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RESEARCH ARTICLE

The Effects of Antifreeze Proteins I and III Supplemented Medium on Cryopreserved Rat Ovaries in Different Durations

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Abstract

Cryopreservation techniques achieved by optimization of the composition and concentration of cryoprotectants not only reduce cryoinjury to ovarian tissue but also enhance survival. Anti-Freeze Proteins (AFPs) have been acknowledged to have positive effects on the survival of oocytes, embryos and ovaries during cryopreservation. AFPs drop the freezing point beneath the balance point of melting by binding ice crystals; restricting their expansion and recrystallization. This study aimed to investigate the protective effects of AFP I and III separately or in combination on the vitrification and warming procedures of ovarian tissues which were vitrified for one week and one month. Ovaries were obtained from rats and randomly assigned to four groups according to the AFP supplements in the media used during freeze/warm procedures: 1) control group (medium without AFP); 2) AFP I; 3) AFP III; 4) AFP I + AFP III groups. The groups were further organized according to the duration of vitrification, 1 week or 1 month. Ovaries were evaluated morphologically by a grading system, TUNEL assay and by transmission electron microscopy. AFP supplementation decreased apoptosis and follicular damage. Supplementation of AFP type I and type III has cryoprotective roles in ovaries which were vitrified for 1 week and 1 month. Additionally, AFP I+AFP III supplementation were shown to have more protection in long term.

Keywords: *Anti-freeze protein, Cryopreservation, Ovary, Vitrification, Rat*

Antifreeze Protein I ve III Eklenmiş Medyumun Farklı Sürelerde Kryoprezerve Edilmiş Sıçan Overi Üzerine Etkisi

Öz

Kriyoprotektanların kompozisyonu ve farklı konsantrasyonlarının eklenmesiyle geliştirilen kriyoprezervasyon teknikleri, over dokusunda sadece dondurma hasarını indirgemekle kalmaz, aynı zamanda over dokusunun devamını da sağlar. Anti-freeze Proteinlerin (AFP'ler) kriyoprezervasyon sırasında oositlerin, embriyoların ve yumurtalıkların hayatta kalması üzerinde olumlu etkileri olduğu kabul edilmiştir. AFP'ler, buz kristallerini bağlayarak donma noktasını erime noktasının altına düşürerek ve buz kristallerinin genişlemelerini ve yeniden oluşumunu sınırlandırır. Bu çalışmada, AFP I ve III'ün ayrı ayrı veya kombinasyon halinde hem bir hafta hemde bir ay süreyle vitrifiye edilen over dokularının çözdürme işleminden sonra koruyucu etkilerinin araştırılması amaçlandı. Sıçanlardan elde edilen overler vitrifikasyon/çözdürme prosedürleri sırasında kullanılan medyumdaki AFP takviyelerine göre rastgele dört gruba ayrıldı: 1) Kontrol grubu (AFP eklenmemiş medyum) 2) AFP I; 3) AFP III; 4) AFP I + AFP III grupları. Gruplar ayrıca vitrifikasyon süresine göre 1 hafta veya 1 ay olarak düzenlendi. Over dokularına; morfolojik olarak skorlama yapıldı. Ayrıca TUNEL metodu ile apoptozis açısından ve geçirimli electron mikroskobu ile ultrasütrüktürel açıdan değerlendirildi. AFP eklenmesi over dokularında apoptozisi ve foliküler hasarı azaltmıştır. AFP tip I ve tip III'ün eklenmesi, 1 hafta ve 1 ay süreyle vitrifiye edilen overlerde kriyo koruma sağlamıştır. Ek olarak, AFP I + AFP III takviyesinin uzun vadede daha fazla korumaya sahip olduğu gösterilmiştir.

Anahtar sözcükler: *Anti-freeze protein, Kriyokoruma, Over, Vitrifikasyon, Rat*

INTRODUCTION

Cryopreservation of oocytes, embryos and ovarian tissues, is a widely used technique particularly preferred for the preservation of fertility in cancer patients [1]. However, damages such as follicle loss, stromal cell destruction and programmed cell death could occur during the cryopreservation of ovarian tissues [2]. With recent advances

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in cryopreservation techniques, cryo-injury rates were decreased and the survival duration of the tissues was increased [3-5].

The efficiency of cryopreservation depends on the composition and concentration of cryoprotectants in the cryopreservation medium [1]. Survival of oocytes can be improved by adding cryoprotectants to the cryopreservation medium [5].

Antifreeze proteins (AFPs) are a group of polypeptides found in the serum of Antarctic fish that can live under sub-zero temperatures which were first identified by DeVries and Wohlschlag in 1969 [6]. An animal studies showed that these proteins had positive effects on cryopreservation of sperm [7], mouse ovaries [1], mouse oocytes [8]. AFPs decrease freezing point beneath the balance point of melting by binding ice crystals. Therefore, they prevent expansion and recrystallization. Additionally, AFPs protect cell membranes from physical damage [9,10]. AFPs have also been shown to have beneficial effects on ovary vitrification [11]. Additionally, it was thought that they can be used to diminish cryo-injury during cryopreservation and increase survival via protection of follicular structure in the mouse ovary [12].

