pISSN 1738-2696 · eISSN 2212-5469
https://doi.org/10.1007/s13770-016-0009-y
ORIGINAL ARTICLE

TERM

The Expression Profile of Angiotensin System on Thawed Murine Ovaries

Yoon Young Kim[1†], Yong Jin Kim[2†], Kyung Mee Cho[1], Soo Hyun Kim[1], Kyung Eui Park[1], Byeong-Cheol Kang[3], Kyung Cheon Jung[4], Moon Suk Kim[5], Seung-Yup Ku[1*]

[1]Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul, Korea
[2]Department of Obstetrics and Gynecology, Korea University Guro Hospital, Seoul, Korea
[3]Biomedical Research Institute, Seoul National University Hospital, Seoul, Korea
[4]Department of Pathology, Seoul National University College of Medicine, Seoul, Korea
[5]Department of Molecular Science, Ajou University, Suwon, Korea

Freezing and thawing is one of the most widely used tissue engineering techniques for the preservation of ovaries. Many cells and tissues demonstrate changes in functional gene expression after thawing. Several studies have reported the important roles of angiotensin (AT) system during the ovarian follicular growth. AT system consists of ATII, and ATII receptors type I (ATII-RI) and type II (ATII-RII). However, little is known whether frozen-thawed ovaries show any alteration of AT system member gene expression when treated with survival-enhancing factors. We aimed to investigate whether mass freezing and thawing with or without the use of Rho-associated kinase (ROCK) inhibitors up- or down-regulate the expression of ATII, ATII-RI, and ATII-RII genes on frozen-thawed ovarian tissues. Significant changes in the expression of ATII, ATII-RI, and ATII-RII genes were observed on thawed ovaries when compared to fresh control. The treatment with ROCK inhibitors did not significantly alter their expression. In conclusion, freezing and thawing of ovarian tissue may affect the mRNA expression levels of intra-ovarian AT system genes, and modulation of ROCK inhibitor activity may not regulate AT system on the frozen-thawed ovarian tissue.
Tissue Eng Regen Med 2016;13(6):724-731

Key Words: Angiotensin; Freezing; Ovary; Rho-associated kinase; Thawing

INTRODUCTION

In vivo and *in vitro* follicular development demands the roles of many intra-follicular regulating factors as well as endocrine female sex steroid hormones. Angiotensin (AT) system consists of ATII, and ATII receptors type I (ATII-RI) and type II (ATII-RII). The presence of prorenin, renin, angiotensinogen, ATII and its receptors has been identified in the ovary. ATII acts as a paracrine and autocrine regulator during the ovarian follicular development. It plays an important role in follicular steroidogenesis [1], oocyte maturation [2], and ovulation [3] through its receptors on granulosa cells. Its action has been suggested in both dominant [4] and atretic follicles [5]. Disorders of the intra-ovarian renin-AT system are involved in the ovarian tumors, polycystic ovary syndrome, and ovarian hyperstimulation syndrome [6].

The long-term preservation of reproductive tissue mostly requires the bioengineering procedures of freezing and thawing [7]. For the therapeutic applications, the thawed ovaries may be transplanted to the recipient, however the *in vitro* follicular growth seems to be a feasible alternative to enhance the fecundability of subfertile couples [8]. Freezing and thawing allows the ovarian tissue to go through various structural [9] and functional [10] changes. The previous studies revealed that frozen and fresh ovarian tissue require different culture media to promote *in vitro* development of bovine pre-antral follicles [11], and a few reports revealed increasing survival of cryopreserved cell with using Rho-associated kinase (ROCK) inhibitors [12-15]. A few studies suggested that ROCK activity augment AT system in renal [16,17] and vascular system [18].

Considering the importance of AT system in the ovary, it is important to know any possible alterations of their expression that occur during or after the freezing and thawing process when *in vitro* follicular growth is attempted using thawed ovaries. However, to date, little is known about the changes in the expression of ATII and its receptors on the frozen-thawed ova-

Received: January 5, 2016
Revised: March 2, 2016
Accepted: March 11, 2016
***Corresponding author:** Seung-Yup Ku, Department of Obstetrics and Gynecology, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea.
Tel: 82-2-2072-1971, Fax: 82-2-762-3599, E-mail: jyhsyk@snu.ac.kr
†These authors contributed equally to this work.

 © The Korean Tissue Engineering and Regenerative Medicine Society

ries. In this investigation, the expression levels of ATII, ATII-RI and ATII-RII on the thawed ovaries were evaluated and analyzed according to the freezing-thawing protocols with or without the addition of ROCK inhibitors (Fig. 1).

MATERIALS AND METHODS

Animal care and collection of ovaries

We used female C57BL6 mice for this study which was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital. Mice were kept under SPF settings on 12-hour interval light:dark cycles with an unlimited access to water and food until sacrificed using cervical dislocation [19]. The ovaries were collected from stunned animals after laparotomy and kept in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

H&E staining of ovaries before and after freezing

Isolated or thawed ovaries were fixed with 10% formalin for overnight at 4°C and processed for embedding. Briefly, samples were dehydrated and paraffin was infiltrated. Embedded paraffin block was sliced with microtome and prepared in slide for staining. Slide was stained using Harris hematoxyline and eosin according to manufacturer's protocol and mounted with Canada balsam solution. The images were captured using inverted microscope (Leica, Wetzlar, Germany).

