	No. of oocytes degenerated
		GV	GVBD	MI	MII		
0	108	1 (1.0)	41 (39.8)	51 (49.5)	10 (9.7)	103	5
0.2	58	1 (1.8)	26 (45.6)	28 (49.1)	2 (3.5)	57	1
1	52	1 (2.0)	23 (46.0)	23 (46.0)	3 (6.0)	50	2
5	123	1 (8.2)	55 (45.1)	60 (49.2)	6 (4.9)	122	1
20	72	0 (0)	24 (33.8)	45 (63.3)	2 (2.8)	71	1
100	85	0 (0)	36 (42.9)	38 (45.2)	10 (11.9)	84	1
200	65	0 (0)	32 (59.3)	15 (27.8)	7 (13.0)	54	11

[†] %: No. of oocytes at each meiotic stage / No. of surviving oocytes. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

factor [39], and estradiol [40]. These factors may increase the level of *IGF-1R* expression during maturation. Therefore, the increased *IGF-1R* expression in post-IVM cumulus cells detected in the present study suggests the function of the IGF pathway within canine COCs; IGF-1 may function via *IGF-1R*, which is expressed during the later stages of culture, and promote the meiotic progression of oocytes from the MI to MII stage. In Experiment 3, we investigated whether bpV supplementation during IVM can improve the nuclear maturation rate of canine oocytes. IGF-1 has been shown to activate two major signal transduction pathways: the PI3K/Akt pathway and the Ras/MAPK pathway [21]. A bisperoxovanadium inhibitor of PTEN, bpV, [25], has been shown to regulate cell survival signaling through the PI3K/Akt pathway. Furthermore, a previous study reported that bpV can stimulate the growth of isolated non-growing primary oocytes in mice [41]. In the present study, although the expression level of *IGF-1R* increased during the later stages of IVM culture, the treatment of canine COCs with bpV (0.2 to 200 $\mu\text{mol/l}$) and IGF-1 (10 $\mu\text{g/ml}$) did not influence the maturation rate of canine oocytes (Table 3). Villa-Diaz *et al.* indicated that an MAPK inhibitor (SB203580) did not affect GVBD, but it decreased the number of oocytes reaching MII, and conversely increased the number of oocytes arrested at MI in the maturation of porcine oocytes [42]. Activation of MAPK leads to the phosphorylation and activation of specific MAPK-activated protein kinases (MAPKAPKs), such as members of the RSK, MSK, and MNK family, as well as MK2/3/5 [43]. These previous studies and the results of the present study indirectly suggest that nuclear maturation of canine oocytes may be promoted by IGF-1 through the activation of the Ras/MAPK pathway rather than the PI3K/Akt pathway; the Ras/MAPK pathway has been shown to be implicated in the maturation of oocytes in various mammalian species [44].

In conclusion, supplementation of 50 $\mu\text{g/ml}$ IGF-1 into IVM medium exerted a positive influence on canine oocytes, with increased maturation to the MII stage. A higher expression level of *IGF-1R* after IVM and no effect of bpV on the promotion of nuclear maturation suggest that IGF-1 may stimulate the Ras/MAPK pathway via increased expression of *IGF-1R* in cumulus cells during IVM, and thus promote the maturation of canine oocytes from the MI to MII stage.

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