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The effect of Aldosterone on Na^+/K^+/ATPase expression and development of embryos derived from vitrified-warmed sheep oocytes

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ABSTRACT

Media supplementation with various compounds in order to increase the oocyte developmental competence is of particular importance. This experiment was conducted to evaluate the effect(s) of media supplementation with aldosterone on embryo development and Na^+/K^+/ATPase expression following sheep oocyte vitrification. The abattoir-derived sheep COCs (cumulus oocyte complexes) were randomly allocated into six experimental groups: IVM of fresh and vitrified COCs in the presence of aldosterone followed by IVF/IVC (F-IVM and Vit-IVM groups, respectively); IVM/IVF of fresh and vitrified COCs followed by IVC wherein the embryos were exposed to aldosterone on Day 4 of IVC (F-D4 and Vit-D4 groups, respectively); and IVM/IVF and IVC of fresh and vitrified COCs in the absence of aldosterone (F-Cont and Vit-Cont groups, respectively). The expression of Na^+/K^+/ATPase α_1 and β_1 subunits in embryos were assessed at morula and blastocyst stages with related primary antibodies. The hatching rate was greater in aldosterone supplemented groups in both fresh and vitrified COCs compared to their controls. The expression of Na^+/K^+/ATPase β_1 subunit was significantly greater in F-D4 and Vit-D4 groups compared to other groups. The ICM / Total ratio in F-IVM was greater compared to the other groups. In conclusion, addition of aldosterone to the IVM and IVC media could improve the hatching rate of blastocysts derived from both fresh and vitrified COCs. These improvements, in D4 supplemented groups, might be related to the greater expression of Na^+/K^+/ATPase β_1 subunit induced by aldosterone supplementation.

Keywords: aldosterone, sheep oocyte, embryo development, Na^+/K^+/ATPase

1. Introduction

Based on previous studies the ovarian renin-angiotensin system (OvRAS) is quite active during the preovulatory period in bovine and human species (Nielsen et al., 2002; Loret de Mola et al., 1999). Aldosterone as an active component of the renin-angiotensin system is tightly regulated by angiotensin II. The same regulation scheme may be active in the ovary. Additionally, OvRAS in association with aldosterone may play a role in folliculogenesis and oocyte maturation. The synthesized mineralocorticoids in primate ovarian granulosa cells play a key role in steroid synthesis that is associated with luteinization of macaque granulosa cells (Fru et al., 2006).

The presence of mineralocorticoids in human ovarian follicles has also been shown by Sneeringer et al (2011). They demonstrated high concentrations of aldosterone and its precursor, corticosterone, in follicular fluid of women undergoing IVF. The concentration was significantly increased in follicular fluid compared to plasma and the maximum concentration was achieved prior to ovulation. Moreover, they reported the primary evidence indicating the local aldosterone synthesis and its receptors in oocyte. Considering the increased concentrations of aldosterone in large follicles compared to small follicles and the more likely presence of mature oocytes in larger follicles, it can be inferred that aldosterone has a role in oocyte development (Sneeringer et al., 2011). Aldosterone as a steroid hormone, which is synthesized in zona glomerulosa of the adrenal cortex, plays a pivotal role in electrolyte and fluid balance (Booth et al., 2002). Several genes have been identified as being directly or indirectly regulated by aldosterone including

71 Na^+/K^+/ATPase and cyclooxygenase-2 (COX-2) (Grossmann and Gekle, 2009; Sugiyama et al.,
72 2004).

73 A few studies have demonstrated a correlation between cryopreservation and the
74 amplitude expression of some specific genes in embryos (Tachatacki et al., 2003; Park et al.,
75 2004; Boonkusol et al., 2006). It has been shown that ovine oocyte vitrification significantly
76 reduces mRNA abundance of some important genes such as Poli A Polimerase (PAP), P34cdc2,
77 Cyclin b and Na^+/K^+/ATPase (Succu et al., 2008).

78 The investigations on the effects of Na^+/K^+/ATPase on the mechanisms directing
79 blastocyst formation in mouse has demonstrated that (a) Na^+/K^+/ATPase has a polarized
80 distribution confined to the trophectoderm basolateral membrane regions just before the onset of
81 cavitation, (b) expression of Na^+/K^+/ATPase subunits genes are up-regulated during the morula
82 to blastocyst transition, (c) Na^+/K^+/ATPase activity is significantly increased during the morula
83 to blastocyst transition for a number of mammalian species, (d) treatment with ouabain (a potent
84 and specific inhibitor of the Na^+/K^+/ATPase) affects cavitation and blastocyst formation in a
85 number of mammalian species, (e) deletion of the Na^+/K^+/ATPase α_1 subunit gene product is
86 linked to aberrant blastocyst formation in vitro and likely peri-implantation lethality in vivo, and
87 (f) Na^+/K^+/ATPase regulates the formation and function of trophectoderm tight junctions (Madan
88 et al., 2007).

89 Considering the greater concentrations of aldosterone in larger follicles of human and the
90 significant role of Na^+/K^+/ATPase during blastocyst formation, we aimed to evaluate the effect
91 of aldosterone supplementation to the IVM and IVC media on sheep oocyte and embryo
92 developmental competence.

93

2. Materials and methods

All experimental procedures were reviewed and approved by the Avicenna Research Institute Bioethics Committee. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise indicated in the text.

2.1. Oocyte collection

Adult sheep ovaries of Moghani breed were collected during the breeding season (September to November) from a local slaughterhouse and transported to the laboratory in saline (30–35^{o}C) within 2 to 3 h following collection. Ovaries were washed three times with pre-warmed fresh saline (37^{o}C) and all visible follicles with a diameter of 2 to 6 mm were aspirated using gentle vacuum (30 mmHg) via a 20 gauge short beveled needle connected to a vacuum pump. Prior to aspiration, 2 mL pre-incubated hepes-modified tissue culture medium (H-TCM), supplemented with 50 IU/mL heparin was added to the collecting tube.