Freezing-thawing of ovaries and ROCK inhibitor treatment

Isolated whole ovaries were washed with PBS and sequentially incubated with vitrification solution I (VS I, DMSO 7.5%, Ethylene glycol 7.5%, culture medium) for 3 minutes and VS II (DMSO 15%, Ethylene glycol 15%, 0.5 M sucrose, culture medium) for 30 seconds. And then, an ovary was put on to a small mesh with VS II solution drop and plunged into liquid nitrogen (LN_2). Frozen ovary in the mesh was individually caped and stored in LN_2 tank.

To thaw, frozen drop containing ovaries was put into thawing solution I (TS I, DMSO 7.5%, Ethylene glycol 7.5%, 1 M sucrose, culture medium) for 5 minutes, and then transferred to TS II (DMSO 15%, Ethylene glycol 15%, 0.5 M sucrose, culture medium) and incubated for 10 minutes. After that, the ovary was transferred to culture medium.

10 μM of ROCK inhibitor (Y-27632, Calbiochem) was treated for 1 hour before and after freezing by adding to incubation medium. Pre-treatment was achieved before ovaries put into VS I and post-treatment was processed in culture medium after incubation with TS II and transfer to fresh culture medium.

Quantitative analysis using FACS (Annexin V analysis)

Isolated ovaries were dissociated into single cells by treatment of Collagenase type IV (5 mg/mL) and DNase I (10 ug/mL) for 45 minutes at 37°C. Dissociated cells were washed and re-suspended in 1x binding buffer. And then, samples were incubated with Annexin V and propidium iodide for 15 minutes

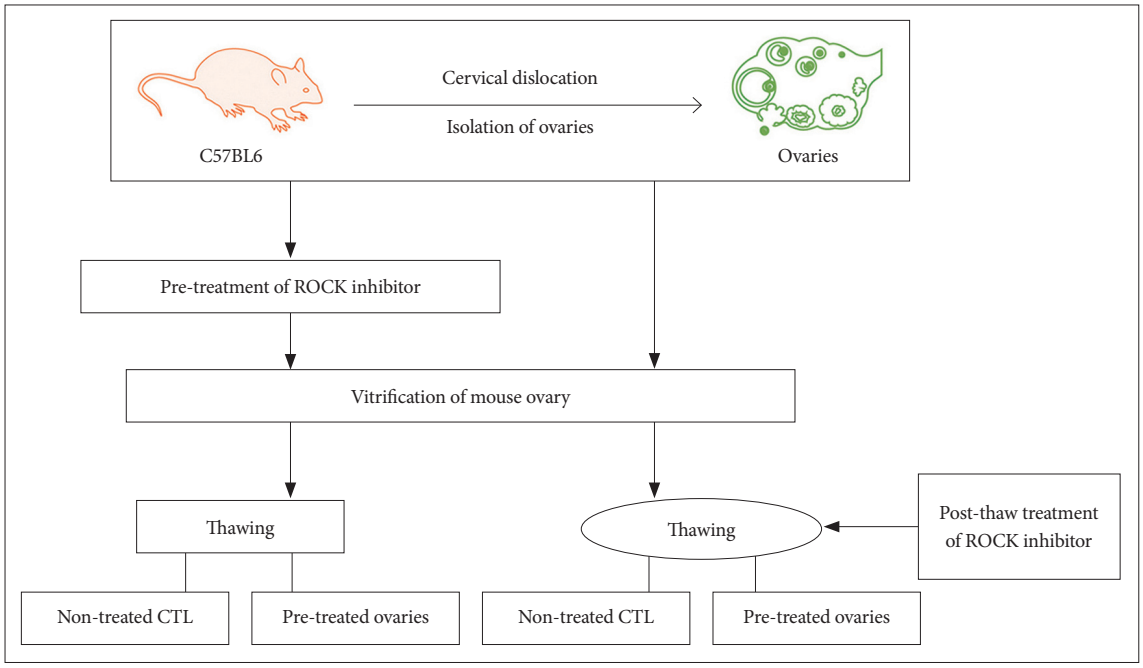

Figure 1. Flow chart of experimental strategy. ROCK: Rho-associated kinase.

at room temperature (RT). Cells were analyzed using FACS Calibur (BD Biosciences, San Jose, CA, USA).

Immunocytochemical staining

To evaluate the expression of ATII-RI genes, fresh and frozen-thawed ovaries were transferred into multi-well dish (Nunc, Denmark). To analyze the samples, media was discarded and the ovaries were washed several times with PBS. And then, samples were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at RT and blocked with 3% bovine serum albumin solution for 12 hours at 4°C. Then, the tissues were incubated with each primary antibody (1:100), rabbit anti-mouse ATII-RI (Novus Biologicals, Littleton, CO, USA) for overnight at 4°C, and washed three times with TBS. Secondary antibodies, goat anti-rabbit 488 IgG (Molecular Probes, Grand Island, NY, USA) was treated for 50 min at 37°C and washed three times with TBS. Finally, 10 μg/mL of DAPI (Sigma-Aldrich) was treated and the images were assessed under fluorescence microscope (EVOS-FL, Life Technologies, Grand Island, NY, USA).

