**Regulation of proteolysis in bovine cumulus cells with possible inclusion of proton pump activators**

Ipek Coruhlu1 and Filiz Tepekoy\*1,2,3

1Department of Histology and Embryology, Faculty of Medicine, Altinbas

University, 34147, Istanbul, Turkey

2Central Research Laboratory, Altinbas University, 34147, Istanbul, Turkey

3School of Human Sciences, College of Science and Engineering, University of Derby, Kedleston Road, Derby, DE22 1GB UK.

\*Corresponding author e-mail: filiztepekoy@gmail.com, ORCID ID: **0000-0003-1901-3787**

**STATEMENTS AND DECLARATIONS**

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**ABSTRACT**

**Purpose:** To reveal the effects of V-ATPase proton pump activation on lysosomal acidity and protein degradation in cultured cumulus cells.

**Methods:** Cumulus cells from bovine ovaries were cultured in the presence of 10and50 µM doses of V-ATPase proton pump activators PIP2, PMA, and DOG for 12 and 24 hours. At the end of the culture period, the level of protein degradation was evaluated through DQ-Red-BSA analysis and the lysosomes were detected through a fluorescent probe. In addition, total and phosphorylated MAPK1/3 and AKT protein levels of cumulus cells were determined through western blotting.

**Results:** PIP2 and PMA were shown to increase protein degradation and lysosomal acidity in cultured bovine cumulus cells, whereas DOG did not have any significant effects on these cells. Total and phosphorylated MAPK and AKT protein levels were higher in PIP2 and PMA groups compared to the control and DOG.

**Conclusion:** Particular proton pump activators can enhance protein degradation and lysosomal acidification in cultured bovine cumulus cells without having detrimental effects on cell signaling members required for cell viability and proper functioning. Due to the cellular interactions, increasing the lysosomal activity in cumulus cells in the culture environment could also affect the removal of protein aggregates in the oocytes. This strategy could be effective for improving in vitro maturation of the oocytes by providing proteostasis.

**Keywords:** cumulus, PIP2, PMA, DOG, lysosome

**INTRODUCTION**

The female gametes, oocytes undergo several molecular regulations until they reach a maturational level which allows them to be competent for fertilization and proper embryonic development. During the oocyte nuclear maturation, immature oocytes arrested at prophase of the first meiotic division continue to metaphase of the second meiotic division after gonadotropin stimulation. The cumulus cells closely associated with the oocytes are assisting this process by supporting the oocytes in terms of various signalling molecules (Dekel, 1988).

In vitro maturation (IVM) of oocytes indicates the process through which immature oocytes (germinal vesicle, GV stage) are cultured and then matured in an appropriate medium to the metaphase II (MII) stage (Escrich et al., 2012). One of the biggest benefits of the IVM procedure is avoiding ovarian hyperstimulation, especially for patients with polycystic ovaries (PCO) and polycystic ovary syndrome (PCOS). Ovarian hyperstimulation syndrome (OHSS) caused by gonadotrophin treatments during the oocyte pick-up process can be prevented by IVM applications (Dahan et al., 2016). IVM can also be preferred for follicle-stimulating hormone (FSH) resistant ovaries or as a substitute for cases in which standard in vitro fertilization (IVF) procedure is irrelevant (Walls & Hart, 2018). The current literature reveals that, in addition to the cellular dynamics within the oocytes, the signalling pathways in accompanying cumulus cells also have a crucial role in the effectiveness of IVM procedure (Lonergan & Fair, 2016).

During the IVM, control of oocyte and accompanying cumulus cell proteostasis may have an impact on a successful maturation process. The Proteosome network that controls protein synthesis, folding, and degradation, eliminates misfolded and aggregated proteins (Sala et al., 2017). It is required for the proper functioning of the germ cells and to avoid the passage of protein damage to the offspring (Wang et al., 2018). Proteosomes also have a crucial role in sustaining proteostasis for the reproduction ability of females and there may exist a connection between aging and the disruption of proteostasis (Labbadia & Morimoto, 2015).

