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Effects of Transforming Growth Factor β on In-vitro Maturation of Porcine Oocytes

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Transforming Growth Factor β가 돼지 난자의 체외성숙에 미치는 영향

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요 약

돼지 수정란의 체외생산은 난자의 체외성숙과 체외수정에 관한 기술의 부족으로 아직까지 만족스럽지 못한 수준이다. 특히 돼지 수정란의 체외생산에는 복잡한 세포질의 성숙과정과 높은 다정자침입율 및 전핵형성의 억제등의 문제점이 있다. 본 연구에서는 돼지 난자의 체외성숙 체계를 개선하기 위하여 transforming growth factorβ(TGFβ)의 첨가가 난자 및 난구세포에 미치는 효과에 대하여 검토하였다. 체외성숙용 배지에 TGFβ를 1~10ng/㎖의 농도로 첨가하여 미성숙 난자를 배양한 결과 성숙율이 높아졌다. TGFβ의 효과는 난구세포가 제거된 난자의 성숙에도 효과적이었다. TGFβ를 첨가하지 않은 배양액 내에서는 배양 24시간 까지 metaphase-II로 성숙된 난자가 관찰되지 않았으나 TGFβ를 첨가한 배양액 내에서는 관찰되었다. 한편, 난구세포가 부착된 난자의 성숙배양시 TGFβ의 첨가시기에 따른 차이는 없었으나, 난구세포를 제거한 난자의 경우에는 성숙배양 전반기(59%) 또는 후반기(57%) 24시간 동안에만 TGFβ를 첨가하는 것이 48시간 동안 계속하여 첨가(27%)하는 경우 또는 비첨가(38%)에 비하여 유의적으로 높은 성숙율을 나타냈다($p<0.05$). 이와 같은 결과는 난구세포가 돼지 난자의 체외성숙에 필수적이지만 TGFβ는 난구세포가 제거된 난자의 체외성숙에 어느정도 유익한 효과를 발휘하는 것으로 추측된다.

Key words : Porcine oocytes, In-vitro, Maturation, TGFβ, Cumulus cells

INTRODUCTION

The maturation of mammalian oocytes is initiated during fetal life and arrested at the diplotene stage of prophase-I until shortly before ovulation[1]. In this arrested stage, the oocyte is incapable of fertilized. Fertilization can occur only after the resumption of meiosis to metaphase-II and formation of the first polar body. Fully grown mammalian oocytes, surrounded by a compact mass of somatic cumulus cells, are maintained in the mature, germinal vesicle (GV) stage in-vivo until a preovulatory gonadotrophin surge provokes a dramatic physiological response. In the hours following the ovulation stimulus, the oocyte resumes nuclear maturation, manifested initially by germinal vesicle break down (GVBD), while the cumulus oophorus undergoes mucification and becomes embedded in a glycosaminoglycan matrix, a process termed cumulus cells expansion. By the time of ovulation, the cumulus cells have fully expanded and encompassed an oocyte that has progressed meiotically to metaphase-II. It is generally accepted that gonadotrophins, specifically LH, provide the stimulus in-vivo that brings about the resumption of meiosis and expansion of the cumulus oophorus.2,3) However, the possibility exists that other types of molecules participate in the mechanism controlling these physiological changes during the preovulatory period.

Recent studies using growth factors have shown that meiotic resumption in cumulus-oocyte complexes or follicle-enclosed oocytes in several species can be induced by *epidermal growth factor*[4,5,6] *transforming growth factor* α (TGFα)[7,8] and *transforming growth factor* β (TGFβ)[9]. TGFβ is a multifunctional cytokine that has multiple forms[10]. It belongs to a large gene family with roles in cell growth, differentiation, and migration, as well as in the formation of extracellular matrix and regulation of the expression of cell surface molecules. There are several species of TGFβ coded for by different genes. In 1985, the cDNA for human TGFβ, now called TGFβ-1, was cloned by Dernck et al., and in 1987 Seyedin et al. isolated a polypeptide factor from bovine bone that induced type II collagen synthesis, exhibited 71% amino acid sequence identity to the mature active form and functional similarity to TGFβ-1, and was later termed TGFβ-2. A third TGFβ, designated as TGFβ-3, showed 76% and 80% identity in the mature polypeptide region with TGFβ-1 and TGFβ-2, respectively, and demonstrated some functional similarities to the other two TGFβ species. Mammals can often express all three types of TGFβs. TGFβ-1 is synthesized as large secretory precursor polypeptides of 390 aminoid [11]. Virtually all cells have TGFβ-1 receptors which control a variety of functions in cells from essentially every lineage. Nine membrane proteins that bind TGFβ have been identified to date.