Quantitative reverse transcription and polymerase chain reaction

RNAs were extracted from the frozen and thawed ovarian tissue using Trizol reagent (Invitrogen). One microgram total RNAs and ACCU premix (Bioneer, Daejeon, Korea) were used for the synthesis of cDNAs that were used for polymerase chain reaction (PCR) reactions. Specific primers (Table 1) were added and the reaction was amplified under the following conditions: incubation for 15 minutes at 95°C, denaturation for 20 seconds at 95°C, annealing for 50 seconds at 58°C and 30 seconds for 72°C for extension. All the reactions were executed three times and the expression of cycle threshold value was re-calculated upon the reference of GAPDH expression.

Statistical analysis

All the experiments were conducted in triplicate. Data were expressed as means and standard deviations and compared using the Student's t-test. A significant difference was statistically deemed with $p<0.05$. Statistical Package for the Social Sciences for Windows (version 12.0, SPSS Inc., Chicago, IL, USA) was used for the data analysis.

RESULTS

Morphological change of mouse ovary before and after freezing (H&E staining)

Follicles at different phases were observed in both groups (Fig. 2). Following freezing and thawing, a general inspection of ovarian tissue sections revealed a significant increase in the proportion of damaged follicles compared to fresh tissue. Some detached spaces were observed between oocyte-granulosa cell complex and basal granulosa cell layer in the section of ovary after preservation. Post-thaw ovaries showed a similar number of antral follicles, the majority of which became atretic. The arrangement of granulosa cells was uneven in thickness, and some granulosa cells were detached in ovaries after freezing compared to ovaries before freezing.

Table 1. Primer sequences used for the experiments

Gene	Sequences (5′ → 3′)	Sequences (3′ → 5′)
GAPDH	GAAGGTCGGTGTGAACGAAT	TTTGATGTTAGCGGGGTCTC
ATII	CCGCATTTAACTGCTCACACA	ATCATGTAGTAGAGAACAGGAATTGCTT
ATII-RI	CCATTGTCCACCCGATGAA	TGACTTTGGCCACCAGCAT
ATII-RII	CCTGGCAAGCATCTTATGTAGTTC	CCGGAAATAAAATGTTGGCAAT
BMP15	CAGTAAGGCCTCCCAGACGT	AAGTTGATGGCGGTAAACCA
GDF9	AACCCAGCAGAAGTCACCTC	AGGGGCTGAAGGAGGGAGG
AMH	GCAGTTGCTAGTCCTACATC	TCATCCGCGTGAAACA GCG
AMH-RII	TGTGTTTCTCCCAGGTAATCCG	AATGTGGTCGTGCTGTAGGC
FSH-R	GCTCACCAAGCTTCGAGTCA	GCCTTAAAATAGACTTGTTGC
LH-R	CCAGAGTTGTCAGGGTCGCGC	AGCATCTGGTTCTGGAGTAC
ALK2	TTAAAAGGCGCAACCAAGA	CGTACAACGATCCCATTTCA
ALK3	TTTATGGCACCCAAGGAAAG	TGGTATTCAAGGGCACATCA
ALK6	CTCAGGGAGCGACCTGGGCA	GCGGCCCCAAATGCAGGGAT

ATII: angiotensin II, ATII-RI: angiotensin II receptor type I, ATII-RII: angiotensin II receptor type II, BMP15: bone morphogenetic protein 15, GDF9: growth differentiation factor 9, AMH: anti-Müllerian hormone, AMH-RII: anti-Müllerian hormone receptor type II, FSH-R: follicle-stimulating hormone receptor, LH-R: luteinizing hormone receptor, ALK2, 3, 6: anaplastic lymphoma receptor tyrosine kinase 2, 3, 6