AFP type I is mostly composed of alanine with 4.3 and 3.3 kDa sized helical structures whereas AFP type II is cysteine rich and its helical structures are 14 kDa in size. On the other hand, AFP type III has only 66 amino acids and it lacks cysteine and alanine [6].

In the present study, we aimed to investigate the protective effects of AFP I and III supplemented cryopreservation medium on vitrified and warmed rat ovarian tissues at two different time points.

Material and Methods

Experimental Animals

Forty-eight Wistar albino female rats were housed with a 12-h-light/dark cycle at 22°C and fed *ad libitum* according to animal care guidelines. The ethical approval was obtained from Yeditepe University, Animal Care Committee with approved number 15.12.2014/435.

Vitrification and Warming of Rat Ovaries

Whole ovaries were obtained from rats after CO_2 inhalation. The ovaries were first randomly assigned into two groups according to the vitrification durations: 1) Ovaries that were warmed after vitrification of one week; 2) Ovaries that were warmed after vitrification for one month. Afterwards, for each of the vitrification durations, the ovaries were further assigned into four groups depending on the AFP supplements used in the vitrification and warming media: 1) Control group (vitrification medium without AFP); 2) AFP I supplemented medium (AFP I Group); 3) AFP III supplemented medium (AFP III Group); AFP I + AFP III supplemented medium (AFP I + AFP III Group).

The vitrification and warming media were supplemented with 10 mg/mL of type I AFP, 10 mg/mL of type III AFP, and the combination of both. The amount of AFP proteins was determined according to previously published data [11]. The chemical features of AFP proteins are shown in *Table 1*.

Ovaries were vitrified by a two-step process as previously described [10,11]. Initially, ovaries were equilibrated for 10 min at room temperature in Dulbecco's phosphate buffered saline (D-PBS) supplemented with 20% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 7.5% dimethylsulfoxide and ethylene glycol (Sigma-Aldrich St. Louis, MO, USA). Afterwards, ovaries were incubated in the vitrification medium (D-PBS containing 20% FBS, 20% dimethylsulfoxide, 20% ethylene glycol, and 0.5 M sucrose) for 5 min at room temperature. Finally, the ovaries were placed into 1.5 mL cryovials (Nunc, Denmark) filled with liquid nitrogen and were stored for either 1 week or 1 month.

One week and 1 month after vitrification, ovaries were warmed as follows. Initially, the ovaries were brought out to air for 10 sec. Afterwards, they were incubated at room temperature by sequential 5-min equilibrations in 1, 0.5, 0.25, and 0 M sucrose (Sigma-Aldrich) solutions respectively. D-PBS with 20% FBS was used as the basal medium for both vitrification and warming solutions. For the AFP-supplemented groups, vitrification and warming media were supplemented with 10 mg/mL AFP type I and/or type III.

Histological Procedure

After the cryopreservation (7 or 30 days), all ovaries were fixed

Table 1. *The chemical features of AFP proteins*

Characteristic	AFP Type I	AFP Type III
Molecular Mass (kDa)	3.3-4.5	6.5
Primary Structure	alanine-rich multiple of eleven aa repeats	general
Secondary Structure	alpha helical amphiphilic	beta sandwich
Tertiary Structure	100% helix	not determined
Biosynthesis	prepro AFP	pro AFP
Protein Components	7	12
Natural Source	right-eyed flounders (winter flounder); sculpins (shorthorn)	ocean pout; wolffish

with 4% formaldehyde. Then, tissues were dehydrated and embedded in paraffin blocks. Five µm sections were taken from each paraffin block. Every fifth section was stained with Hematoxylin and Eosin and examined under light microscope (Leica DM 2500, Germany). The follicles were graded as tertiary, secondary, primary, or primordial follicles. Morphology of each follicle was evaluated according to the previously reported data [13,14] and presented in *Fig. 1* and *Fig. 2*. A total of 10 sections were evaluated for each animal. Percentages of follicles were compared statistically.

1- Primary and Primordial Follicles

Grade 1 (G1): Spherical shaped follicle with commensurate arrangement of granulosa cells,

Grade 2 (G2): Disarranged granulosa cells and a spherical oocyte in the follicle,

Grade 3 (G3): A spherical oocyte or vacuolation with pyknotic nuclei, abnormal granulosa cells