The major sites of action of growth factors that regulate the maturation of oocyte are cumulus cells[12]. It was reported that somatic cells supply nutrients and other substances to the oocytes and communicate with each other and the oocyte via gap junction[13]. Communications through these junctions are important for cellular interaction, especially those regulated by endocrine and paracrine signaling[14]. Therefore, the objective of the present studies were to examine the effects of TGFβ on in-vitro maturation of porcine oocytes and cumulus cells in our culture system.

MATERIALS AND METHODS

Oocyte preparation

Porcine ovaries were collected from a local

slaughter-house in Chunchon city and kept in saline (NaCl, 0.9% W/V ; Penicillin 100.000 IU/ℓ ; Streptomycin 100mg/ℓ and Amphotericine B 250μg/ℓ ; Sigma Chemical, St. Louis, MO, USA) at 30 to 32℃. Cumulus-oocytes complexes were aspirated from 2 to 6 mm follicles using 10 mℓ syringe with 18-G needle. The oocytes collected were washed three times in Hepes-buffered Tyrode's medium(TLH) and once in maturation medium. The oocytes with a compact and complete cumulus cells were introduced into droplets of maturation medium (10 oocytes/50μℓ droplet) and covered with mineral oil. The oocytes were cultured under the atmosphere of 5% CO_2 in air at 39℃ for 42~44 hrs. The maturation medium was consisted of TCM-199 with Earle's salts (Gibco Lab., NY, USA) supplemented with 3.05mM glucose, 0.32mM Ca-lactate, 2.5 mM Na-pyruvate (Sigma), 50μℓ/mℓ gentamycin (Sigma), 1μg/mℓ FSH (Sigma), 5μg/mℓ LH (Sigma), 1μg/mℓ estradiol 17β (Sigma) and 10% (V/V) porcine follicular fluid (PFF)

Experimental design

In the first experiments, the effects on different concentrations of TGF β-1 (0, 1, 5 and 10ng/mℓ) during in-vitro maturation were determined using the oocyte culture system described above.

In the second experiments, porcine oocytes with or without cumulus cells were cultured in presence or absence of TGF β-1 (1ng/mℓ). At 24 and 48 hrs after culture, the oocytes were examined for maturation status.

In the third series of experiments, oocytes with or without cumulus cells were cultured in medium with or without TGF β-1 (1ng/mℓ). TGF β-1 were added for various periods (0~24, 24~48 or 0~48 hrs after culture) during in-vitro maturation.

Evaluation of oocyte maturation

At the end of the experiments, the oocytes were mounted, fixed (acetic acid 1 : ethanol 3) for 2~3 days and stained with 1% acetic-orcein in 40% acetic acid solution. The oocytes were examined under a phase-contrast microscope at ×200 and ×400 magnification.

The maturation stages of the oocytes were classified as germinal vesicle (GV), prophase-I (P-I), metaphase-I (M-I), anaphase-I (A-I), telophase-I (T-I) and metaphase-II (M-II).

Statistical analyses

Chi-squaer (χ^2) analysis with the Yates correction was used to compare the rates of individual maturation status.

RESULTS

In the first experiments, when the cumulus-oocytes complexes were cultured with TGFβ at different concentrations, the proportions of oocytes matured to metaphase-II were 53 (42/79), 69 (61/88), 64 (57/89) and 52% (31/60) at 0, 1, 5 and 10ng/mℓ of TGFβ, respectively (Table 1).

Table 1. Effect of TGFβ concentration on in-vitro maturation of porcine oocytes

Concentrations of TGF β (*ng/ml*)	No of oocytes examined	No of oocytes matured*		
		GV	P-I~T-I	M-II (%)
0	79	0	37	42(53)
1	88	0	27	61(69)
5	89	0	32	57(64)
10	60	0	29	31(52)

* GV : germinal vesicle, P-I : prophase-I, T-I : telophase-I, M-II : metaphase-II

To examine the effects of TGFβ-1 and cumulus cells on the in-vitro maturation, oocytes with or without cumulus cells were cultured in medium with various addition times of TGFβ. At the 24 hrs after culture, the maturation rates were slightly higher in oocytes cultured with (5 and 4%) than without (0 and 0%) TGFβ-1 regardless of presence of cumulus cells (Table 2). However, the proportions of oocytes matured to metaphase-II at 48 hrs after culture were significantly ($p\%0.05$) higher in medium with (70 and 52% for with or without TGFβ-1) than without (35 and 26 % for with or without TGFβ-1) cumulus cells(Table 3).