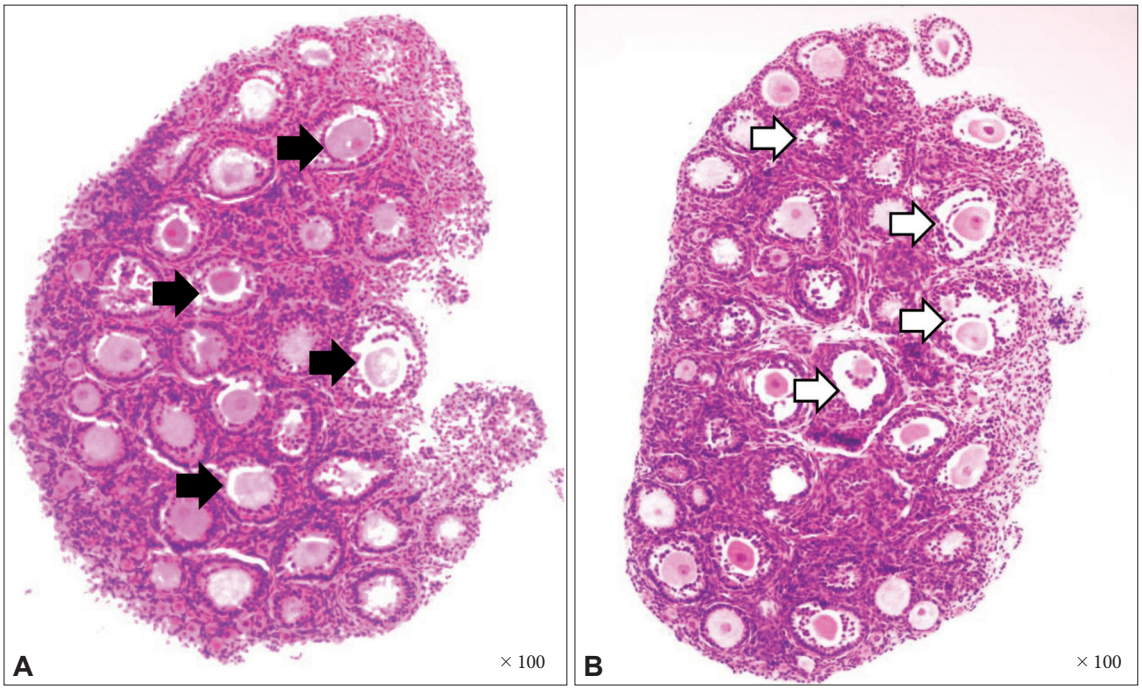

Figure 2. Representative H&E-stained section images showing morphology of (A) fresh and (B) frozen-thawed ovary. Follicles with intact structure (black arrows) were found frequently in the fresh ovary, while those with arrangement of uneven or detached granulosa cell layer were seen in the frozen-thawed ovary (white arrows).

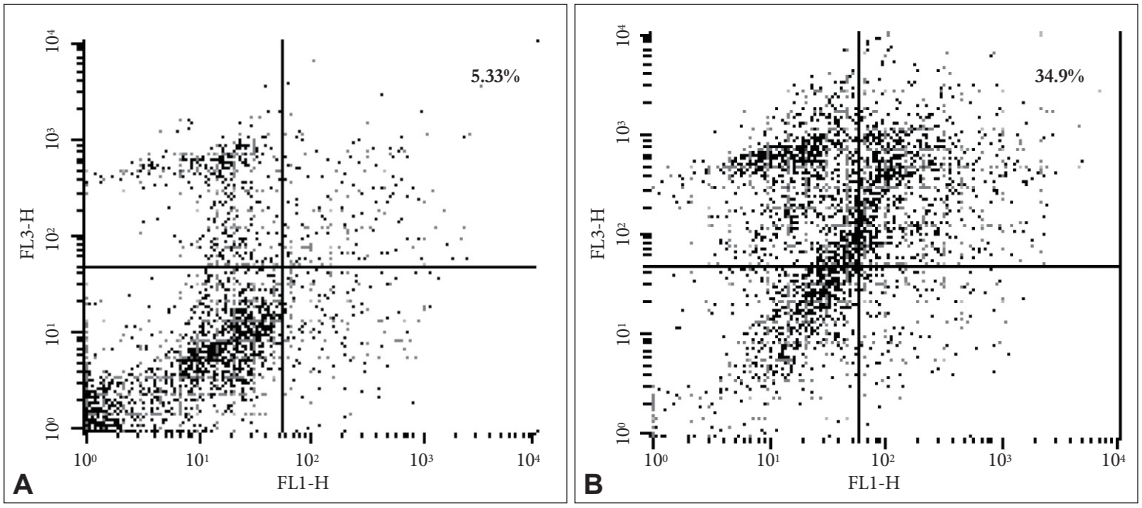

Figure 3. Apoptosis in the mouse ovaries before and after cryopreservation using FACS analysis. (A) Fresh ovaries. (B) Frozen-thawed ovaries.

FACS analysis of mouse ovaries before and after freezing

We performed quantitative analysis for apoptosis using FACS (Annexin V analysis) for the comparison between fresh and cryopreserved ovaries. In both groups, analyzed populations were heterogeneous (Fig. 3). Ovaries after freezing showed more enhanced apoptotic population compared to ovaries before freezing (34.9% vs. 5.33%, $p<0.001$).

Immunocytochemical analysis

To assess the localization of ATII-RI expression on ovary, immunocytochemical staining was performed. ATII-RI expressions were shown in the granulosa cell layer of follicles (Fig. 4). The follicle of fresh ovaries showed a higher expression of ATII-RI compared to follicles of frozen-thawed ovaries.

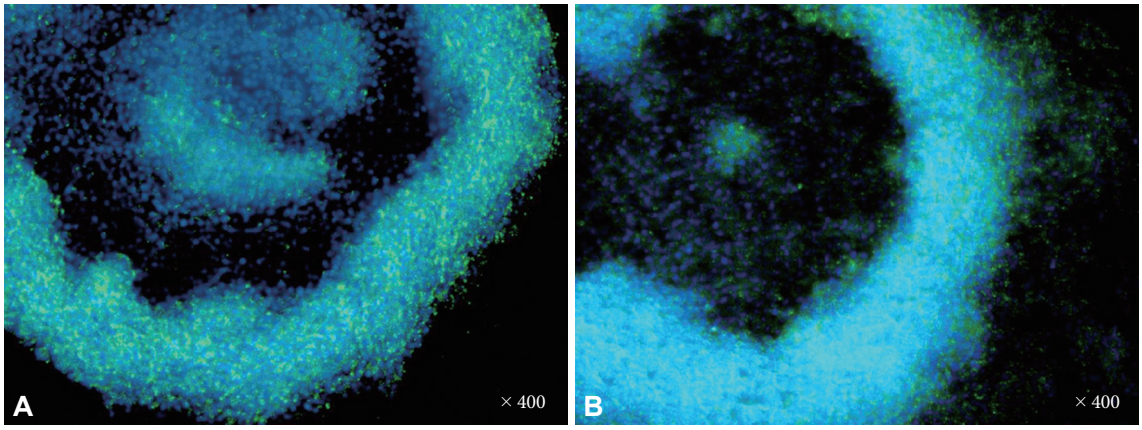

Figure 4. Localization of ATII-RI. Follicles of fresh ovaries (A) shows higher expression of ATII-RI compared to those of frozen-thawed ovaries (B). ATII-RI: angiotensin II receptor type I.