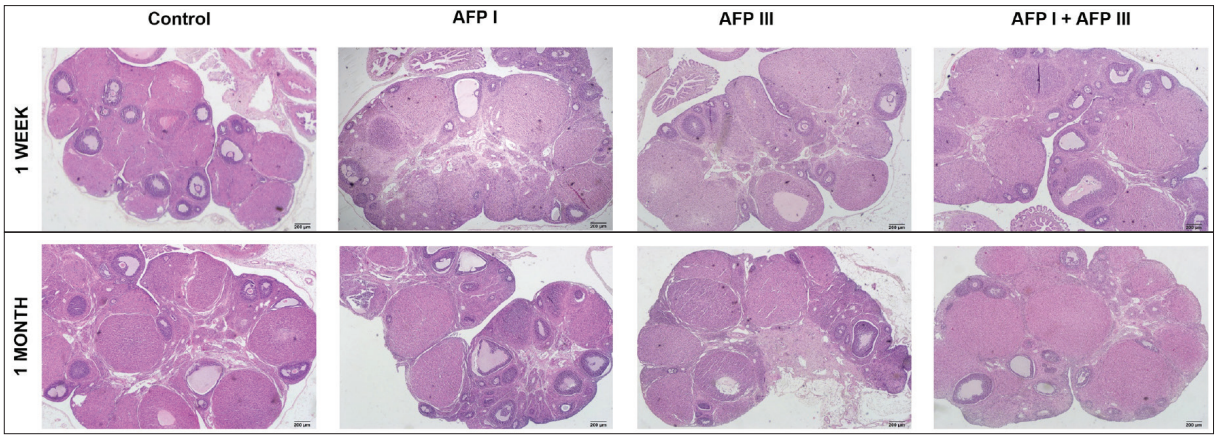

Fig 1. Morphology of rat ovaries in vitrification control, AFP I, AFP III, and AFP I+III groups after 1 week and 1 month. Hematoxylin and Eosin staining. Bar: 200 µm

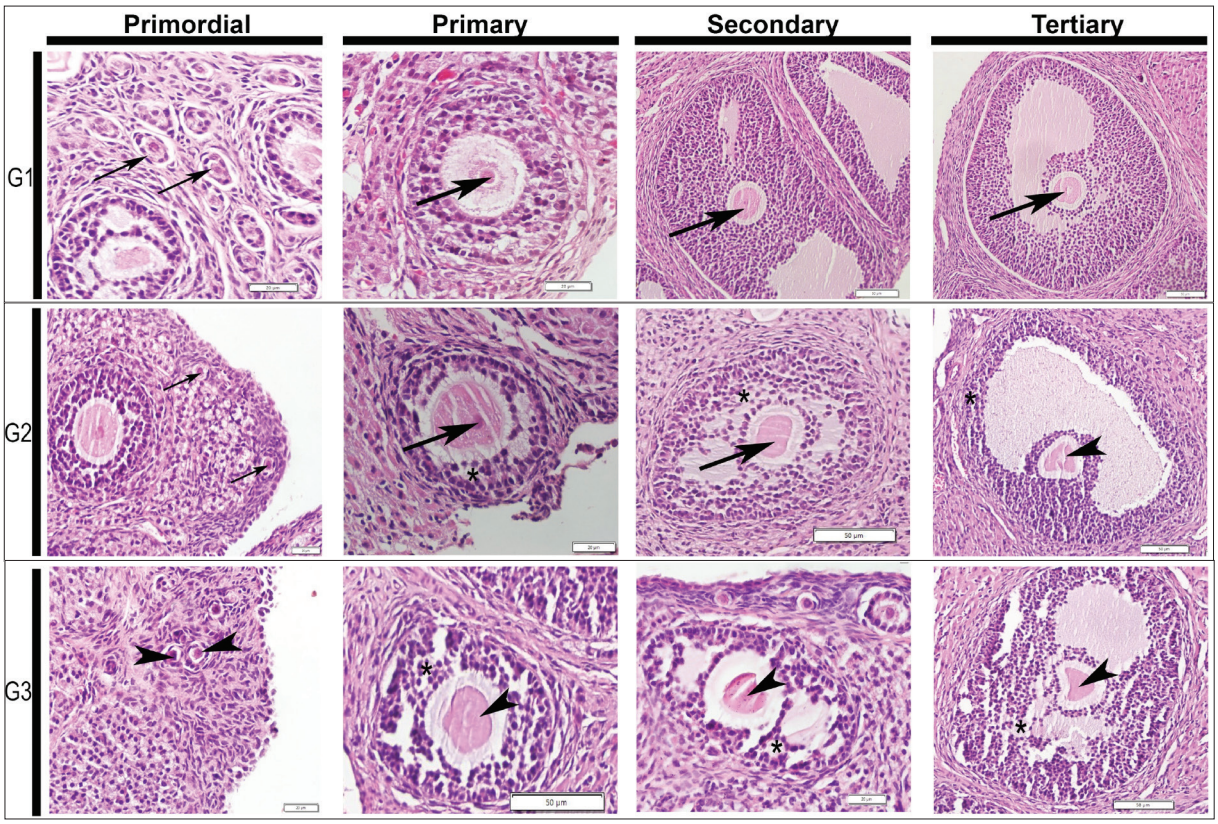

Fig 2. Morphological differences in rat ovarian follicles according to their grading scores. Spherical oocyte in primordial follicle *(thin arrow);* spherical oocyte *(thick arrow)* in primary, secondary and tertiary follicle; abnormal oocyte *(arrow head);* disarranged or abnormal granulosa cells (*). Hematoxylin and Eosin staining

2- Tertiary and Secondary Follicles

Grade 1 (G1): Intact spherical shaped follicle with commensurate arrangement of theca and granulosa cells and aspherical oocyte,

Grade 2 (G2): Intact morphology of theca cells, abnormal granulosa cells and a spherical oocyte,

Grade 3 (G3): Loss of or abnormal theca and granulosa cells without an oocyte or abnormal oocyte in the follicle.