2.2. In vitro maturation

After aspiration, only oocytes with evenly granulated cytoplasm surrounded by more than three layers of unexpanded cumulus cells (COCs) were selected for in vitro maturation (IVM). Before culturing, oocytes were washed in H-TCM supplemented with 10% FBS (v / v; Fetal bovine serum, Gibco BRL, Grand Island, NY, USA), and 2 mM L-glutamine. The oocyte maturation medium consisted of bicarbonate-buffered TCM 199 with 2 mM L-glutamine

supplemented with 0.05 U/ mL FSH, 100 IU／mL penicillin, 100 μg／mL streptomycin, 0.2 mM Na. Pyrovate and 10% FBS (v / v). The medium was adjusted to 275 mOsm. The selected COCs were pooled and randomly distributed in six experimental groups. In each group the selected COCs were placed in maturation droplets (10 oocytes／50 μL) and were covered by sterile paraffin oil in a 60-mm Petri dish (Falcon 3004; Becton & Dickinson, Franklin Lakes, NJ, USA) and were then incubated under an atmosphere of 5% CO_2 and 95% air with 100% humidity at 39^{o}C for 24 h.

2.3. Experimental groups

The selected COCs were randomly allocated into two classes of fresh and vitrified oocytes. The COCs and the resulting embryos was received 1000 ng／mL aldosterone either during IVM (F-IVM and Vit-IVM groups, in fresh and vitrified COCs, respectively) or Day 4 of IVC (F-D4 and Vit-D4 groups, in fresh and vitrified COCs, respectively). The COCs and the resulting embryos without aldosterone supplementation were considered as controls (F-Cont and Vit-Cont, in fresh and vitrified COCs, respectively). Each group consisted of at least four replicates.

(Table 1)

The presumptive zygotes were cultured at 39^{o}C under condition of 7% O_2, 5%CO_2, and 88% N_2 in humidified air for 8 days. The cleavage and blastocyst / hatching rates were recorded on Days 3 and 6 to 8, respectively (Day 0 was defined as day of fertilization). To evaluate the

effects of aldosterone on Na^+/K^+/ATPase subunits expression, the Day 8 embryos at morula and blastocyst stages were immunostained with specific primary and common FITC-conjugated secondary antibodies (~ 10 morula and blastocysts / replicate with at least three replicate / group). The mean fluorescence intensity of the subunits was measured with Image J 1.37v software (National Institutes of Health, Bethesda, MD, USA). In each group, the rest of resulting blastocysts were then subjected to differential cell staining (totally 83 blastocysts; three replicates / experimental group).

2.4. Vitrification and Warming

The selected immature COCs prior to vitrification were cultured in IVM media for 2h. Vitrification was performed following the method of minimum essential volume (MEV) using cryotop (Kitazato Ltd., Tokyo, Japan) as cryodevice (Succu et al., 2008). Briefly, group of five immature COCs were initially transferred into the holding medium (HM) consisting of 20 mM H-TCM supplemented with 20% (v / v) FBS for 1 min. The COCs were then incubated in 10% (v / v) ethylene glycol (EG) and 10% (v / v) dimethyl sulfoxide (DMSO) dissolved in HM for 30 s and successively transferred into 20% (v / v) EG, 20% (v / v) DMSO and 0.5 M sucrose for 20 s. The COCs were then loaded on cryotop and immediately plunged into liquid nitrogen (LN2) for storage. Warming was performed by directly plunging the cryotops in the HM supplemented with 1.25M sucrose for 1 min. The COCs were then transferred into HM with decreasing sucrose concentrations (0.62 M and 0.31 M for 30 s of each) and then washed in HM and used for further experiments.

2.5. Preparation of sperm and in vitro fertilization

After IVM, the oocytes were washed four times in HSOF [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-synthetic oviductal fluid] and once in fertilization medium and were then transferred into the fertilization droplets. A frozen semen pool from a single batch of Shal breed ram, with approved fertility, was used in all experiments. Semen was fractionated on discontinuous Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradients as previously described (Stefanello et al., 2006). Briefly, 700 µL of each percoll 90 % and percoll 40% were poured at a bottom of a 15 mL Falcon tube and 350 µL of thawed semen was slowly added on the top and tube was then centrifuged at 700 × g for 10 min. The fertilization medium was synthetic oviductal fluid (SOF), as originally described by Tervit et al. (1972), enriched with 20% heat inactivated estrous sheep serum and 10 mg / mL heparin. A 5 µL aliquot of sperm suspension, 1.0×10^6 sperm/mL, was added into the fertilization droplets (45 µL) containing 10 oocytes. Fertilization was carried out by co-incubation of sperm and oocytes in an atmosphere of 5% CO_2 in humidified air at 39°C for 18 h.

2.6. In vitro culture

After IVF, presumptive zygotes were vortexed for 2 to 3 min to remove the cumulus cells and then washed in H-SOF to remove spermatozoa and cellular debris. They were then allocated to the 20 µL culture drops (5 embryos / 20µl IVC medium) containing SOF supplemented with 2% (v/v) BME- essential amino acids, 1% (v/v) MEM-non essential amino acids, 1 mM glutamine and 8 mg/mL fatty acid free bovine serum albumin (BSA). They were then cultured

at 39°C under conditions of 7% O_2, 5% CO_2 and 88% N_2 in humidified air. On the third and fifth day of culture, 10% (v/v) charcoal stripped FBS was added to the medium. The osmolarity was maintained at 270 to 285 mOsmol. The percentage of cleaved embryos at Day 3 and the percentage of blastocysts at Day 6 to 8 were expressed on the basis of the number of oocytes at the onset of culture, and the percentage of hatched blastocysts at Days 7 and 8 was expressed based on the total number of blastocysts at the same day.