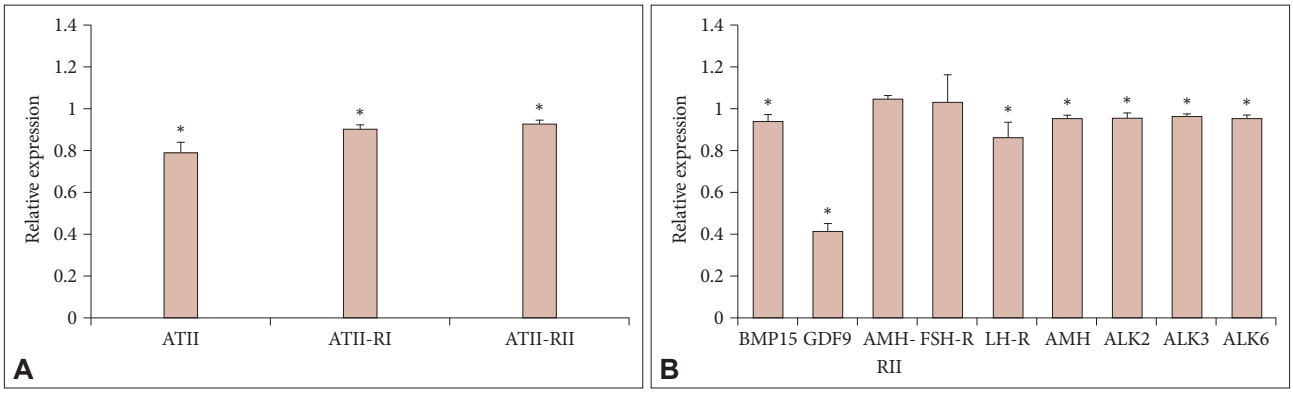

Figure 5. Post-thaw changes in the relative gene expression of (A) angiotensin system member genes and (B) genes related to the follicular development (*p<0.05). ATII: angiotensin II, ATII-RI: angiotensin II receptor type I, ATII-RII: angiotensin II receptor type II, BMP15: bone morphogenetic protein 15, GDF9: growth differentiation factor 9, AMH: anti-Müllerian hormone, AMH-RII: anti-Müllerian hormone receptor type II, FSH-R: follicle-stimulating hormone receptor, LH-R: luteinizing hormone receptor, ALK2, 3, 6: anaplastic lymphoma receptor tyrosine kinase 2, 3, 6.

Post-thaw changes in the relative expression of angiotensin system member genes and of those related to follicular development

We analyzed the mRNA expression of AT system member genes and those genes related to the follicular development in the fresh and cryopreserved ovaries by Quantitative reverse transcription (qRT)-PCR in order to evaluate the effects of freezing process on these genes. The expression of ATII, ATII-RI, and ATII-RII in ovaries was down-regulated after freezing compared to ovaries before freezing (Fig. 5A). In contrast to the genes related to follicular development such as bone morphogenetic protein 15 (BMP15), growth differentiation factor 9 (GDF9), luteinizing hormone receptor (LH-R), anti-Müllerian hormone (AMH), anaplastic lymphoma receptor tyrosine kinase (ALK) 2, ALK3, and ALK6 that showed decreased expression, AMH-RII and follicle-stimulating hormone receptor (FSH-R) revealed similar expression when the post-thaw ovaries were compared to those before freezing (Fig. 5B).

Effects of ROCK inhibitor treatment on post-thaw ovaries

To evaluate the effects of ROCK inhibitor treatment during freezing process on the relative expression of AT system member genes and those genes related to follicular development, mRNA expression from cryopreserved ovaries with or without ROCK inhibitor treatment was analyzed by qRT-PCR. After freezing, the expression of ATII, ATII-RI, and ATII-RII was similar in ROCK inhibitor treatment group compared to non-treatment group (Fig. 6A). While BMP15, GDF9, LH-R, AMH, ALK3, and ALK6 showed increased expression, AMH-RII, FSH-R, and ALK2 revealed similar expression in ROCK inhibitor treatment group compared to non-treatment group (Fig. 6B).

