Localization of Angiotensin II in Korean Bovine Follicles and Its Effects on IVM/IVF of Oocytes

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한우 난소 내 Angiotensin II의 분포와 이의 첨가가 체외성숙 및 수정에 미치는 영향에 관한 연구

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ABSTRACT

1. The concentrations of Ang. II were 7.20.91 × 10^3, 3.80.34 × 10^3, 3.50.30 × 10^3, 2.80.22 × 10^3 pg/ml in bovine follicular fluids from 1~3 mm, 3~5 mm, 5~7 mm and 8~10 mm follicles, respectively. The concentrations of Ang. II decreased in follicular fluids from large follicles.

2. When oocytes were cultured in media containing various concentrations of Ang. II, a higher proportion of oocytes developed to MII stage in medium with 100 ng/ml (79.5%) Ang II compare to that without Ang. II (58.8%). When oocytes from different sizes of follicles were separately cultured in media containing 100 ng/ml Ang. II, maturation rates were higher in oocytes from small and medium follicles those from controls.

3. GSH content in oocytes cultured for 24 hrs in TCM-199 medium containing 10 and 100 ng/ml of Ang. II was also higher than that of oocytes cultured in medium containing 0 or 10 ng/ml Ang. II. When oocytes were cultured in media containing 0, 10, 100, 1,000 ng/ml of Ang. II, the concentrations of GSH were 5.1M, 5.5M, 7.2M, 8.7M, respectively.

4. When oocytes were cultured in media containing various concentrations of 10, 100, 1,000 ng/ml Ang. II, *in vitro* maturation and developmental rates were 84.0%, 90.0%, 78.0% and 28.0%, 36.0%, 20.0%, respectively. When oocytes were cultured with an addition of Ang. II in media, *in vitro* maturation rates higher than that of their controls (76.0%).

(Key words : Korean bovine follicle, Angiotensin concentration, Developmental rate)

Ⅰ. INTRODUCTION

The renin-angiotensin system (RAS), including Angiotensin II (Ang. II) and its different receptors, is present in mammalian ovaries. However, its physiological role is largely unclear. Angiotensin II (Ang. II) found in pig ovary influences the oocyte maturation *in vitro*. While, the distribution of Ang. II in Korean bovine follicles has not been reported.

It is well known that RAS has many roles in mammals, including regulation of blood pressure, metabolization of water and salts, maintaining body fluid balance and stimulating proliferation of blood vessels in muscular tissue. In addition, there is a good evidence that RAS plays important roles in synthesis and secretion of prostaglandins and estrogen. Yoshimura. et al. found that Ang. II administration at 2 h intervals induced oocyte maturation and ovulation in rabbit

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ovaries (Yoshimura, et al., 1993). Ang. II secretion in perfused rabbit ovaries is enhanced during the ovulatory process by exposure to human chorionic gonadotropin (hCG), and the addition of saralasin, an inhibitor of Ang. II, inhibits hCG-induced ovulation *in vitro* in a dose-dependent manner. Ang. II is closely related to follicular atresia, oocyte maturation and ovulation *in vivo* in mice. Even the factors affecting bovine *in vitro* maturation including cumulus cells, oviductal epithelial cells, uterine epithelial cells, hormones, EGF (epidermal growth factor), glucose, SOD (superoxide dimutase), catalase, beta-mercaptoathanol, hyaluronic acid may also be related to RAS.

In the present study, the localization of Ang. II in Korean bovine follicles was examined and its effects on IVM/IVF of oocytes were determined.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* maturation and fertilization

Ovaries from Korean cows were collected immediately after slaughter and ovaries were kept at 35℃ physiological saline containing 100 IU/ml penicillin G and 100 ㎍/ml streptomycin sulfate. Upon arrival at the laboratory, ovaries were washed three times with physiological saline.

The follicle solution was collected by syringe and then the cumulus oocyte complexes (COCs) were collected in petri dishes under a stereomicroscope. The sperm and oocytes were co-incubated in TCM-199 medium supplemented with 10% (v/v) FCS (Sigma, U.S.A.), 1 ㎍/ml FSH (Sigma, U.S.A.), 2 IU/ml hCG (Sigma, U.S.A.), 1 ㎍/ml estradiol (Sigma, U.S.A.), 100 IU/ml penicillin G and 100 ㎍/ml streptomycin sulfate under the conditions of 38.5℃, 5% CO_2, and 95% O_2.