Vacuolar adenosine triphosphatase (V-ATPase) proton pumps are known to activate the lysosomes (Ohkuma et al., 1982). V-ATPase is composed of a transmembrane V0 domain and cytosolic V1 domain in addition to several subdomains (Cipriano et al., 2008). As a result of ATP hydrolysis, the proton pump which includes the proteolipid-C ring rotates and proton flow movement occurs, and H+ concentration is regulated (Breton & Brown, 2013). Many studies conducted on various cell types proved the induction of V-ATPase proton pumps by protein kinase C (PKC) activators with the aid of hormone-dependent and hormone-independent pathways (Breton & Brown, 2013). In addition, phosphatidylinositol 4,5-bisphosphate (PIP2) is known to activate the V-ATPase proton pump by stabilizing the V1-V0 domain interaction (Li et al., 2014).

Mitogen-activated protein kinases (MAPKs) have crucial roles in the oocyte maturation process, since the absence of these kinases results in an interruption of the maturation, and oocytes remain in the GV stage (Ye et al., 2003). In addition to triggering meiosis, MAPK can also modulate microtubular activity (Dedieu et al., 1996). Phosphatidylinositol-3-kinase (PI3K)/Akt and MAPK pathways activated by FSH have crucial functions in mitosis and cell cycle in granulosa cells as well (Law et al., 2017).

Since control of cytoplasmic dynamics within the cumulus cells might have a critical contribution to the IVM of oocytes, the purpose of the current study was to reveal the effect of V-ATPase activation on cultured bovine cumulus cells. The culture was conducted in the presence of different doses of V-ATPase activation factors; PIP2, PKC activators Phorbol 12-myristate 13-acetate (PMA) and diacylglycerol (DOG) which are related to lysosomal acidification. We aimed to determine an optimal culture environment for the cumulus cells in terms of proteostasis regulators, by evaluating the lysosomal acidification as well as MAPK and AKT protein levels.

**MATERIALS and METHODS:**

1. **Bovine ovary collection**

 The ovaries from 13-16 years old bovines were collected after slaughter in a local abattoir (Narmanlar ET, Istanbul, TURKEY) under the control of a veterinarian (Inclusion of slaughtered bovine ovarian samples in an experimental study was not subjected to ethical approval). After dissection, the ovaries were placed into a thermos bottle including phosphate-buffered saline (PBS) at 38.50C and transferred to the laboratory within two hours.

1. **Cumulus-oocyte complex (COC) retrieval**

The bovine ovaries were transferred to the M199 medium which includes HEPES, amphotericin B (2.5 mg/ml), pyruvic acid (25 mg/ml), penicillin G (75 mg/ml), and streptomycin (50 mg/ml). The cumulus-oocyte complexes (COCs) from antral follicles of the ovaries were pulled into the injector together with the follicle fluid with an 18G needle.

1. **Cumulus cell isolation and culture**

After obtaining the COCs, cumulus cells were mechanically isolated from the oocytes under the stereomicroscope. The cells within M199 medium were centrifuged at 5000 rpm for 5 minutes. Then, the cells were cultured at 370C within M199 medium which contains penicillin/streptomycin, fetal calf serum (FCS), insulin-transferrin-selenium (ITS), L-Glutamine, sodium pyruvate in 24 well plates above circular coverslips. After incubation for 24 hours, a group of cells was separated from wells by trypsin and transferred to Laemmli buffer solution for western blot analysis, while another group was subjected to DQ-Red BSA Analysis and stained with Lysotracker.

1. **Lysosomal Activation and DQ-Red BSA Analysis**

To obtain proteostasis through the removal of protein aggregates, activators that have been known to induce V-ATPase proton pumps within the lysosome membrane and achieve lysosomal acidification were used. To activate V-ATPase proton pumps during the cumulus cell culture, 10 µM and 50 µM of subsequent activators were applied for 12- and 24-hour intervals.