Comparison between non-frozen and frozen-thawed ROCK inhibitor-treated ovaries

We analyzed the relative expression of AT system member genes and those genes related to follicular development between

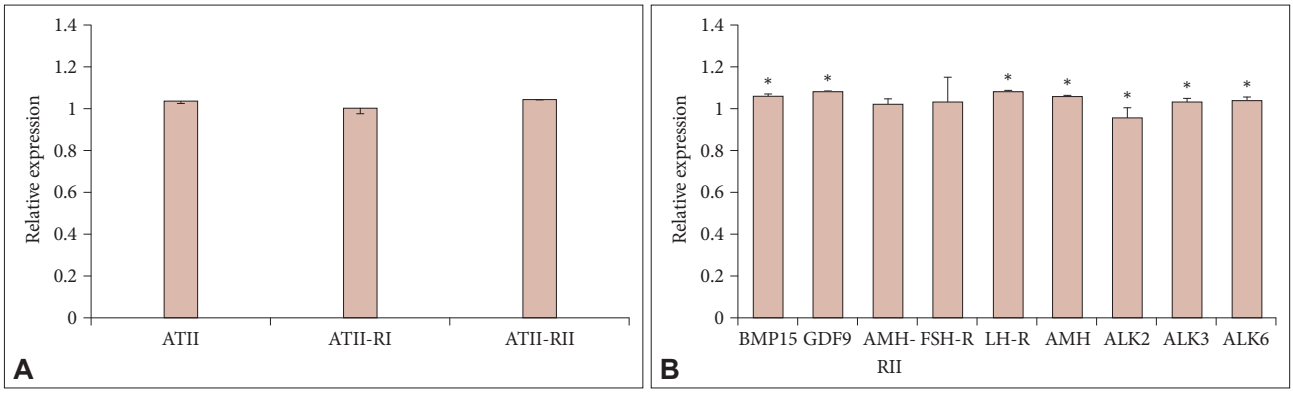

Figure 6. The effects of ROCK inhibitors on the expression of (A) angiotensin system member genes, (B) genes related to the follicular development in the post-ovaries (*p<0.05). ATII: angiotensin II, ATII-RI: angiotensin II receptor type I, ATII-RII: angiotensin II receptor type II, BMP15: bone morphogenetic protein 15, GDF9: growth differentiation factor 9, AMH: anti-Müllerian hormone, AMH-RII: anti-Müllerian hormone receptor type II, FSH-R: follicle-stimulating hormone receptor, LH-R: luteinizing hormone receptor, ALK2, 3, 6: anaplastic lymphoma receptor tyrosine kinase 2, 3, 6.

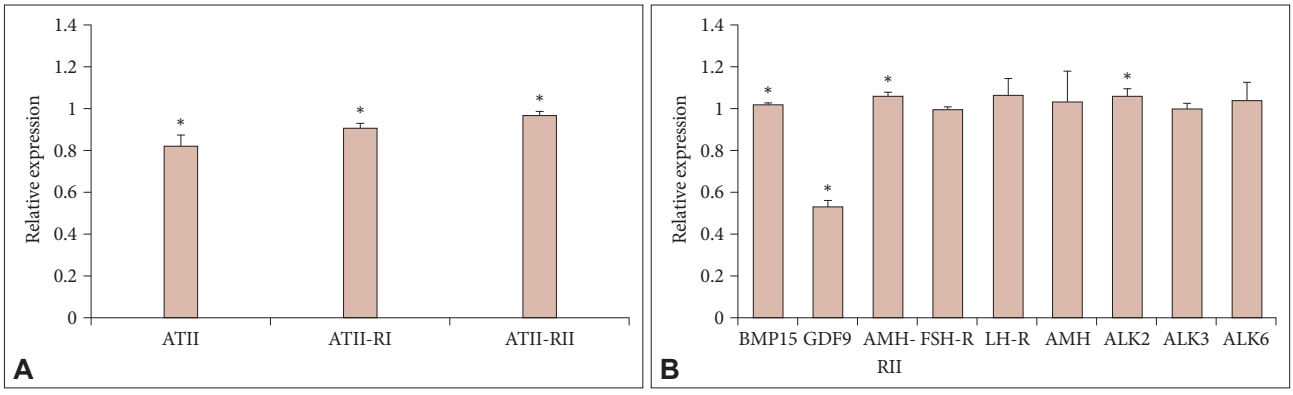

Figure 7. Comparison of relative gene expression between non-frozen and frozen-thawed ROCK inhibitor-treated ovaries (*p<0.05). (A) Angiotensin system member genes, (B) Genes related to the follicular development. ATII: angiotensin II, ATII-RI: angiotensin II receptor type I, ATII-RII: angiotensin II receptor type II, BMP15: bone morphogenetic protein 15, GDF9: growth differentiation factor 9, AMH: anti-Müllerian hormone, AMH-RII: anti-Müllerian hormone receptor type II, FSH-R: follicle-stimulating hormone receptor, LH-R: luteinizing hormone receptor, ALK2, 3, 6: anaplastic lymphoma receptor tyrosine kinase 2, 3, 6.

non-frozen and frozen-thawed ROCK inhibitor-treated ovaries. The expression of ATII, ATII-RI, and ATII-RII was down-regulated in cryopreserved ovaries with ROCK inhibitor treatment compared to fresh non-cryopreserved ovaries (Fig. 7A). The expression of BMP15, AMH-RII, and ALK2 was increased, and GDF9 showed a decreased expression in the cryopreserved ovaries with ROCK inhibitor treatment group compared to fresh non-cryopreserved ovary groups (Fig. 7B). The expression of FSH-R, LH-R, AMH, ALK3, and ALK6 was not different between the two groups.