2.7. Differential staining

The blastocysts were incubated in Triton X-100 prepared in the base medium (H-TCM supplemented with 10% FBS) for 15 s. The blastocysts were then stained in base medium containing 30 μg / mL propidium iodide (PI) for 1 min and after two washes in base medium were then transferred into ice-cold ethanol containing 10 μg / mL Hoechst for 15 min. The blastocysts were then directly mounted into a small droplet of glycerol on a glass slide and examined under a Nikon TE 300 epifluorescent microscope (Nikon Corporation, Melville, U.S.A.). Inner cell mass (ICM) nuclei were appeared blue, caused by DNA labeling with Hoechst, while trophectoderm (TE) cells appeared pink due to staining of nuclear DNA with the membrane impermeable PI.

2.8. Immunostaining and quantification of fluorescence intensity

The embryos at compact morula / blastocyst stages were washed 3 times with base medium (PBS supplemented with 1 mg / mL polyvinyl alcohol: PVA) and then fixed with 4% (w

/ v) Paraformaldehyde for 10 min at 37°C. After rinsing with base medium, the embryos were blocked for 30 min at room temperature in PBS supplemented with 10 mg / mL BSA and 10% (v ⁄ v) sheep serum. Blocking solution was removed and embryos were then transferred to the primary antibody solution at 37°C for 4 h and then kept overnight at 4°C. The primary antibodies against Na^+/K^+/ATPase α_1 and β_1 subunits (Na^+/K^+/ATPase anti-α_1 Monoclonal Antibody, Thermo scientific, Rockford, USA and Na^+/K^+/ATPase anti-β_1 Monoclonal Antibody, Abcam, Cambridge) were prepared in antibody diluent (PBS supplemented with 10 mg / mL BSA and 3%; v ⁄ v; sheep serum). The embryos after several washes were transferred to the secondary antibody solution at 37°C for 4 h. The secondary antibody was sheep anti-mouse antibody conjugated with fluorescein isothiocyanate (Avicenna Research Institute, Tehran, Iran). In negative controls, the primary antibodies were removed. The primary antibodies against Na^+/K^+/ATPase α_1 and β_1 subunits as well as the secondary antibody were diluted at 1:50, 1:100, and 1:50, respectively.

After immunostaining, the embryos were directly mounted into a small droplet of glycerol on a glass slide and examined under an epifluorescent microscope. Observations were performed by a Nikon TE 300 (Nikon Corporation, Melville, U.S.A.) epifluorescence microscope with excitation wavelengths of 488 nm (for FITC). All images were recorded digitally with a high-resolution CCD camera (Nikon Corporation) and duration of exposure for acquiring each type of fluorescence was kept constant. After subtracting the background, mean fluorescence intensity was measured by manually outlining all embryos with ImageJ 1.37v software (National Institutes of Health, Bethesda, MD, USA).

2.9. Statistical analysis

Data were collected over at least four replicates. The differences in developmental competence in COCs and embryos as well as cells number in derived blastocyst and morula / blastocyst expression of Na^+/K^+/ATPase subunits between groups were analyzed by one-way ANOVA followed by post hoc Fisher LSD method using SigmaPlot (version 11.0). When equal variance test was failed, the treatments were compared by Student–Newman–Keuls Method. When normality test was failed the Kruskal–Wallis on ANOVA ranks was applied. Differences were considered significant when $P < 0.05$. Data were expressed as mean ± SEM.

3. Results

3.1. Effect of Aldosterone supplementation on embryo development in vitro

In fresh and vitrified COCs, the addition of 1000 ng/mL aldosterone to the IVM and Day 4 of IVC media had no significant effect on the rates of cleavage, expanded and total blastocysts, during days 6 to 8 of IVC. Though, the hatchability of blastocysts in aldosterone supplemented groups in both classes of COCs on Day 8 were greater than controls ($p = 0.002$ and $p = 0.01$; Tables 2 and 3, respectively).

(Table 2)

(Table 3)

3.2. Effect of Aldosterone supplementation on blastocyst cells number

In fresh COCs, the blastocyst cell numbers, ICM, TE, and total cells were not influenced by the presence of aldosterone in media during either IVM or Day 4 of IVC. However, the ICM / Total ratio was significantly increased by addition of aldosterone to the IVM medium (Fig. 1; Table 4; $p = 0.002$).

(Fig. 1)

(Table 4)

3.3. Effect of Aldosterone supplementation on Na^+/K^+/ATPase subunits expression in embryos

There was a significant difference in Na^+/K^+/ATPase subunits expression among treatment groups (Fig. 2). The expression of Na^+/K^+/ATPase β_1 subunit was significantly greater when aldosterone was added to Day 4 of IVC in both fresh (F-D4) and vitrified (Vit-D4) COCs (Fig. 3; $p = 0.003$ and $p < 0.001$, respectively) compared to the controls. Media supplementation with aldosterone during IVM or IVC in both fresh and vitrified COCs, however, had no influence on expression of Na^+/K^+/ATPase α_1 subunit (Fig. 3).

(Fig. 2)

(Fig. 3)

4. Discussion

It has been indicated that sheep oocyte vitrification significantly reduces mRNA abundance of some important genes including Na^+/K^+/ATPase. In mouse, the expression of Na^+/K^+/ATPase has been increased during morula to blastocyst transition and hatching process (Madan et al., 2007). Considering the increased concentration of aldosterone in larger follicles, it seems there is a positive role for aldosterone in oocyte development (Fru et al., 2006). Therefore, considering the reduction of Na^+/K^+/ATPase mRNA abundance after vitrification (Succo et al., 2008) and the inductive role of aldosterone on Na^+/K^+/ATPase expression (Oguchi et al., 1993), it could be inferred that the greater hatchability of blastocysts in current study might be related to the greater expression of Na^+/K^+/ATPase β_1 subunits.

