Pyridoxine supplementation during oocyte maturation improves the development and quality of bovine preimplantation embryos

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Abstract.

Recently, inhibition of cathepsin B (CTSB) activity during in vitro maturation (IVM) and culture (IVC) improved the developmental competence and quality of bovine oocytes and embryos. E-64 is a widely used inhibitor to inhibit CTSB activity, however, E-64 inhibits not only CTSB activity but also the activities of other proteases including cathepsin L (CTSL), papain, calpain, and trypsin. Pyridoxine, the catalytically active form of vitamin B6, plays a crucial role in several cellular processes and has the ability to inhibit CTSB activity. However, whether pyridoxine has an improving effect during IVM of bovine oocytes is still unknown. In this study, we investigated the effect of pyridoxine supplementation during IVM on the developmental competence of bovine oocytes and the quality of the produced blastocysts. Supplementation of pyridoxine to the maturation medium significantly decreased the activity of CTSB in both bovine cumulus cells and oocytes. Moreover, pyridoxine improved both the blastocyst and hatched blastocyst rates. In addition, the presence of pyridoxine during IVM also significantly improved the quality of the produced embryos by increasing the total cell number as well as decreasing the CTSB mRNA expression and apoptotic rate. These results indicate that pyridoxine is a promising tool to improve the developmental competence of bovine oocytes and subsequent embryo quality.

Keywords: cathepsin B, pyridoxine, in vitro maturation, developmental competence, bovine oocytes

1. Introduction

Many studies have been performed to improve the developmental competence of bovine oocytes and embryos [1-4]. However, the quality of *in vitro* produced (IVP) embryos remains incomparable to that of *in vivo* produced embryos [5-7]. Many stressors during *in vitro* maturation (IVM), either extrinsic such as medium composition or culture conditions, or intrinsic such as oocyte quality itself, affect the developmental competence and quality of IVP embryos [8, 9]. As a result, it is necessary to find optimal conditions to overcome the negative factors affecting the developmental competence of bovine oocytes.

Cathepsin B (CTSB) is an abundant and ubiquitously expressed cysteine protease found in a wide variety of cells, including bovine oocytes and cumulus cells [10, 11]. CTSB is involved in many physiological processes, including intracellular protein degradation in lysosomes, initiation of the apoptotic pathway [12, 13], stress-induced response [14], autophagy [15], and differentiation of cancer cells [16]. We have shown that CTSB activity is inversely correlated with the quality and developmental competence of bovine oocytes [3, 10]. Thus, inhibiting CTSB activity during *in vitro* maturation (IVM) has emerged as a new strategy to improve the developmental competence of bovine oocytes *in vitro* [10].

l-trans-Epoxysuccinyl-Leucylamido-(4-guanidino) Butane (E-64) and its derivative compounds (CA-030 and CA-074) are the most commonly used inhibitors to inhibit CTSB activity [17, 18]. Unfortunately, the affinity of E-64 to bind an active thiol group in many cysteine proteases renders it a non-selective CTSB inhibitor [19]. In fact, it can also inhibit many cysteine proteases including CTSL, papain, calpain, as well as trypsin [20]. In addition, application of oral administration of E-64 has been achieved for protection from bacterial and viral infections [21-23], that raises the possible improvement of oocyte quality by *in vivo* administration of CTSB inhibitor. However, large scale application of CTSB inhibitor such as *in vivo* administration is impractical because of the cost and the possible toxicity. Thus, it is necessary to find a natural alternative inhibitor that could be used to regulate CTSB activity in mammalian oocytes either *in vivo* or *in vitro*.

Vitamin B_6 coenzymes such as pyridoxine is very similar to vitamin B_6 enzyme in terms of amino acid metabolism and synthesis of nucleic acids [24]. Vitamin B_6 has an essential role in antioxidant activities [25]. Singlet oxygen resistance 1 (*SOR 1*) is involved in *de novo* vitamin B_6 biosynthesis. Pyridoxine quenches singlet oxygen at a rate comparable to that of vitamin C and E, two of the most highly efficient biological antioxidants [26]. Interestingly, vitamin B_6 coenzyme has an active aldehyde at position 4 of the pyridine ring, which has a binding affinity for the active SH-site of cysteine residues in CTSB. This unique structure can explain the inhibitory effect of pyridoxine on CTSB activity in helper T lymphocyte type-2 [27]. Using pyridoxine as a CTSB inhibitor outperforms others by its natural origin from whole-grain products (including cereals), starchy vegetables, fish, liver and organ meats [28, 29]. In this study, we investigated the effect of pyridoxine on CTSB activity during oocyte maturation, and its effect on the subsequent development and quality of embryos.