1) Phosphatidylinositol 4,5-bisphosphate (PIP2) (Sigma Sigma-Aldrich, 850174P, Steinheim, Germany)

2) PKC activator phorbol 12-myristate 13-acetate (PMA) (Abcam, ab120297, Cambridge, UK)

3) PKC activator diacylglycerol (DOG) (Abcam, ab143805, Cambridge, UK)

The control group that activators were not applied and the groups that different doses of activators were applied were named as follows: **C:** control group, **P1:** 10 µM PIP2**, P2:** 50 µM PIP2**, M1:** 10 µM PMA**, M2:** 50 µM PMA**, D1:** 10 µM DOG**, D2:** 50 µM DOG

To detect the effect of V-ATPase activation in lysosomes, live cumulus cells were subjected to the DQ-Red BSA proteolytic analysis. DQ-Red-BSA is a synthetic substrate used for evaluating lysosomal proteolytic degradation (Marwaha & Sharma, 2017). DQ-Red-BSA (10 μg/mL) (Life Technologies Corporation, Eugene, Oregon, USA) was applied to the cumulus cells at 37°C for 2 hours. After extracellular DQ-Red-BSA removal, cumulus cells were incubated in a medium that does not contain amino acids (aa) for 4 hours. After DQ-Red-BSA undergoes destruction, it generates red fluorescence. In the current study, lysosome activity was determined by detecting the red fluorescent light density generated by the DQ-Red-BSA.

1. **LysoTracker Analysis**

To determine the effect of V-ATPase activation on lysosomes, the probe (“Lysotracker”) was applied to the cumulus cells in culture. “Lysotracker” is a probe that enables the visualization of acidic organelle density in living cells. By applying this probe to the cells, acidic lysosomes can be detected by the red fluorescent. After DQ-Red-BSA analysis, cumulus cells washed with PBS were transferred into the prepared solution by adding “Lysored indicator” (20μL) to the “Live cell stain buffer” (10 mL) in the Lysotracker kit (Abcam, ab112137, Cambridge, UK) and incubated at 37 °C for 30 minutes. After washing with PBS, cumulus cells were observed and photographed on a Zeiss Axio Zoom.V16 fluorescent stereomicroscope with the appropriate filter. The density of red fluorescent which lysosomes generate in live cumulus cells was determined from the fluorescent micrographs by using NIH image analysis software (ImageJ Version 1.36b, National Institutes of Health, Bethesda, MD, USA).

1. **Determining the protein concentrations in cumulus cells with Western Blot**

At 12 and 24 hours of cumulus cell culture, the cells were washed with M199 medium twice. Cumulus cell lysate was obtained with lysis buffer which includes 63.5 mM Tris-HCl, 10% glycerol, 4% SDS, 20 mM Na3VO4, 10 mM NaF, and 1% (v/v) protease inhibitor cocktail. The samples were boiled for 5 minutes, and polyacrylamide gels were prepared according to theirweight (kDa) of intended proteins. To each well 20 μL sample was placed in addition to the protein marker (Amersham GERPN800E, GE Healthcare, Buckinghamshire, UK) and electrophoresis was run. Proteins that were in the gels were transferred to the membrane through the immunoblotting technique following the electrophoresis. After the transfer of proteins to the polyvinylidene fluoride (PVDF) membrane, the membranes were washed with Tris Buffer solution (pH 7.2-7.4) which includes 0.1% Tween-20 and were blocked with 5% bovine serum albumin (BSA) for 1 hour at room temperature. Membranes were incubated with MAPK1-MAPK3 (137F5), pMAPK1-pMAPK3 (4370S), Akt (9272S), pAkt (9271S) (Cell Signaling Tech., Danvers, MA, USA) primary antibodies at optimum temperature and time above the shaker. After incubation, the membranes were washed with tris-buffered saline (TBS-T) solution including tween-20 3 times for 10 minutes. Membranes were incubated with the proper seconder antibody (anti-rabbit, HRP-linked secondary antibody 7074S, Cell Signaling Tech., Danvers, MA, USA) at RT above shaker for 1 hour. After incubation, the membranes were washed with TBS-T solution 3 times for 10 minutes. Membranes were developed with Chemiluminescence (20X LumiGLO, 7003S, Cell Signaling Tech., Danvers, MA, USA) for a proper time and transferred to x-ray films. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, 14C10, Cell Signaling Tech., Danvers, MA, USA) was used as an internal control. The optical intensity of bands obtained from the western blot experiments was determined by using NIH image analysis software (ImageJ Version 1.36b, National Institutes of Health, Bethesda, MD, USA).