As shown, in fresh groups (F-IVM and F-D4), media supplementation with aldosterone was significantly increased the hatching rate of Day 8 blastocysts compared to the control (F-Cont). Similarly, addition of aldosterone to the IVM and IVC media in vitrified COCs was significantly increased the rate of hatched blastocysts on Day 8 of culture compared to the control (Vit-Cont). It has been shown that Na^+/K^+/ATPase has a fundamental role in regulating mouse trophectoderm tight junction formation and function (Madan et al., 2007). On the other, blastocyst expansion only occurs when the tight junction permeability seal fully forms, to restrict the leakage of fluid via paracellular routes, ensuring the expansion of the cavity as fluid accumulates (Bell and Watson, 2013). Furthermore, previous studies have indicated that aldosterone can significantly increase the expression of Na^+/K^+/ATPase α_1 and β_1 subunits in a various cell types (Olivera et al., 2000; Verrey et al., 1989; Geering et al., 1982; Oguchi et al.,

1993). Therefore, in current study it could be hypothesized that the increase in the rates of Day 8 hatched blastocysts in aldosterone supplemented groups, in both fresh and vitrified oocytes might be related to the inductive role of aldosterone on Na^+/K^+/ATPase expression (Olivera et al., 2000; Verrey et al., 1989; Geering et al., 1982; Oguchi et al., 1993) during blastocyst to hatched blastocyst transition.

Apart from the inductive role of aldosterone in expression of Na^+/K^+/ATPase and the subsequent embryo development, this question that whether this compound can exert its effect via other pathway (s) remains to be elucidated. There are evidences indicating the inductive role of aldosterone on expression of several pro-inflammatory genes, including COX-2 (Sugiyama et al., 2004). The crucial role of cyclooxygenase-2 (COX-2) as a potential regulator of mammalian hatching has been established in many species (Huang et al., 2007; Liszewska et al., 2009; Kim et al., 2010; Wang et al., 2002). In this context, it has been shown that COX-2 can modulate hatching process via regulation of zonalytic proteases which rather exclusively occur in trophectodermal projections at the abembryonic pole of the blastocyst. (Roy and Seshagiril, 2013). Therefore, one possibility for the greater hatching rate in the presence of aldosterone might be related to the induction of COX-2 pathway.

In this study, the expression of Na^+/K^+/ATPase β_1 subunit was significantly increased when IVC medium was supplemented with aldosterone, in both fresh (F-D4) and vitrified (Vit-D4) COCs. As demonstrated, Na^+/K^+/ATPase β_1 subunit protein was required to support mouse early embryo development to the morula (16–32 cells) and blastocyst formation (Madan et al., 2007).

The Na^+/K^+/ATPase β_1 subunit gene products display a dramatic up-regulation just before blastocyst formation, suggesting up-regulation of this gene is required for cavitation to

occur. Thus it could be inferred that the up-regulation of Na^+/K^+/ATPase β_1 subunit might have a positive effect on hatched blastocyst formation in aldosterone treated groups. The up regulation of Na^+/K^+/ATPase β_1 subunit in our study, however, had no positive effect on blastocyst formation.

Concerning to the Na^+/K^+/ATPase α_1 subunit, its mRNAs are present throughout preimplantation development and display a much more gradual increase as the embryo progresses to the blastocyst stage (Madan et al., 2007).Considering the physiologic up-regulation of β_1 subunit just before cavitation and its specific role in cavitation, the positive effect of aldosterone in the hatching process might be exerted through the β_1 subunit compared to the α_1 subunit.

Addition of aldosterone during IVM, however, had no effect on expression of Na^+/K^+/ATPase α_1 and β_1 subunits (Fig. 3). One possibility for this issue might be related to the relatively longer time interval between aldosterone supplementation (during IVM) and the assessment of Na^+/K^+/ATPase α_1 and β_1 expression (blastocyst stage on Day 8).

Considering the importance of TE and ICM cells in placenta and epiblast formation, respectively, the numbers of TE, ICM, total cells, and ICM/total cell ratio are considered as qualitative indices in evaluation of embryos produced in vitro.

In fresh oocytes, following aldosterone supplementation despite the lack of significant alteration in ICM, TE, and total cells numbers, the ICM/Total cells ratio was significantly increased by aldosterone supplementation during IVM. In this context, cell numbers of mouse embryos were significantly decreased following treatment with ouabain as a Na^+/K^+/ATPase inhibitor (Violette et al., 2006). In lymphocytes and prostatic smooth muscle, there was an association between higher expression of Na^+/K^+/ATPase and inhibition of apoptosis (Olej et al., 1998; Chueh et al.,

2001). From above, It can be inferred that aldosterone by induction of Na^+/K^+/ATPase expression, could reduce apoptosis in ICM which in turn increased ICM / Total ratio (Kuran et al., 2002; Van Soom et al., 1996).

5. Conclusion

Addition of aldosterone to the IVM and Day 4 of IVC media was significantly increased the hatching rates of blastocysts derived from both fresh and vitrified COCs. The expression of the Na^+/K^+/ATPase β_1 subunit in embryos derived from both classes of oocytes was significantly increased when aldosterone was added during IVC. The ICM/Total ratio was also increased by addition of aldosterone to the IVM medium.

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Fig. 1. Differential staining of blastocysts derived from fresh oocytes supplemented with (during IVM and Day 4 of IVC; a and b, respectively) and without aldosterone (c).

Inner cell mass nuclei were appeared blue, caused by DNA labeling with Hoechst, while trophectoderm cells appeared pink due to staining of nuclear DNA with the membrane impermeable PI.