Five oocytes were transferred to 50 ㎕ drops of maturation medium under mineral oil, cultured in CO_2 incubator for 24 h and transferred to each droplet of 50 ㎕ fertilization medium. Frozen sperm were thawed in 1.2 ml of BO-semen solution (5:1). After swim-up treatment in CO_2 incubator, the supernatant was added to fertilization medium and centrifuged at 500 rpm for 10 min. The sperm pellet was diluted with 100 ㎍/ml heparin (Sigma, U.S.A.) solution and incubated for 15 min in CO_2 incubator. 2 ul suspension of capacitation-sperm (~1.5 × 10^6) was added in the fertilization medium containing matured oocytes. Fertilized oocytes were cultured in TCM-199 medium supplemented with 10% (v/v) FCS and the developmental rate of embryos was investigated.

2. Ang. II assay in follicular fluid

Follicular fluid was aspirated from different sizes of follicles and divided into four groups according to the follicle diameter classifications. Samples were stored at -20℃ until assay. Ang. II assay was performed with Ang. II radioimmuoassay kit (Amersham, U.S.A.) according to the method described previously (Yoshimura et al., 1996).

3. GSH assay

Cumulus cells were removed from oocytes by treatment with 0.1% hyaluronidase. Cumulus-free oocytes were washed three times with stock solution (0.2 mM sodium phosphate supplemented with 10 mM sodium EDTA). Groups of 30 oocytes were transferred into a tube containing 5 ml stock solution and mixed well. 1.25 mM phosphoric acid was added to the tube. Tubes containing samples were kept frozen (-20 ℃) until assay. The concentration of glutathione in oocytes was determined by the 5,5'-dithiobis-2-nitrobenzoic acid and glutathione disulfide reductase recycling assay method described previously (Anderson, 1985).

4. Immunohistochemical examination

Oocytes with cumulus cell were treated with 0.2% sodium hyaluronidase (Sigma, USA) for 1~5 min and cumulus-free oocytes were fixed with acetic acid:ethanol (1:3) for 24h. The fixed oocytes were stained with 1% aceto-orcein or 10 ㎍/ml bisbenzimide (Hoechst 33342, Sigma, U.S.A.). Cell and nuclear maturation was judged depending on cell division and formation of male pronucleus.

5. Statistical analysis

Data were analyzed by Student's *t*-test using GLM procedures (SAS package. 1996) and Duncan's multiple range test.

Ⅲ. Results and Discussions

1. Ang. II concentrations in follicular fluid

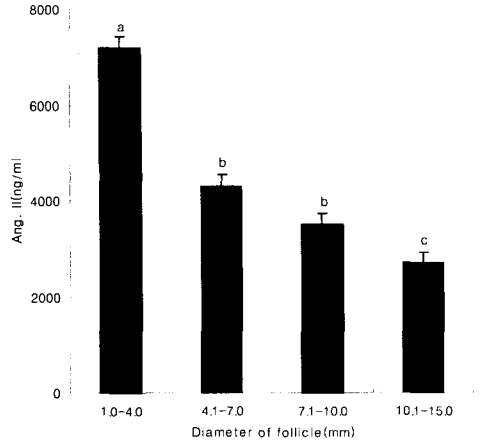

Fig. 1. Mean concentration of Ang. II in bovine follicular fluid from different size of follicles. Columns without a common superscript (a-c) are different ($p<0.05$).

Mean concentrations of Ang. II in follicular fluid from different sizes of follicles were described in Fig. 1.

The concentrations of Ang. II were 7.2±0.91 × 10^3, 3.8±0.34 .× 10^3, 3.5±0.30 × 10^3, 2.8±0.22 × 10^3 ng/ml in bovine follicular fluids from 1~3 mm, 3~5 mm, 5~7 mm and 8~10 mm follicles, respectively. The concentrations of Ang. II decreased gradually in follicular fluids from small to large follicles. These concentrations were similar to or slightly higher than the Ang. II concentration in porcine follicular fluid representing 6.951 1.30 × 10^3 ng/ml ~ 2.545 0.41 × 10^3 ng/ml. Palumbo et al. (1989) found that Ang. II existed in theca and stroma cells of human ovaries. In granular cells, before ovulation, renin-angiotensin system was stimulated by gonadotropin surge, and then Rennin and Ang. II were produced.

2. Ang. II influence on MII development

Effect of addition of Ang. II to media on *in vitro* nuclear maturation of bovine oocytes was described in Fig. 2.

When oocytes were cultured in media containing 0, 10, 100, 1000 ng/ml Ang. II for 24 h. the rates of oocytes developed to MII were 58.8%, 79.5%, 87.6%, 62.5%, respectively. When comparing maturation rate of oocytes from different sizes of follicles cultured in media with or without Ang. II, the maturation rate of oocytes collected from small and medium size follicles was significantly higher in Ang. II-containing medium than in medium without Ang II, However, the results obtained from large follicles were of an opposite way. Our results were consistent with previous results obtained from porcine oocytes (Li et al., 2004). In porcine oocytes, maturation rates were between 60.0% and 80.2% and the best results were obtained from the medium containing 100 ng/ml Ang. II. Overall, when cultured in Ang. II-containing medium, the significantly higher maturation rate was achieved.