TUNEL Assay for Apoptosis

Five µm sections from all groups were placed on positively charged slides for TUNEL assay. TUNEL assay was utilized according to the manufacturer's instructions (Biotium, CF™ 488A TUNEL Assay Apoptosis Detection Kit). Sections were incubated with proteinase K (5 min), washed with distilled water and incubated with 3% hydrogen peroxide in PBS (5 min). Then the slides were washed with PBS, put in the equilibrium buffer (30 min) and incubated in recombinant terminal transferase TdT enzyme (at 37°C for 1 h). The sections were then washed in buffer with agitation (15 s), followed by a wash in PBS and incubated with anti-digoxigenin conjugate (30 min). After washing in PBS, the sections were incubated with peroxidase solution for 6 min. After a final wash in distilled water, the slides were covered with Entellan (Merck, Darmstadt, Germany). Five randomly selected areas from each section were observed under fluorescence microscope (Leica DM 2500, Leica MC 170, Germany) and TUNEL positive cells were counted. Average values were evaluated.

Transmission Electron Microscopy

Vitrified and warmed ovaries from all groups were fixed with 2.5% glutaraldehyde in PBS (pH=7.4) and then incubated with osmium tetroxide for 1 h. All tissues were dehydrated in ascending ethanol series and then incubated in propylene oxide and embedded into Epoxy epon blocks. Semi-thin sections were taken from epon blocks by an ultramicrotome (Reichert, Germany) and stained with toluidine blue. Thin sections were contrasted with uranyl acetate and lead citrate and examined under transmission electron microscope (Jeol 1011, Japan, Olympus-Veleta TEM camera, Tokyo, Japan).

Statistical Analysis

Sample size is determined by power analysis. The result of the calculation was determined as n=8 for histological examinations. All calculations were calculated as type I error $P<0.05$, type II error (power) 0.20.

All results were compared with Mann-Whitney U or ANOVA test. Tukey's test was used for post-hoc test. Data were analyzed by Graphpad 3.0 prism. $P<0.05$ is considered as statistically significant.

Results

Morphological Evaluation

All ovaries were examined morphologically *(Fig. 1)* and follicles in ovaries were graded *(Fig. 2)*. In the control group (without supplementation of AFP), follicles in ovaries were significantly damaged after 1 week of vitrification. The damage became severe after 1 month of vitrification. Vitrification and warming caused granulosa cell degeneration and shrinkage of oocytes in the vitrification control group and there were less G1 follicles when compared to AFP supplemented groups ($P<0.01$, *Fig. 3)*. Ovaries of AFP I and AFP III groups were less damaged when compared to the control group. There was less degeneration in granulosa cells and oocytes. Additionally, there were more numbers of G1 follicles when compared to the time-matched control group. Ovarian damage was found to be the highest in the control group after 1 month of vitrification ($P<0.05$, *Fig. 3)*.

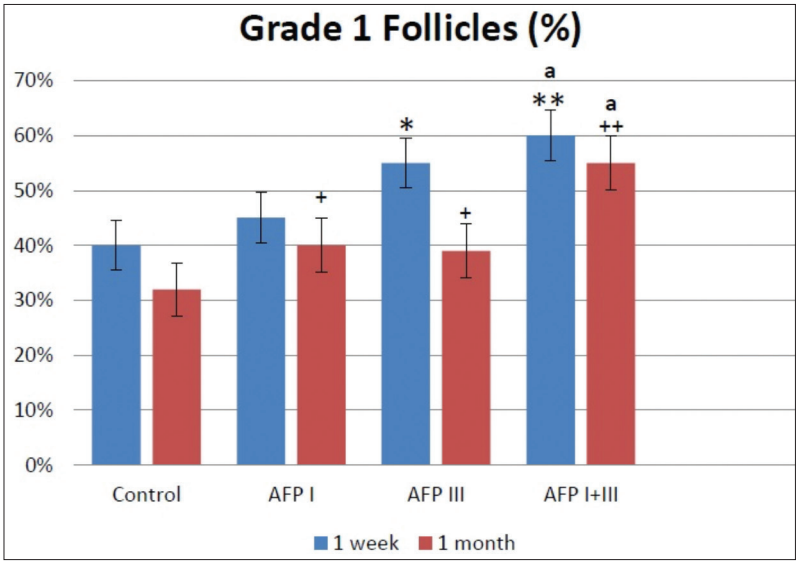

Fig 3. Percentage of all grade 1 follicles in vitrification control, AFP I, AFP III and AFP I + AFP III groups after 1 week and 1 month vitrified and warmed ovaries. * $P<0.05$, ** $P<0.01$ compared with time-matched vitrification control group, +: $P<0.05$, ++: $P<0.01$ compared with time-matched vitrification control group; a: $P<0.05$, compared with time-matched AFP I and AFP III