Fig. 2. Immunostaining of Na^+/K^+ /ATPase α_1 and β_1 subunits in morula and blastocysts derived from vitrified oocytes. The presence of α_1 and β_1 subunits was identified as green color areas. a and b) Expression of Na^+/K^+ /ATPase α_1 subunit in blastocysts derived from the oocytes supplemented with and without aldosterone during IVM, respectively; c and d), Expression of Na^+/K^+ /ATPase β_1 subunit in blastocysts derived from the oocytes supplemented with and without aldosterone during IVM, respectively; e and f) Expression of Na^+/K^+ /ATPase α_1 subunit in blastocysts derived from the embryos supplemented with and without aldosterone during Day 4 of IVC, respectively; g and h) Expression of Na^+/K^+/ATPase β_1 subunit in blastocysts derived from the embryos supplemented with and without aldosterone during Day 4 of IVC, respectively.

Fig. 3. Effect of aldosterone in culture medium during IVM or IVC (Day 4) on expression of α_1 and β_1 Na^+/K^+/ATPase subunits in derived blastocysts. The values, expressed as mean ± SEM. Bars with different letters indicate significant difference ($p \leq 0.01$).

Table 1

Experimental groups' nomenclature. The COCs received aldosterone during IVM or Day 4 of IVC in both classes of fresh and vitrified COCs.

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Fresh groups			Vitrified groups		
Aldosterone supplemented		Without aldosterone	Aldosterone supplemented		Without aldosterone
IVM (F-IVM)	IVC-D4 (F-D4)	Control (F-Cont)	IVM (Vit-IVM)	IVC-D4 (Vit-D4)	Control (Vit-Cont)

Table 2

The presence of aldosterone in culture media during IVM or Day 4 of IVC on embryo development of fresh oocytes.

Group	Oocyte N	Cleavage N (%)	Blastocyst % (mean±SEM)						
			Day 6		Day 7			Day 8	
			Expanded	Total	Expanded	Total	Hatch	Total	Hatch
F-Cont	122	108(86.6±4.9)	14(15.2±8.9)	24(23.6±12)	30(27.1±6)	52(44.4±6.6)	10(12±12.1)	50(41.3±3.3)	12(24.2±5.4)[a]
F-IVM	192	182(94.8±0.9)	20(10±5.8)	34(17.4±8.7)	40(20.8±2.6)	68(35.6±2.1)	20(29.8±6.5)	84(43.6±3.5)	44(52.8±4.7)[b]
F-D4	168	156(92.8±1)	4(1.8±1.8)	14(7.8±4.6)	38(21±3.7)	56(31±5.2)	10(13.2±7.1)	68(36.5±7.6)	42(63.2±2.3)[b]

[a,b] Numbers with different lowercase superscript letters in the same column differ significantly ($p \leq 0.01$). The percentage of expanded and total blastocysts at Days 6 to 8 were expressed based on oocytes number at the onset of culture, and the percentages of hatched blastocysts at Days 7 and 8 were expressed based on the total number of blastocysts at the same day. IVM: In vitro maturation in the presence of 1000ng / ml aldosterone; D4: In vitro embryo culture in the presence of 1000ng / ml aldosterone on Day 4 of culture

Table 3

The presence of aldosterone in culture media during IVM or Day 4 of IVC on embryo development of vitrified oocytes.

Group	Oocyte N	Cleavage N (%)	Blastocyst % (mean±SEM)						
			Day 6		Day 7			Day 8	
			Expanded	Total	Expanded	Total	Hatch	Total	Hatch
Vit-Cont	212	106(50.3±4.7)	0(0±0)	1(0.4±0.4)	2(0.8±0.8)	7(2.9±1.5)	0(0±0)	8(6.2±1.4)	2(8.3±6)[a]
Vit-IVM	227	133(58.3±7)	3(1.3±0.6)	3(1.3±0.6)	8(3.9±0.8)	11(4.5±2.3)	3(10.6±5.8)	11(7.1±3.2)	9(79.1±15)[b]
Vit-D4	168	79(48.7 ±3.8)	1(0.4±0.4)	2(1 ±0.6)	3(1.7±1.1)	9(1.7±0.6)	2(17±8.5)	6(8.2±2.6)	4(72.2±14.7)[b]

[a,b] Numbers with different lowercase superscript letters in the same column differ significantly ($p \leq 0.01$). The percentage of expanded and total blastocysts at Days 6 to 8 were expressed based on oocytes number at the onset of culture, and the percentages of hatched blastocysts at Days 7 and 8 were expressed based on the total number of blastocysts at the same day. Vit-IVM: In vitro maturation in the presence of 1000ng / ml aldosterone; Vit-D4: In vitro embryo culture in the presence of 1000ng / ml aldosterone on Day 4 of culture

519 **Table 4**

520 The presence of aldosterone in culture media during IVM or Day 4 of IVC on blastocyst cells number in embryos

521 derived from fresh oocytes

Group	Blastocyst	Blastocysts cell numbers (mean±SEM)			Ratio
	N	ICM	TE	Total	%±SEM
F-Cont	24	19.9±4.1	115.9±17.1	135±20.8	14±0.01[a]
F-IVM	32	23.8±2.6	86.6±6.8	110.3±8.8	21.1±0.01[b]
F-D4	27	19.6±2.5	97.9±7.9	117.5±9.5	16.5±0.01[a]

522 [a,b] Numbers with different lowercase superscript letters in the same column differ significantly ($p \leq 0.01$). IVM: In vitro
523 maturation in the presence of 1000ng / ml aldosterone; D4: In vitro embryo culture in the presence of 1000ng / ml aldosterone
524 on Day 4 of culture
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Conflict of interest statement

The study was financed by Avicenna Research Institute, which had no influence on study design, data evaluation or manuscript preparation. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