1. **Statistical Analysis**

The data obtained by the ImageJ from fluorescent images and western blot bands were analysed by Sigma Stat program (Sigma Stat for Windows, version 3.0, Jandel Scientific Corp., San Rafael, CA, USA). Parametric One-way ANOVA, Holm Sidak method was applied and the values were presented as mean ± SEM. Statistical significance was defined as P <0.05.

**RESULTS**

**The effect of V-ATPase proton pump activators on protein degradation and lysosome acidification in cultured cumulus cells**

DQ-Red BSA is an artificial substrate used to assess lysosomal proteolytic degradation (Marwaha & Sharma, 2017). The degradation of this substrate is indicative of increased lysosomal acidity and increased proteolytic activity. Smaller peptides resulting from the hydrolysis of DQ-Red BSA are converted to intensely fluorescent products. Therefore, high fluorescence intensity indicates an increase in proteolytic activity and lysosomal acidity. During the assays, this substrate was added to the culture medium of the cumulus cells (10 μg/mL) and applied to the cumulus cells for 2 hours at 37°C. After removal of extracellular DQ-Red BSA, cumulus cells were incubated for 4 hours with aa-free and serum-free medium. Unfixed, viable cumulus cells were observed under a fluorescent stereomicroscope (Zeiss Axio Zoom.V16, Oberkochen/ Germany) with the appropriate filter, and fluorescent images were obtained at the time of application of the DQ-Red BSA substrate (DQ-Red BSA-1) and after a 4-hour culture period after application (DQ-Red BSA-2). Fluorescent staining intensity was measured through the Image-J (National Institutes of Health, Bethesda, MD, USA) program.

**DQ-Red BSA and Lysotracker analysis in cumulus cells treated with PIP2**

DQ-Red fluorescent intensity for P1-24h-DQ2 was higher than P1-24h-DQ1. A similar increase was also observed in P2-12-DQ2 when compared to P2-12-DQ1. P1 lysotracker analysis showed that the maximum lysosome acidity was obtained after 24 hours of P1 application. Although, for all the cases, DQ2 values were higher, it was statistically significant for P1-24h and P2-12-hour groups **(Fig. 1 and 4)**.

**DQ-Red BSA and Lysotracker analysis in cumulus cells treated with PMA**

M1-12H-DQ2 fluorescent intensity was higher than M1-12H-DQ1. M1-24H-DQ2 also showed a higher fluorescence intensity compared to M1-24H-DQ1. Interestingly, fluorescent intensity in M2-12H-DQ1 was higher than in M2-12H-DQ2. When the lysotracker intensity values for lysosome acidity were compared, it was determined that M2-24H-lysotracker intensity was higher than that of M2-12H **(Fig. 2 and 4).**

**DQ-Red BSA and Lysotracker analysis in cumulus cells treated with DOG**

Among the DOG groups, DQ analyses showed that the fluorescence intensity of DQ1s was higher than DQ2s, except for the D2-24H-DQ2 group. The lysotracker analysis revealed a similarity in lysosome acidity of 12H and 24 H groups **(Fig. 3 and 4).**

**DQ-Red BSA and Lysotracker analysis in the control group of cumulus cells**

DQ2 fluorescence intensity was not significantly increased in the C-12h group and was even determined to be lower in the C-24h group when compared to the DQ1. C-12h-Lysotracker intensity was also significantly higher when compared to C-24h **(Fig. 4).**

**The effect of V-ATPase proton pump activators on Akt and MAPK1/3 protein levels in cultured cumulus cells**

Akt and MAPK1/3 protein levels were determined by Western Blot in control and experimental groups. After 12 hours of culture, Akt protein concentration was measured to be the highest in P1 and P2 groups. At the end of the 24-hour culture, maximum protein concentrations were measured in P2, M1, and M2 groups and these levels were the highest among all time and dose groups. MAPK1/3 protein level was the highest in the M2 group among 12-hour groups, whereas it was the highest in the M1 and M2 groups during 24 hours of culture. MAPK1/3 level was also the highest in the 24-hour culture groups **(Fig. 5).**

The levels of phosphorylated forms of Akt and MAPK1/3 proteins in cultured cumulus cells were also determined by western blot. After a 12-hour culture period, both p-Akt and p-MAPK1/3 levels were shown to be the highest in the P1 group. After 24 hours of culture, the highest p-Akt level was observed in the M1 group, while the highest p-MAPK level was in the P1 group. Cumulus cells cultured in the presence of PIP2 at 10 µM of dose (P1) for 12 hours had the highest p-Akt and also p-MAPK1/3 levels among all groups **(Fig. 5)**.