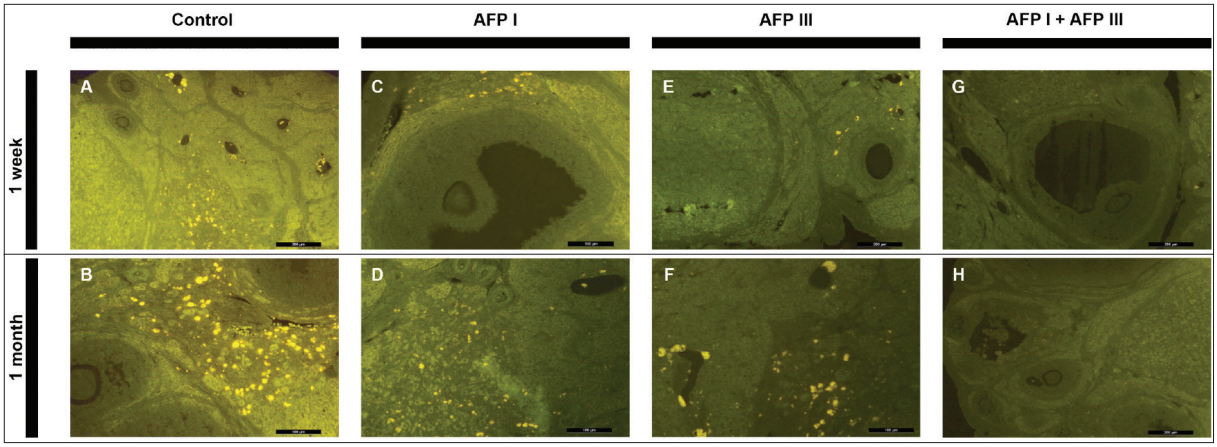

Fig 4. Apoptotic cells in ovarian follicles. **A** and **B)** Vitrification control group; **C** and **D)** AFP I; **E** and **F)** AFP III; **G** and **H)** AFP I + AFP III groups. TUNEL assay. A, E, G, H Bar: 200 µm, B, C, D, F Bar: 100 µm

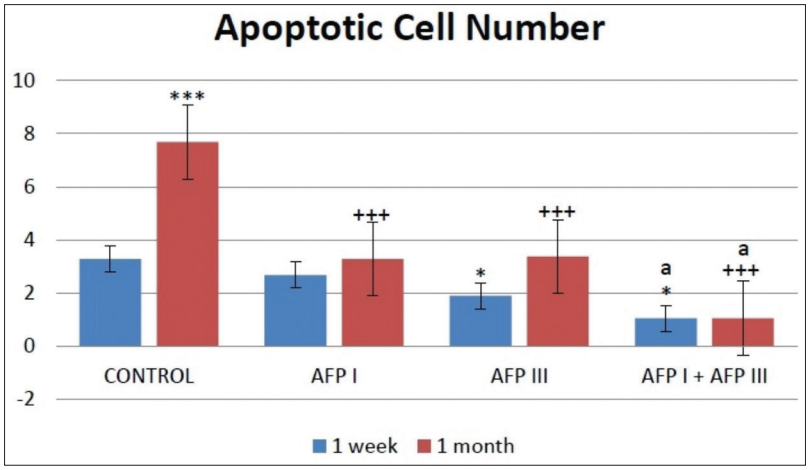

Fig 5. The graph of apoptotic cell numbers in vitrification control, AFP I, AFP III and AFP I + AFP III groups after 1 week and 1 month vitrified and warmed ovaries. * P<0.05 compared with time-matched vitrification control group, *** P<0.001 compared with 1week vitrification control group, +++: P<0.001 compared with time-matched vitrification control group, a: P<0.05 compared with time-matched AFP I and AFP III groups

The morphology of the ovaries of AFP I + AFP III groups were more intact. There was no or very minimum damage in granulosa cells and oocytes. Percentage of G1 follicles were the highest in AFP I + AFP III group when compared to the other groups (P<0.01, *Fig. 3)* in both durations. The protective effects of AFP I and AFP III on ovarian tissue, was higher when used in combination in both of the vitrification durations. Moreover, this protective effect was higher when compared to the control, AFP I and AFP III groups. Percentage of G1 follicles *(Fig. 3)* were higher in AFP III group when compared to AFP I group after 1 week of vitrification. Morphological differences in groups are shown in *Fig. 1* and *Fig. 2*.