In the third experiments (Table 4), oocytes were cultured in medium with or without TGFβ (1ng/mℓ) for various durations of culture. In oocytes with cumulus cells, there were not significant differences in the maturation rates (60, 65, 71 and 54%) in oocytes exposed with various durations during the in-vitro maturation. However, the proportions of the oocytes matured to metaphase-II were significantly ($p<0.05$) higher in medium with TGFβ for 0~24 (59%) hrs or 24~48 (57%) hrs than in medium with (27%) or without (38 %) TGFβ for 48 hrs of culture.

Table 2. Effect of TGFβ on in-vitro maturation of oocytes with or without cumulus cells at 24 hrs after culture

Presence of cumulus cells	Addition of TGFβ	No of oocytes examined	No (%) of oocytes matured*		
			GV	P-I~T-I	M-II
+	+	96	8(8)	83(86)	5(5)
	−	104	13(13)	91(88)	0(0)
−	+	74	11(15)	60(81)	3(4)
	−	97	17(18)	80(82)	0(0)

* GV : germinal vesicle, P-I : prophase-I, T-I : telophase-I, M-II : metaphase-II

Table 3. Effect of TGFβ on in-vitro maturation of oocytes with or without cumulus cells at 48 hrs after culture

Presence of cumulus cells	Addition of TGFβ	No of oocytes examined	No (%) of oocytes matured*		
			GV	P-I~T-I	M-II
+	+	96	1(1)	28(29)	67(70)[a]
	−	106	11(10)	40(38)	55(52)[ab]
−	+	69	6(9)	39(57)	24(35)[b]
	−	62	6(10)	40(65)	16(26)[b]

* GV : germinal vesicle, P-I : prophase-I, T-I : telophase-I, M-II : metaphase-II a,b : $P<0.01$

Table 4. Effect of exposure time of TGFβ during the in-vitro maturation in oocytes with or without cumulus cells

Presence of	Duration of the presence TGFβ(hrs of culture)		No of oocytes	No of oocytes matured*		
cumulus cells	0~24	24~48	examined	GV	P-I~T-I	M-II(%)
+	+	+	58	5	18	35(60)[a]
	+	−	60	3	18	39(65)[a]
	−	+	63	3	15	45(71)[a]
	−	−	63	3	26	34(54)[a]
−	+	+	67	6	43	18(27)[b]
	+	−	63	1	25	37(59)[a]
	−	+	69	5	35	39(57)[a]
	−	−	79	6	43	30(38)[b]

* GV : germinal vesicle, P-I : prophase-I, T-I : telophase-I, M-II : metaphase-II a, b, $p<0.05$

DISCUSSION

In preparation of the oocytes for fertilization, not only meiotic maturation must occur, but the cytoplasm of the oocyte must undergo critical changes in order to achieve competency to support spermatozoon chromatin decondensation and subsequent male pronucleus formation. Although nuclear maturation of oocytes can be achieved spontaneously in-vitro, these in-vitro matured oocytes may lack the ability to decondense spermatozoon chromatin and subsequently to form male pronuclei. Cumulus cells play a very important role in the cytoplasmic maturation[15]. Yoshida et al.[16] and Zheng and Sirard[17] showed enhancement of cytoplasmic maturation when cumulus-enclosed oocytes were cultured in medium supplemented with follicular fluid or follicular shells. These results indicate that follicular cells secrete factors regulating cytoplasmic maturation of oocyte via paracrine and autocrine mechanism.

Growth factors such as transforming growth factors (TGFβ[9], TGFβ[7]) have been demonstrated to stimulate or enhance nuclear maturation of oocytes. Current research focuses on the elucidating the TGFβ-1 signal transduction pathway. Evidence suggests that the Type I and Type II receptors form a heteromeric complex upon binding TGFβ-1. TGFβ-1 binding to the Type I /Type II receptor complex activates the serine/threonine protein kinase domain of the Type II subunit. This kinase activity stimulates phospholipase C (PLC) to breakdown membrane inositol phospholipids to form inositol triphosphate (IP3). This ligand : receptor complex is necessary for transducing the signals which result in growth inhibition in some cell types and the activation of "early gene" responces and proliferation in other cells. IP3 stimulates the release of calcium from intracellular stores within the endoplasmic reticulum.18) Calcium does have considerable effects on mammalian oocytes maintained in meiotic arrest. High levels of extracellular calcium can override meiotic arrest maintained by dibutyryl cyclic AMP in isolated cumulus-free mouse oocytes [19] and in both isolated cumulus-free and cumulus-enclosed hamster oocytes[20]. Whereas meiotic progression appears to be dependent

upon exogenous calcium, more recent studies have focussed on intracellular calcium mobilisation and meiotic resumption. Indirect evidence for this relationship has been shown in studies using neomycin, a putative inhibitor of phosphoinositide turnover, which can block GVBD in both cow[21] and pig[22]. This suggests that IP3 may be responsible for triggering meiotic resumption.