**DISCUSSION**

Oocyte maturation rates are greatly affected by the interactions with the cumulus cells. These interactions are bidirectional and are important in the formation of embryos with high developmental potential (Machado et al., 2015). In our study, the addition of V-ATPase activators PIP2 and PMA to the culture environment was revealed to have significant effects on cumulus cells by increasing lysosomal activity that could have an impact on the proteostasis of these cells. Additionally, Akt and MAPK signaling which are important regulators of oocyte maturation were also protected at an adequate level by the addition of these V-ATPase activators to the culture. We suggest that this effect could be reflected in the oocyte in a co-culture environment, based on the significant interaction between oocyte and cumulus cells increasing the fertilization potential of oocytes with the help of achieving a proper cytoplasmic environment.

Throughout the oocyte maturation process, lysosomal physiology shift has a significant role in the removal of aggregates within the developing embryo. The sperm-derived signals were revealed to initiate lysosome acidification destroying the aggregates morphologically through a microautophagy-like process during and after fertilization in previous studies (Bohnert & Kenyon, 2017). Since there might be another lysosomal physiology shift from the cumulus cells to the oocytes, in the current study, the effect of particular proton pump activators on cultured cumulus cells was examined. Through DQ-Red BSA analysis, it was found that PIP2 and PMA had significant effects on protein degradation, whereas DOG did not have an impact on this process. The findings regarding the lysotracker also revealed that both PIP2 and PMA significantly affected lysosome acidification in cumulus cells, but DOG did not have this effect on these cells. In one of the recent studies, Samaddar et. al. revealed that suppression of lysosomal V-ATPase, vesicle-transport, or proteasome function increases the speed of age-dependent protein aggregation in the soma and preserves proteostasis in the aging soma which links the processes that improve proteostasis in the immortal germ lineage (Samaddar et al., 2021). Our study, proving the assistance of PIP2 and PMA in protein degradation, raised the question of whether these factors could also cause a degradation of vital proteins which take important roles for both oocytes and cumulus cells. Previous studies revealed that, in the IVM procedure, the application of growth factors to COCs elevated MAPK and mTOR signaling, in addition the genes involved in cumulus expansion and cumulus-oocyte interaction were upregulated**.** In the current study, to suggest an optimal culture environment for the cumulus cells and eventually the oocytes, we investigated the effects of these proton pump activators on MAPK and Akt protein levels, which are known to affect cumulus cell viability and functions.

According to the findings of our study, PIP2 was found to be effective only on Akt protein levels during the 12-hour culture period. It was observed that protein phosphorylation levels increased especially at low doses of PIP2 (10 µM) during the 12-hour culture period. PMA at both low (10 µM) and high (50 µM) doses, was found to cause an increase in Akt and MAPK protein levels especially at the end of the 24-hour culture period. In the DOG-applied groups, the level of these proteins in the cumulus cells was found to be similar to the control groups.

In cumulus cells MAPK activation is important to maintain cumulus expansion and resumption of meiosis by gonadotropin induction (Su et al., 2002). In the current study, it was crucial to sustain appropriate levels of MAPK in addition to Akt proteins in the presence of proton pump activators to avoid impairment of cumulus cell survival and function during the controlled activation of protein degradation for proteostasis of the cumulus cells.

The results of the current study showed that PIP2 and PMA have positive effects on both lysosome activation and MAPK and Akt protein levels in cumulus cells**.** On the other hand, DOG addition to the culture medium did not show the same positive effect.When the data we obtained from this study were analyzed, it can be suggested that during in vitro maturation of oocytes, PIP2 and PMA application to the culture medium in the presence of cumulus cells

could increase the cytoplasmic competency and fertilization potential of the oocytes.