Evaluation of Apoptosis

Apoptotic granulosa cell numbers decreased after 1 week of vitrification in AFP I+III group compared to vitrification control and AFP I and AFP III alone groups (P<0.05, *Fig. 4* and *Fig. 5)*. Furthermore, the apoptotic cell numbers were higher in the control group which had been vitrified for 1 month compared to the control group which had been vitrified for 1 week (P<0.001). AFP supplementation decreased apoptosis in both of the vitrification durations. Nevertheless, the decrease in apoptotic cell numbers was more prominent in AFP I + III groups in both of the vitrification durations when compared to the time-matched control groups *(Fig. 5)*. There was a significant decrease in apoptotic cell numbers particularly in the AFP I + AFP III group in which ovaries were vitrified for 1 month (P<0.001, *Fig. 5)*. The apoptotic cell numbers in this group were almost as close to the numbers in the AFP I + AFP III group in which ovaries were vitrified for 1 week. Apoptotic cell numbers were decreased in AFP I and AFP III alone groups underwent 1 month of vitrification when compared to time-matched vitrification control group (P<0.001, *Fig. 5)*.

Electron Microscopic Findings

The cellular organelles were damaged in the vitrification control group. There were more round mitochondria and the cell membranes of granulosa cells were disrupted with big vacuoles. The zona pellucida was abnormal and the area between the oocyte and follicular cells was increased. These findings were reversed in AFP groups. The zona pellucida between oocytes and cellular junctions among granulosa cells appeared normal in both AFP I and AFP III groups at both of the vitrification durations, whereas

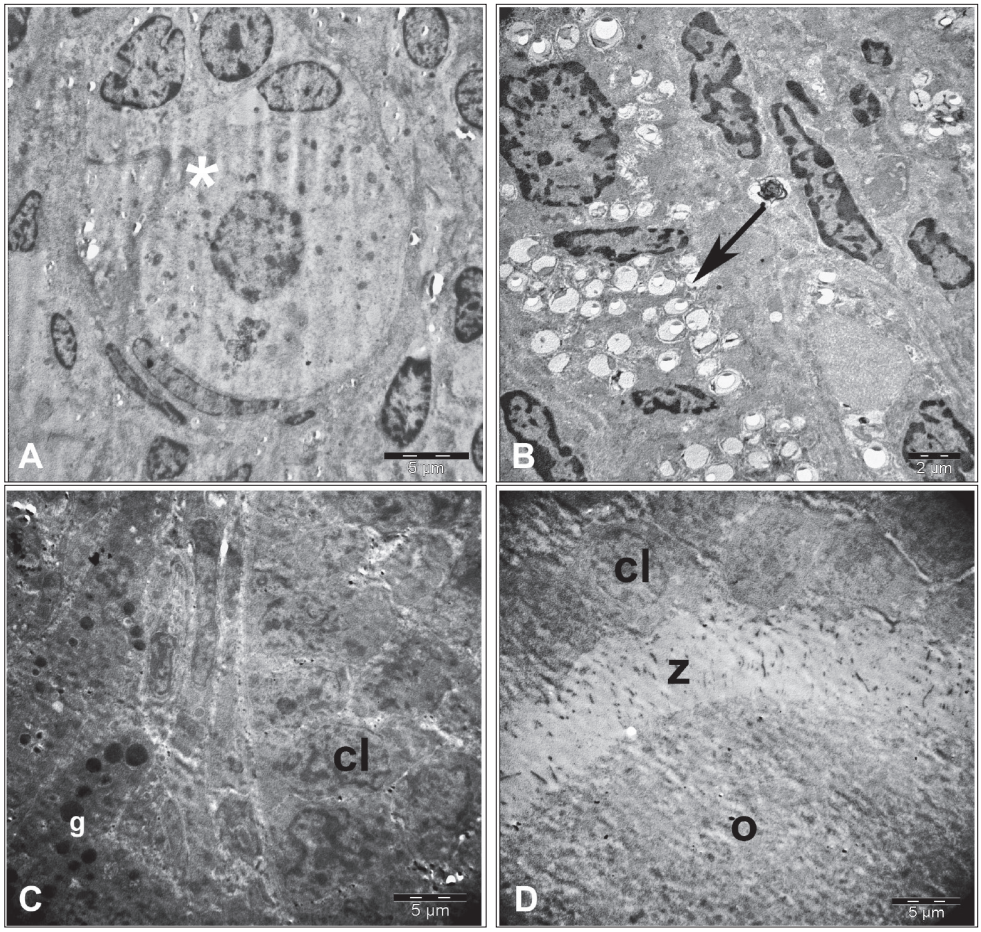

Fig 6. A, B) Vacuoles in granulosa cells (*) and damaged oocyte *(arrow)* in vitrification control group; **C, D)** less damages in granulosa (g) and cumulus cells (cl) and normal zona pellucida (z) and oolemma (o) in AFP I + AFP III group. Transmission electron micrographs. A, C, D Bar: 0.5 µm, B Bar: 0.2 µm

the cellular organelles and cell membranes of granulosa cells were slightly damaged. Moreover, the mitochondria were elongated and the size and amount of vacuoles were decreased in both AFP I and AFP III groups *(Fig. 6)*.