Highlights

- Sheep oocyte vitrification reduces mRNA abundance of Na^+/K^+/ATPase gene
- The increased concentration of aldosterone in larger ovarian follicles
- Aldosterone induces Na^+/K^+/ATPase expression
- Hatching rates improvement induced by aldosterone
- Greater expression of Na^+/K^+/ATPase β_1 subunits in aldosterone supplemented group

Figure 1

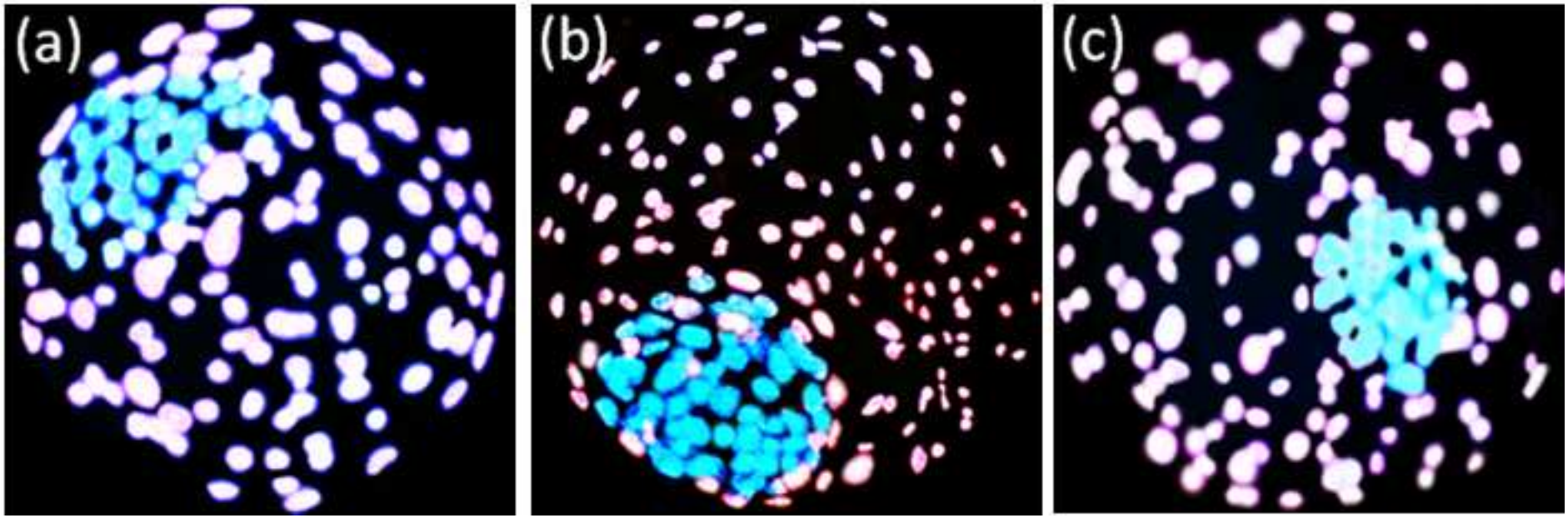
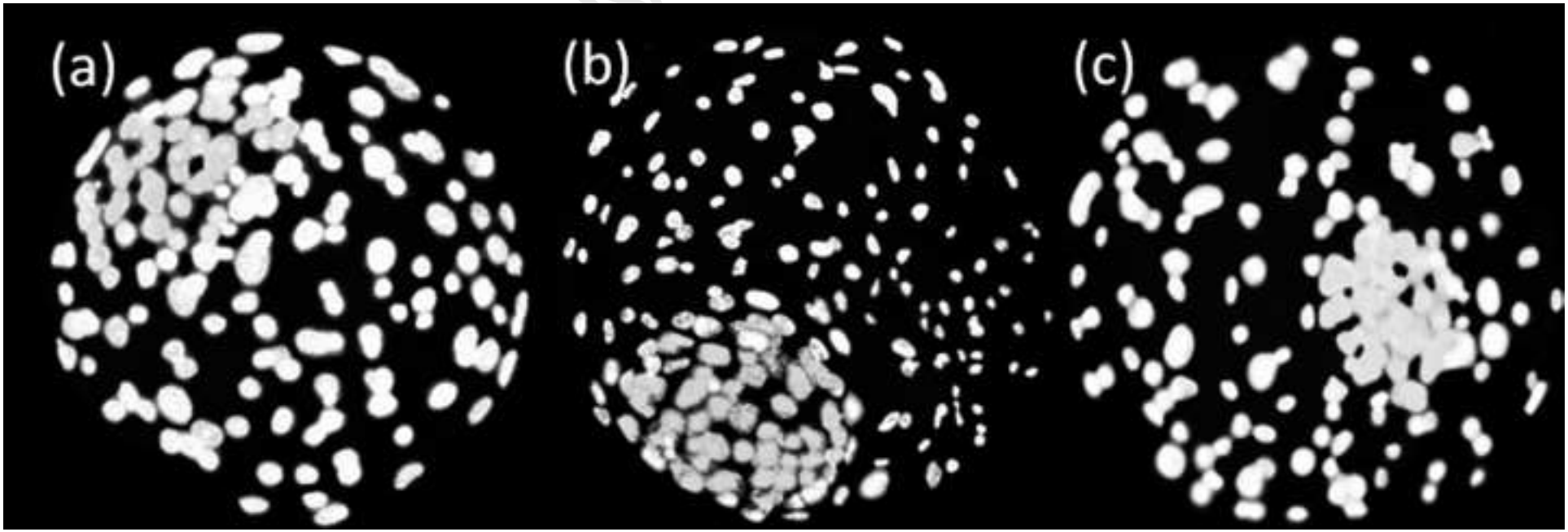
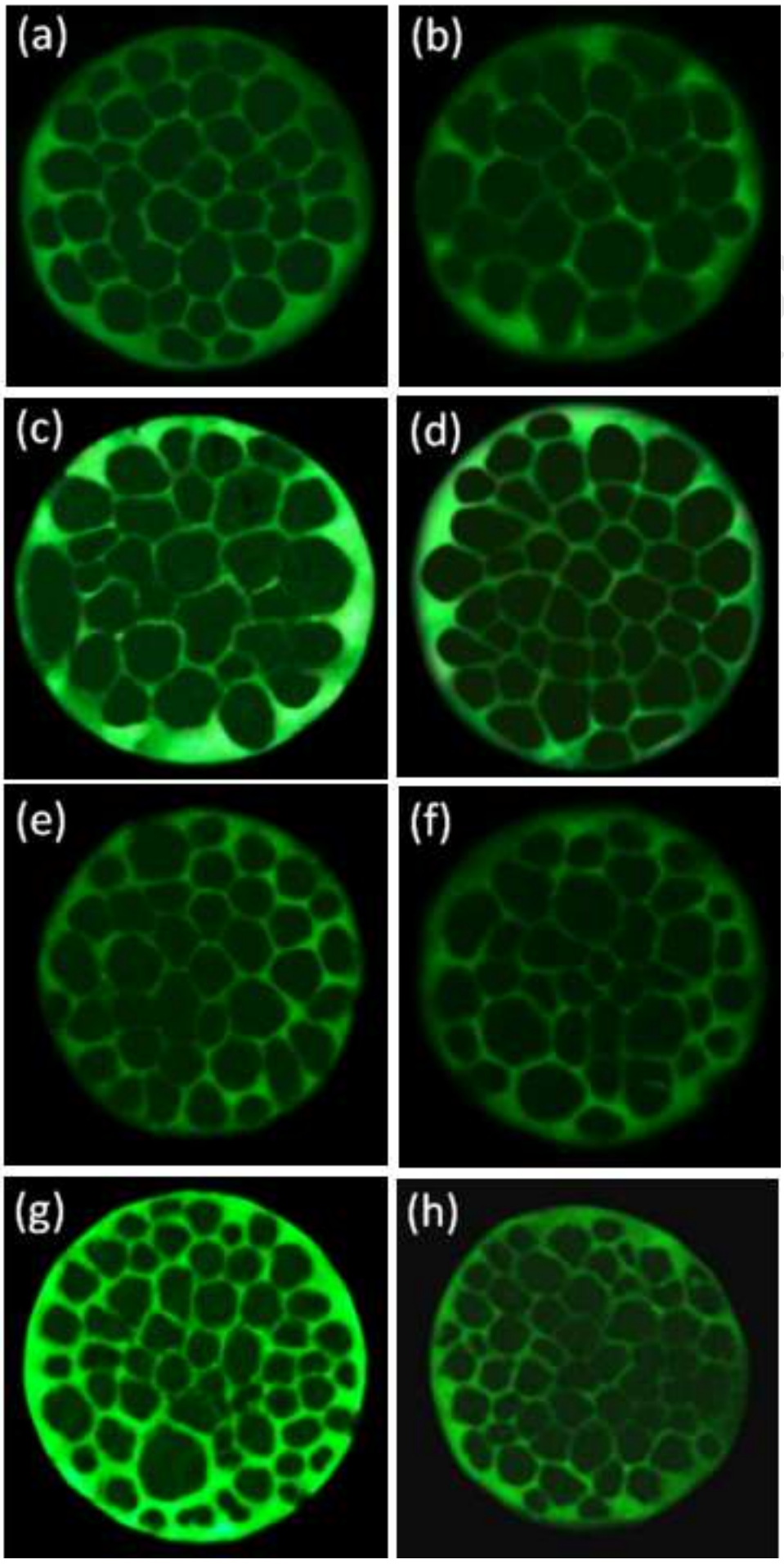