DISCUSSION

Freezing and thawing is one of the widely used tissue engineering techniques for the preservation of ovaries. Many cells and tissues demonstrate changes in functional gene expression after thawing. We aimed to investigate whether mass freezing with or without the use of ROCK inhibitors up- or down-regulate the expression of ATII, and ATII-RI and -RII genes on thawed ovarian tissues.

From the morphologocal comparison, our results revealed that freezing affects micro-environment of preantral and antral follicles in ovaries. Morphological damage of ovarian tissue from freezing has been reported by many previous studies of murine [20], bovine [11], pig [21], and human [22]. Consistent with the previous reports, the detachment between oocyte-granulosa complex and basal granulosa cell layer was found in our study. This present study showed the trends with similar number of antral follicles with atresia in the ovaries after freezing compared to those before freezing. It is suggested that freezing process could induce "unordered" follicular developmental capacity without numerical changes in the follicle counts. When compared to our morphological findings of apoptosis, the FACS analysis provided more discriminating results suggesting that freezing induced

apoptosis in the more increased cell-population.

In the analysis of gene expression, all AT system member genes and majority of genes that are related to the follicular development were decreased after freezing except AMH-RII and FSH-R. Our immunocytochemical analysis results for ATII-RI were consistent with the mRNA expression. Several studies have reported the important roles of AT system during the ovarian follicular growth [1-5]. Affected AT system could be a pivot cause of decreased developmental capacity of follicles in thawed ovaries.

In our results, ROCK inhibitor treatment showed no additional protective effects against decreasing AT system gene expression between fresh and thawed without ROCK inhibitor treatment groups during the freezing process. Similarly, in comparison between fresh and thawed ovaries with ROCK inhibitor treatment, AT system member gene expression was decreased after freezing, albeit with ROCK inhibitor treatment. However, the genes related to follicular development such as BMP15, AMH-RII, and ALK2 showed an increased expression which is comparative to the data from the comparison between fresh and thawed without ROCK inhibitor treatment ovaries in our study. Some previous studies reported that ROCK inhibitors could modulate BMP and ALK families, which is consistent with our results [23-25]. Recently, a few reports revealed an increasing survival of cryopreserved cells with the use of ROCK inhibitors [12-14]. Although many questions remain, the activity of ROCK inhibitors is involved in various cellular function such as the regulation of phosphorylation of myosin light chain and many other kinases and cytoskeletal binding proteins [26], and plays a central role in the apoptosis, migration, cytokinesis, proliferation [27]. A few studies suggested that the ROCK inhibitor activity augmented the function of AT system in renal [16,17] and vascular system [18]. Our results suggest that the modulation of ROCK inhibitor activity may not regulate AT system during freezing of ovarian tissue. However, considering the previous reports on the effects of microRNAs [28] or sex hormones [29] in the *in vitro* follicular growth, the efficacy of ROCK inhibitors should be re-evaluated in combination with these factors.

In conclusion, freezing and thawing of ovary tissue may affect the mRNA expression levels of intra-ovarian AT system genes and modulation of ROCK inhibitor activity may not regulate AT system gene expression during the freezing of ovarian tissue. Further studies are necessary for the confirmation of efficacy of other anti-apoptotic agents during freezing and thawing procedures.

Acknowledgements

This research was funded by the grants of Ministry of Health and Welfare (HI14C2259), and Seoul National University Hospital (0320130270), Republic of Korea. The authors would like to express sincere thanks to the technical assistance of Ye Seul Hwang.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

The study was approved by the IACUC of the Seoul National University Hospital (15-0016-S1A0).

REFERENCES

1. Shuttleworth G, Broughton Pipkin F, Hunter MG. In vitro development of pig preantral follicles cultured in a serum-free medium and the effect of angiotensin II. Reproduction 2002;123:807-818.
2. Siqueira LC, Barreta MH, Gasperin B, Bohrer R, Santos JT, Buratini J Jr, et al. Angiotensin II, progesterone, and prostaglandins are sequential steps in the pathway to bovine oocyte nuclear maturation. Theriogenology 2012;77:1779-1787.
3. Viana GE, Pereira VM, Honorato-Sampaio K, Oliveira CA, Santos RA, Reis AM. Angiotensin-(1-7) induces ovulation and steroidogenesis in perfused rabbit ovaries. Exp Physiol 2011;96:957-965.
4. Ferreira R, Gasperin B, Rovani M, Santos J, Barreta M, Bohrer R, et al. Angiotensin II signaling promotes follicle growth and dominance in cattle. Endocrinology 2011;152:4957-4965.
5. Portela VM, Gonçalves PB, Veiga AM, Nicola E, Buratini J Jr, Price CA. Regulation of angiotensin type 2 receptor in bovine granulosa cells. Endocrinology 2008;149:5004-5011.
6. Yoshimura Y. The ovarian renin-angiotensin system in reproductive physiology. Front Neuroendocrinol 1997;18:247-291.
7. Bellew EA, Green DL, Law EW, Rizzuto PJ, McGaughey RW, Gallicano GI. Ovaries on ice: freezing fertility in preadolescent female cancer patients. Biopreserv Biobank 2012;10:485-492.
8. Vanacker J, Luyckx V, Amorim C, Dolmans MM, Van Langendonckt A, Donnez J, et al. Should we isolate human preantral follicles before or after cryopreservation of ovarian tissue? Fertil Steril 2013;99:1363-1368.
9. Gerritse R, Beerendonk CC, Westphal JR, Bastings L, Braat DD, Peek R. Glucose/lactate metabolism of cryopreserved intact bovine ovaries as a novel quantitative marker to assess tissue cryodamage. Reprod Biomed Online 2011;23:755-764.
10. Qu J, Godin PA, Nisolle M, Donnez J. Distribution and epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and after cryopreservation. Hum Reprod 2000;15:302-310.
11. Castro SV, Carvalho AA, Silva CM, Santos FW, Campello CC, de Figueiredo JR, et al. Frozen and fresh ovarian tissue require different culture media to promote in vitro development of bovine preantral follicles. Biopreserv Biobank 2014;12:317-324.
12. Ji YT, Chen DY, Jiang SL, Qu CQ. Effect of fasudil hydrochloride on the post-thaw viability of cryopreserved porcine adipose-derived stem cells. Cryo Letters 2014;35:356-360.
13. Claassen DA, Desler MM, Rizzino A. ROCK inhibition enhances the recovery and growth of cryopreserved human embryonic stem cells and human induced pluripotent stem cells. Mol Reprod Dev 2009;76:722-732.
14. Ichikawa H, Yoshie S, Shirasawa S, Yokoyama T, Yue F, Tomotsune D, et al. Freeze-thawing single human embryonic stem cells induce e-cadherin and actin filament network disruption via g13 signaling. Cryo Letters 2011;32:516-524.
15. Kim YY, Ku SY, Liu HC, Cho HJ, Oh SK, Moon SY, et al. Cryopreserva-