Discussion

In the present study, the cryoprotective effects of AFP proteins were evaluated in ovarian tissues which were vitrified for 1 week and 1 month. We observed that when AFP I and AFP III were added to the vitrification and warming media either separately or in combination, they exerted protective effects at the tissue, cellular and sub-cellular levels.

The combined supplementation of AFP I and AFP III to the vitrification medium and the warming medium, protects vitrified (1 week and 1 month) and warmed ovaries when compared to AFP supplementation alone.

AFPs can act in cryoprotection through two mechanisms. The first one is by inhibiting recrystallization, ice growth and nucleation of ice. The second one is by protecting the cell membrane and reducing cytotoxicity during cooling. AFPs can also inhibit recrystallization and ice nucleation at the warming stage [15]. Moreover, it was shown that the same AFP concentrations that we used in our study for vitrification and warming procedures protected frozen and warmed whole ovaries [1,15]. Based on this knowledge, in this study, we added AFPs into both vitrification and warming media and examined the protective effects of this AFP supplemented media on the structure of vitrified and warmed ovarian tissue at the light microscopic and the ultrastructural levels. We demonstrated that adding AFPs in vitrification and warming media has beneficial effects on the cryoprotection of whole ovaries in terms of both oocytes and granulosa cells.

Most studies showed that higher concentrations of AFP have degenerative effects on cells and tissues [16]. Other studies emphasized that lower concentrations of AFP have beneficial effects by regulating the survival of oocytes, whereas higher concentrations decreased the survival rates [12]. Moreover, Lee et al.[12] explained that a concentration of 20 mg/mL of AFP had better effects than 5 mg/mL of AFP. A synergistic effect that may have counterbalanced each other is caused by higher concentration of AFPs which are a mixture of AFP type I and III. As shown by previous studies, high concentrations of cryoprotectants cause osmotic stress and damage in follicles of cryopreserved mouse ovaries [16].

Furthermore, AFP supplemented cryopreservation media improved the integrity of follicles in vitrified and warmed

ovaries. Additionally, our results demonstrated that AFP I and AFP III when combined had better effects on vitrified and warmed ovaries even in long term vitrifications.

In our present study, we vitrified ovaries for one week and one month. During vitrification, ovaries may be damaged due to ice crystal formation and/or recrystallization. AFP I and AFP III supplementation to the vitrification and warming media also had beneficial effects at longer vitrification times. AFP I and AFP III, when supplemented together had more protective effects at longer vitrification durations when compared to AFP I or AFP III supplemented alone.

Two different AFP types were used at the same concentration instead of using only one type of the AFPs. And it was shown that the cryoprotectant solution supplemented with two equal concentrations of two different AFPs is more protective than only one type of AFP.

AFPs protect the membrane integrity in mouse oocytes [9,15]. Follicular integrity of ovaries after vitrification with AFP supplemented media is better than that of AFP lacking media [1]. Consistent with our study, it was shown that vitrification solutions have beneficial ultrastructural effects on cellular membrane and organelles as well as on the protection of mitochondria [17]. The protection of cellular organelles, cell membrane and mitochondria after vitrification is very crucial for normal physiological function. AFP supplemented vitrification medium provides protection of primary oocytes and granulosa cells in follicles ultrastructurally.

In conclusion, to the best of our knowledge, our work is unique in describing the cryoprotective effects of AFP type I and III, and the combination of these proteins on the ovaries that were vitrified for different durations. We demonstrated that supplementation of the vitrification and warming media with AFP I and AFP III, and the combination of the two, had beneficial effects on follicle integrity, enhancement in whole ovaries, as well as in primordial follicles with primary oocytes, specifically after 1 week and 1 month vitrification. This study shows the AFPs' cryoprotective effects in long term and the comparison between different durations of vitrification. Further studies are required to clarify the exact mechanisms of how AFPs prevent cryo-injury.

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Conflict of Interest

Authors declare that they have no conflict of interest.

Author Contributions

Gozde Erkanli Senturk: Hypothesis, manuscript writing, animal procedure and histological procedures, statistics; Halil Ibrahim Saygı: Transmission electron microscopic procedure and evaluation, statistics.

Availability of Data and Materials

The authors declare that data supporting the findings of this study are available within the article.