Figure 1 Gray





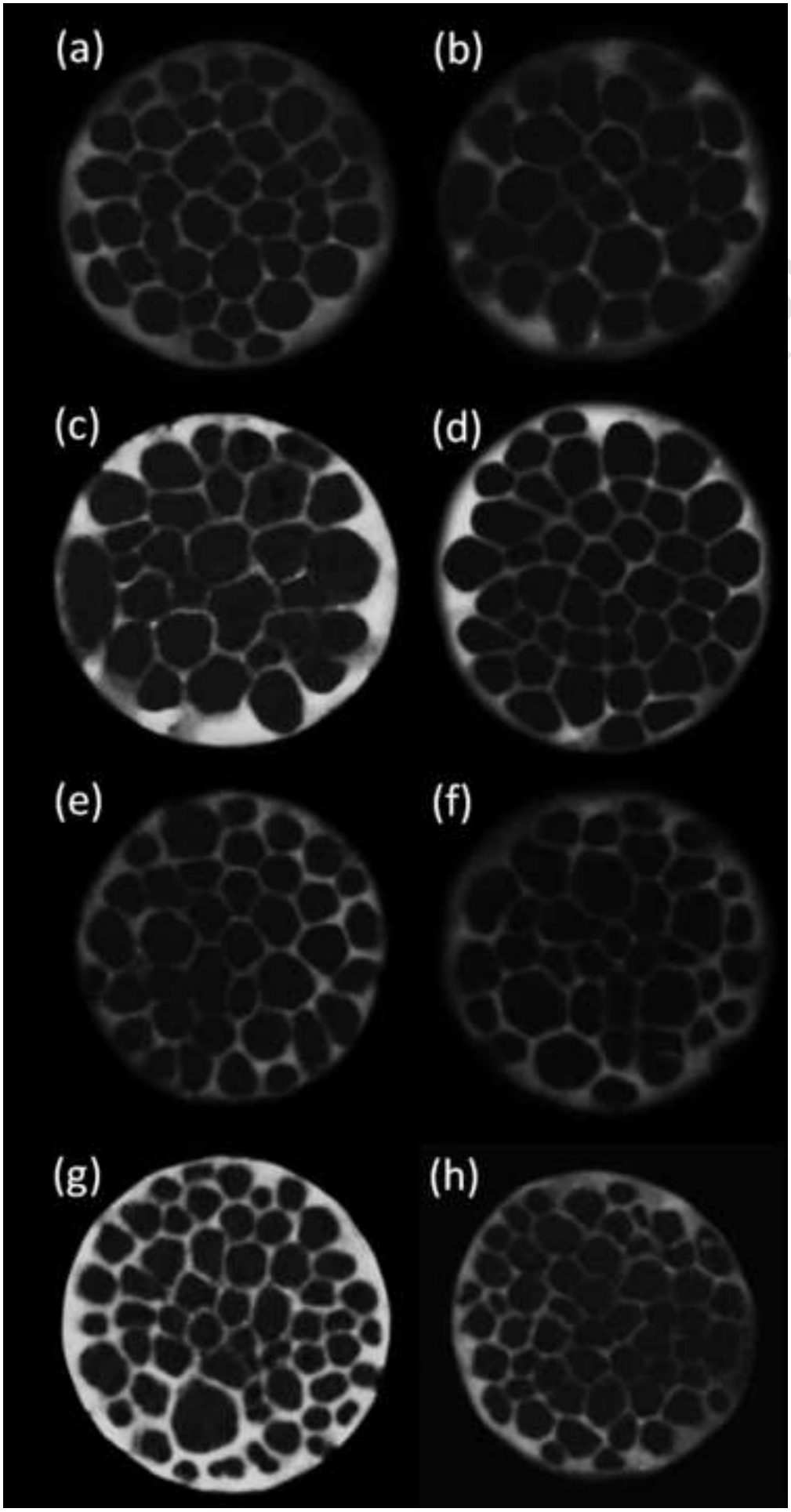
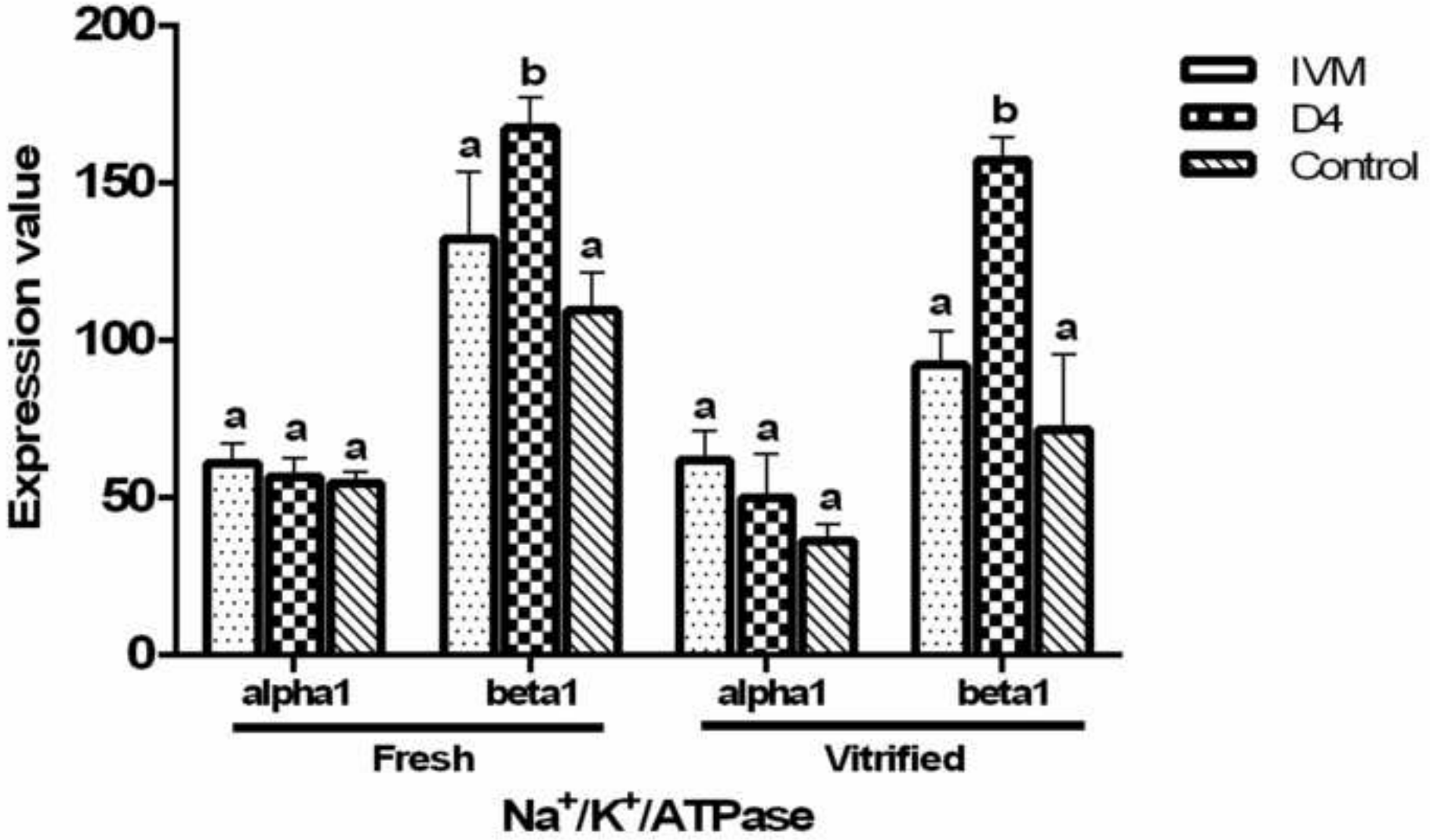


Figure-3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Fresh
Vitrified
Na+/K+/ATPase
IVM
D4
Control