tion of human embryonic stem cells derived-cardiomyocytes induced by BMP2 in serum-free condition. Reprod Sci 2011;18:252-260.
16. Miyata K, Satou R, Shao W, Prieto MC, Urushihara M, Kobori H, et al. ROCK/NF-κB axis-dependent augmentation of angiotensinogen by angiotensin II in primary-cultured preglomerular vascular smooth muscle cells. Am J Physiol Renal Physiol 2014;306:F608-F618.
17. Wang S, Chen C, Su K, Zha D, Liang W, Hillebrands JL, et al. Angiotensin II induces reorganization of the actin cytoskeleton and myosin light-chain phosphorylation in podocytes through rho/ROCK-signaling pathway. Ren Fail 2016;38:268-275.
18. Ruiz-Ortega M, Lorenzo O, Rupérez M, König S, Wittig B, Egido J. Angiotensin II activates nuclear transcription factor kappaB through AT(1) and AT(2) in vascular smooth muscle cells: molecular mechanisms. Circ Res 2000;86:1266-1272.
19. Jeong JH, Jin ES, Min JK, Jeon SR, Choi KH. Wnt3a-producing fibroblasts in ovariectomy-induced osteoporosis in a rat model. Tissue Eng Regen Med 2015;12:306-313.
20. Asgari F, Valojerdi MR, Ebrahimi B, Fatehi R. Three dimensional in vitro culture of preantral follicles following slow-freezing and vitrification of mouse ovarian tissue. Cryobiol 2015;71:529-536.
21. Lotz L, Hauenstein T, Nichols-Burns SM, Strissel P, Hoffmann I, Findeklee S, et al. Comparison of whole ovary cryotreatments for fertility preservation. Reprod Domest Anim 2015;50:958-964.
22. Rimon E, Cohen T, Dantes A, Hirsh L, Amit A, Lessing JB, et al. Apoptosis in cryopreserved human ovarian tissue obtained from cancer patients: a tool for evaluating cryopreservation utility. Int J Oncol 2005;27:345-353.
23. Onishi M, Fujita Y, Yoshikawa H, Yamashita T. Inhibition of Rac1 promotes BMP-2-induced osteoblastic differentiation. Cell Death Dis 2013;4:e698.
24. Bokobza SM, Ye L, Kynaston H, Jiang WG. Growth and differentiation factor 9 (GDF-9) induces epithelial-mesenchymal transition in prostate cancer cells. Mol Cell Biochem 2011;349:33-40.
25. Liu Y, Ren W, Warburton R, Toksoz D, Fanburg BL. Serotonin induces Rho/ROCK-dependent activation of Smads 1/5/8 in pulmonary artery smooth muscle cells. FASEB J 2009;23:2299-2306.
26. Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 2003;4:446-456.
27. Pirone DM, Liu WF, Ruiz SA, Gao L, Raghavan S, Lemmon CA, et al. An inhibitory role for FAK in regulating proliferation: a link between limited adhesion and RhoA-ROCK signaling. J Cell Biol 2006;174:277-288.
28. Kim YJ, Ku SY, Kim YY, Liu HC, Chi SW, Kim SH, et al. MicroRNAs transfected into granulosa cells may regulate oocyte meiotic competence during in vitro maturation of mouse follicles. Hum Reprod 2013;28:3050-3061.
29. Kim YJ, Park KE, Kim YY, Kim H, Ku SY, Suh CS, et al. Effects of estradiol on the paracrine regulator expression of in vitro maturated murine ovarian follicles. Tissue Eng Regen Med 2016 [E-pub]. http://dx.doi.org/10.1007/s13770-016-0006-1.