References

1. Kim MK, Kong HS, Youm HW, Jee BC: Effects of supplementation with antifreeze proteins on the follicular integrity of vitrified-warmed mouse ovaries: Comparison of two types of antifreeze proteins alone and in combination. *Clin Exp Reprod Med*, 44 (1): 8-14, 2017. DOI: 10.5653/cerm.2017.44.1.8

2. Faustino LR, Santos RR, Silva CM, Pinto LC, Celestino JJ, Campello CC, Figueiredo JR, Rodrigues APR: Goat and sheep ovarian tissue cryopreservation: Effects on the morphology and development of primordial follicles and density of stromal cell. *Anim Reprod Sci*, 122, 90-97, 2010. DOI: 10.1016/j.anireprosci.2010.08.001

3. Jee BC, Lee JR, Youm H, Suh CS, Kim SH, Moon SY: Effect of sphingosine-1-phosphate supplementation on follicular integrity of vitrified-warmed mouse ovarian grafts. *Eur J Obstet Gynecol Reprod Biol*, 152, 176-180, 2010. DOI: 10.1016/j.ejogrb.2010.06.019

4. Santana LN, Van den Hurk R, Oskam IC, Brito AB, Brito DC, Domingues SF, Santos RR: Vitrification of ovarian tissue from primates and domestic ruminants: An overview. *Biopreserv Biobank,* 10 (3): 288-294, 2012. DOI: 10.1089/bio.2011.0048

5. Youm HW, Lee JR, Lee J, Jee BC, Suh CS, Kim SH: Optimal vitrification protocol for mouse ovarian tissue cryopreservation: effect of cryoprotective agents and *in vitro* culture on vitrified-warmed ovarian tissue survival. *Hum Reprod*, 29, 720-730, 2014. DOI: 10.1093/humrep/det449

6. Kim HJ, Lee JH, Hur YB, Lee CW, Park SH, Koo BW: Marine antifreeze proteins: Structure, function, and application to cryopreservation as a potential cryoprotectant. *Mar Drugs,* 15 (2): 27, 2017. DOI: 10.3390/md15020027

7. Hossen S, Sharker MR, Cho Y, Sukhan ZP, Kho KH: Effects of antifreeze protein III on sperm cryopreservation of pacific abalone, *Haliotis discus hannai*. *Int J Mol Sci*, 22 (8): 3917, 2021. DOI: 10.3390/ijms22083917

8. Lee HH, Lee HJ, Kim HJ, Lee JH, Ko Y, Kim SM, Lee JR, Suh CS, Kim SH: Effects of antifreeze proteins on the vitrification of mouse oocytes: Comparison of three different antifreeze proteins. *Hum Reprod,* 30, 2110-2119, 2015. DOI: 10.1093/humrep/dev170

9. Leinala EK, Davies PL, Doucet D, Tyshenko MG, Walker VK, Jia Z: A beta-helical antifreeze protein isoform with increased activity: Structural and functional insights. *J Biol Chem*, 277 (36): 33349-33352, 2002. DOI: 10.1074/jbc.M205575200

10. Jo JW, Jee BC, Suh CS, Kim SH: The beneficial effects of antifreeze proteins in the vitrification of immature mouse oocytes. *PLoS One*, 7:e37043, 2012. DOI: 10.1371/journal.pone.0037043

11. Lee J, Kim SK, Youm HW, Kim HJ, Lee JR, Suh CS, Kim SH: Effects of three different types of antifreeze proteins on mouse ovarian tissue cryopreservation and transplantation. *PLoS One*, 10 (5): e0126252, 2015. DOI: 10.1371/journal.pone.0126252

12. Lee JR, Youm HW, Lee HJ, Jee BC, Suh CS, Kim SH: Effect of antifreeze protein on mouse ovarian tissue cryopreservation and transplantation. *Yonsei Med J*, 56 (3): 778-784, 2015. DOI: 10.3349/ymj.2015.56.3.778

13. Wang D, Geng M, Gan D, Han G, Gao G, Xing A, Cui Y, Hu Y: Effect of resveratrol on mouse ovarian vitrification and transplantation. *Reprod Biol Endocrinol*, 19:54, 2021. DOI: 10.1186/s12958-021-00735-y

14. Gandolfi F, Paffoni A, Papasso Brambilla E, Bonetti S, Brevini TA,

Ragni G: Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: Comparative analysis between human and animal models. *Fertil Steril*, 85 (1): 1150-1156, 2006. DOI: 10.1016/j.fertnstert.2005.08.062

15. Mahatabuddin S, Tsuda S: Applications of antifreeze proteins: Practical use of the quality products from japanese fishes. **In**, Iwaya-Inoue M, Sakurai M, Uemura M (Eds): Advances in Experimental Medicine and Biology, 321-337, Springer, Singapore, 2018.

16. Wang JH: A comprehensive evaluation of the effects and mechanisms of antifreeze proteins during low-temperature preservation. *Cryobiology*, 41, 1-9, 2000. DOI: 10.1006/cryo.2000.2265

17. Mahmoudi Asl M, Rahbarghazi R, Beheshti R, Alihemmati A, Aliparasti MR, Abedelahi A: Effects of different vitrification solutions and protocol on follicular ultrastructure and revascularization of autografted mouse ovarian tissue. *Cell J,* 22 (4): 491-501, 2021. DOI: 10.22074/cellj.2021.6877