3. GGH concentrations followed by Ang. II addition

When oocytes were cultured in media containing 0, 10, 100, 1000 ng/ml Ang. II, the concentrations of GSH were described in Fig. 2.

When the oocytes were cultured in TCM-199 medium

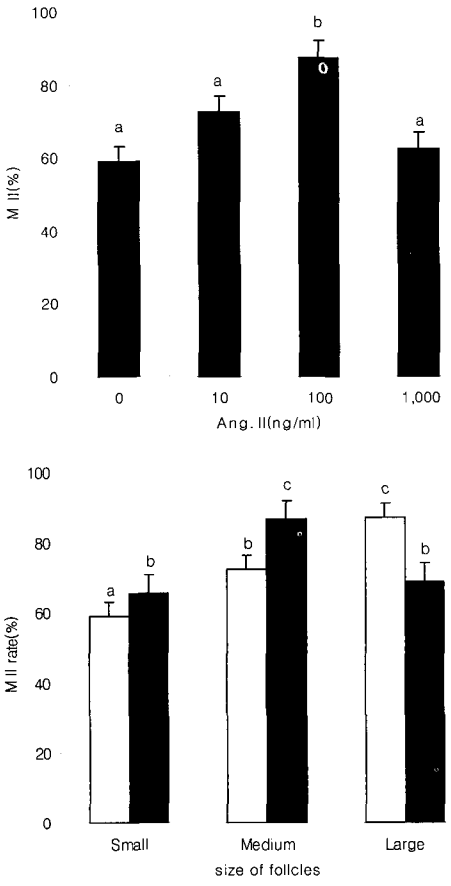

Fig. 2. Effect of addition of Ang. II to media on nuclear maturation of bovine oocytes *in vitro* (24 h). Effect of addition(□) and non-addition(■) of Ang. II to media on nuclear maturation of oocytes. Columns without a common superscript (a-c) are different ($p<0.05$).

Table 1. Rates of fertilization and male pronucleus formation of bovine oocytes

Addition of Ang.II (ng/ml)	No. of oocytes examined	No. of oocytes matured(%)	No. of oocytes cleaveged(%)
0	50	38(76.0)	8(16.0)
10	50	42(84.0)	14(28.0)
100	50	45(90.0)	18(36.0)
1000	50	39(78.0)	10(20.0)

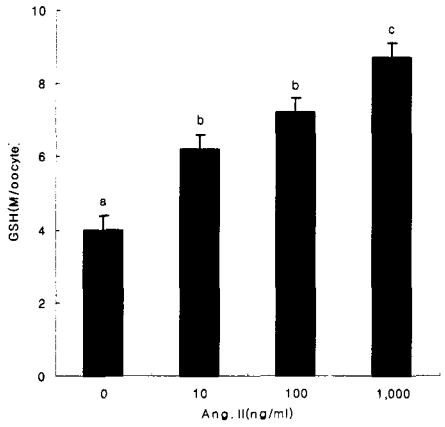

Fig. 3. Effect of addition of Ang. II to media on GSH content in bovine oocytes. Columns without a common superscript (a, b) are different ($p<0.05$).

containing 0, 10, 100 and 1000 ng/ml Ang. II, the concentrations of GSH in oocytes were 5.1 M, 5.5 M, 7.2 M and 8.7 M, respectively. The GSH concentration in oocytes increased along with Ang. II concentration in the medium. Our results were consistent with that obtained from porcine oocytes (Li et al., 2004).

4. *In vitro* fertilization rates from different concentrations of Ang. II

When oocytes were cultured in media containing various concentrations of Ang. II, the *in vitro* fertilization rates were described in Table 1.

When oocytes were cultured in media containing various concentrations of 0, 10, 100, 1,000 ng/ml Ang. II, the *in vitro* maturation and developmental rates were 76% and 16%, 84.0% and 28.0%, 90.0% and 36.0%, 78.0% and 20.0%, respectively. The *in vitro* maturation and developmental rate were significantly higher in Ang. II containing culture groups comparing with no Ang. II culture group.

In porcine oocytes (Li et al., 2004), when cultured in media containing 100 ng/ml Ang. II for 44 h, higher maturation rate (87.0%) was obtained comparing with those (61%) cultured in no Ang. II medium. Even there are differences between bovine and porcine oocytes in their maturation rate, the best results were obtained from the culture containing Ang. II.

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