The present study were examined the effect of TGFβ in the stimulation of both oocyte maturation and cumulus cells. However, it was not different at various concentrations of TGFβ during in-vitro maturation (Table 1). In some study, TGF was previously shown to stimulate oocyte maturation in rats and mice[7,8]. TGF is structurally and functionally related to EGF[23], a factor show to stimulate porcine oocyte maturation in-vitro[6,12,24,25].

TGFβ can stimulate or inhibit aspects of cellular growth and differentiation depending on species, culture conditions, and the steroid being evaluated.26) It is believed that the ability of TGFβ to inhibit cell growth allows for a more differentiated state of the cell[27]. Failure of TGFβ to alter morphological and steroidogenic responses, as well as cell cycle distribution of bovine granulosa cell function in-vitro, may reflect the absence of TGFβ receptor on bovine granulosa cells after 4 days in these culture conditions or the insensitivity of these granulosa cells to TGFβ. Difference in amino acid sequence between porcine and bovine TGFβ can not account for the lack of response, as these sequences are identical in human, bovine and porcine TGFβ[28].

In this study, TGFβ did not significantly affect maturation of porcine oocytes with or without cumulus cells at 24 hrs after culture (Table 2). However, it has showed that TGFβ is a potent stimulator of meiotic resumption in both cumulus-intact and cumulus-free oocytes at 24 hrs after culture. In contrast, M llerian inhibiting substance, which has close homology to TGFβ[29], inhibited spontaneous rat oocyte maturation[30]. Although TGFβ alone had no effect on spontaneous rat oocyte maturation, it suppressed LH-induced rat oocyte maturation in-vitro[31].

Cumulus-oocytes complexes are comprised of cumulus cells and oocytes that are morphologically and functionally associated with each other through heterologous gap junctions[32]. It has been reported that communication via gap junctions between cumulus cells and oocytes provide avenues for the bidirectional transfer of information[33]. Two theories concerning the relationship of gap junctions to meiotic maturation have been proposed. The first theory is that gap junctions may mediate the transfer of inhibitory substances from cumulus cells to the oocyte[34]. The second theory suggests that a positive stimulus is generated by cumulus cells in response to hormones, and this stimulus is then transferred into oocytes via gap junctions to stimulate maturation [35]. Since TGFβ was unable to enhance spontaneous maturation of denuded oocytes alone or in coculture with cumulus cells, this data seem to support the latter hypothesis.

The present study confirmed that TGFβ can stimulate nuclear maturation of porcine oocytes in-vitro in an apparently exposure time-dependent manner in oocytes without cumulus cells (Table 4). These results suggests that because of the interaction between TGFβ and cumulus cells, TGFβ was required for 24 hrs only during in-vitro maturation in porcine oocytes. Immunohistochemical studies have confirmed that TGFβ and TGFβ are present in the thecal/interstitial cells of rat and bovine ovaries[36,37] and granulosa cells of rat preovulatory follicles[38].

In summary, the present study shows that

TGFβ can stimulate nuclear maturation in porcine oocytes. These results indicated that cumulus cells is essential for in-vitro maturation in porcine oocytes but TGF β can promote oocytes maturation in cumulus-free oocytes.

SUMMARY

This study was undertaken to evaluate the interaction between cumulus cells and TGFβ on in-vitro maturation in porcine oocytes. No differences were found in maturation rates when follicular oocytes were cultured in medium with various concentrations of TGFβ. At 24 hrs after maturation, the oocytes matured to metaphase-II were found in medium with TGFβ regardless of cumulus cells. On the other hand, the maturation rates were significantly ($p<0.01$) higher in cumulus-enclosed oocytes (70 and 52%) than that of cumulus-denuded oocytes (35 and 26%) in medium with or without TGFβ at 48 hrs after culture. In another experiment, the same maturation rates (54~71%) were observed when cumulus-enclosed oocytes were cultured with various addition time of TGFβ. However, the maturation rates in cumulus-denuded oocytes were significantly ($p<0.05$) higher in medium added at 0~24 hrs (59%) or 24~48 hrs (57%) of culture than in medium with (27%) and without (38%) TGFβ for 48 hrs. These results indicated that cumulus cells is essential for in-vitro maturation in porcine oocytes but TGFβ can promote oocytes maturation in cumulus-free oocytes.

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