Collectively, thoroughly recognizing the molecular network and inter-proteomic communication that has been thought to be responsible for the perpetuation of homeostasis (the so-called proteostasis) between V-ATPase proton pump-dependent lysosomal/proteasomal biodegradation and protein biosynthesis/accumulation in cumulus cells stemming from bovine ex vivo-matured COCs might be inevitable for recognizing the intracellular conditions related to expansion and intercellular mucification of cumulus oophorus and corona radiata layers surrounding metaphase II-stage heifer/cow oocytes. Furthermore, this seems to be positively correlated with molecular symptoms of PI3K/Akt and MAPK-dependent cytoplasmic maturation and its synchronization with nuclear/meiotic maturation of in vitro cultured bovine oocytes. Therefore, comprehensively deciphering the above-indicated molecular pathways characteristic of IVM-derived COCs in cattle and other mammalian species might provide an abundant source of nuclear recipient oocytes for such novel assisted reproductive technologies

(ARTs) as in vitro fertilization by either gamete co-incubation or intracytoplasmic sperm injection (Ashibe et al., 2019; Gorczyca et al., 2022; Jang et al., 2010; Santos et al., 2014) and cloning by somatic cell nuclear transfer (SCNT) (Moradi-Hajidavaloo et al., 2023; Nadri et al., 2022; Samiec et al., 2012; Skrzyszowska & Samiec, 2021).

**Figure Captions**

**Fig. 1:**DQ-Red BSA and Lysotracker fluorescence in cultured cumulus cells treated with 10 µM (P1) and 50 µM (P2) of PIP2 for 12 and 24 hours. Fluorescent images were obtained at the time of application of the DQ-Red BSA substrate (DQ1) and after a 4-hour culture period (DQ2) and 30 minutes after the culture with Lysotracker.

**Fig. 2:** DQ-Red BSA and Lysotracker fluorescence in cultured cumulus cells treated with 10 µM (M1) and 50 µM (M2) of PMA for 12 and 24 hours. Fluorescent images were obtained at the time of application of the DQ-Red BSA substrate (DQ1) and after a 4-hour culture period (DQ2) and 30 minutes after the culture with Lysotracker.

**Fig. 3:** DQ-Red BSA and Lysotracker fluorescence in cultured cumulus cells treated with 10 µM (D1) and 50 µM (D2) of DOG for 12 and 24 hours. Fluorescent images were obtained at the time of application of the DQ-Red BSA substrate (DQ1) and after a 4-hour culture period (DQ2) and 30 minutes after the culture with Lysotracker.

**Fig. 4**:DQ-Red BSA and Lysotracker fluorescence in cultured cumulus cells for 12 and 24 hours. Fluorescent images were obtained at the time of application of the DQ-Red BSA substrate (DQ1) and after a 4-hour culture period (DQ2) and 30 minutes after the culture with Lysotracker. The fluorescence intensity of DQ-Red-BSA and Lysotracker in cultured cumulus cells for 12 and 24 hours at different concentrations of V-ATPase proton pump activators PIP2 (P1:10 µM, P2:50µM), PMA (M1:10µM, M2:50µM) and DOG (D1:10 µM, D2:50µM). C: Control group. \*(P < 0.05) (One-way ANOVA, Holm Sidak method).

**Fig. 5:**Western blot bands and graphs of ImageJ evaluations of Akt, MAPK1/3, p-Akt, and p-MAPK1/3 proteins of cultured cumulus cells for 12 and 24 hours at different concentrations of V-ATPase proton pump activatorsPIP2 (**P1:**10 µM, **P2:**50µM), PMA (**M1:**10µM, **M2:**50µM) and DOG (**D1:**10 µM, **D2:**50µM). **C:** Control group. For 12-hour groups: #(P < 0.05) (One-way ANOVA, Holm Sidak method). For 24-hour groups: \*(P < 0.05) (One-way ANOVA, Holm Sidak method).

**Data availability statement**

The authors confirm that the data supporting the findings of this study are available in the article

**Author contributions**

F.T. conceived the original idea and supervised the project. I.C. and F.T. carried out the experiments. I.C. wrote the manuscript with support from F.T.

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