2. Material and Methods

2.1. Oocyte collection and IVM

According to the strict regulation of Sapporo slaughterhouse and after BSE screening test result, bovine ovaries were brought to our laboratory from a local abattoir within 12 h after slaughter. The ovaries were washed several times in a sterile saline. IVM was performed as described previously [30]. In brief, cumulus–oocyte complexes (COCs) were aspirated from follicles (2–8 mm in diameter) using an 18-gauge needle attached to a 10-ml syringe and washed three times in tissue culture medium (TCM)-199 medium (Invitrogen, Grand Island, NY, USA). Ten COCs were matured in a 50-µl drop of TCM-199 supplemented with 10% fetal calf serum (FCS; Invitrogen), follicle stimulating hormone (FSH; 0.02 units/ml; Kyoritsu Seiyaku Corp., Tokyo, Japan), estradiol-17β (1 µg/ml; Sigma-Aldrich, St. Louis, MO, USA), and gentamycin (50 µg/ml; Sigma-Aldrich) covered with mineral oil (Sigma-Aldrich) for 22–24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. In vitro fertilization (IVF)

IVF was conducted according to a procedure described previously [31]. Briefly, after thawing of frozen semen in warm water (37 °C) for 20 sec, motile sperm were separated using percoll gradients (45 and 90%) (Sigma-Aldrich). COCs were co-incubated with motile sperm (5×10^6 cells/ml) in droplets (10 COCs/100 µl) of modified Brackett and Oliphant's isotonic medium containing 3 mg/ml fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich) and 2.5 mM theophylline for 18 h at 38.5 °C under a humidified atmosphere of 5% CO_2, and 90% N_2.

2.3. In vitro culture (IVC)

IVC of presumptive zygotes was performed as described previously [32]. Briefly, after fertilization, cumulus cells were removed by mechanical pipetting (internal diameter of the pipette, 150–180 mm) [33], and the presumptive zygotes were transferred to 50 µl drops (20–30 zygotes/drop) [34] of modified synthetic oviduct fluid (SOF) medium supplemented with amino acid solution (Sigma-Aldrich), 10 µl/ml insulin, 1 mM glucose, and 3 mg/ml fatty acid-free BSA at 38.5 °C under 5% CO_2, 5% O_2 and 90% N_2.

2.4. RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA from twenty blastocysts per replication was extracted using ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The extracted RNA was then immediately used for RT-PCR or stored at -80 °C until analysis. cDNA was synthesized with the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Conventional PCR was performed using the GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA). Primers specific for *CTSB* were designed and commercially synthesized (Eurofins Genomics, Co., Ltd., Tokyo, Japan). The primer information is presented in Table 1. The reactions were carried out in 96-well PCR plates, in a total volume of 10 µl containing 1 µl of 10 pmol/µl of each primer, 5 µl of the Thunderbird Sybr qPCR Mix (Toyobo), and 3 µl of cDNA. After centrifugation, the plates were placed in a Roche Light Cycler 480 II (Roche, Basel, Switzerland) and subjected to the following cycling conditions: a denaturation step at 95 °C for 30 sec, an amplification step of 50 cycles at 95 °C for 10 sec, 57 °C for 15 sec, 72 °C for 30 sec, a melting-curve step using a gradient of 55–95 °C with an increment of 2.2 °C/sec and continuous fluorescence acquisition, and a cooling step at 4 °C. Amplicons that consisted of PCR products were confirmed to have a single band for each target gene by electrophoresis and sequenced to verify their authenticity. The expression levels of the target genes were determined relative to that of the histone H2A family member Z (*H2AFZ*) [35].

2.5. Detection of CTSB activity

CTSB activity was measured by using Magic Red Detection Kit (MR-RR) 2 (Immunochemistry Technologies, LLC, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, IVM oocytes or COCs were stained in 250 µl of serum-free Dulbecco's modified Eagle medium (DMEM) containing 1 µl of reaction mix in a humidified atmosphere of 5% CO_2 at 38.5 °C for 30 min. For nuclei staining, Hoechst (H 33342; Sigma-Aldrich) was added and incubated in the same culture conditions for 5 min. After rinsing in phosphate-buffered saline (PBS), the stained oocytes or COCs were mounted onto a glass slide and observed under a fluorescence microscope BZ-9000 Biorevo (Keyence, Osaka, Japan). An excitation filter of 590 nm was used for CTSB detection (red), while an excitation filter of 365 nm was used for observing the cumulus cell nuclei (blue).

2.6. Differential staining

Differential staining for bovine embryos was carried out as described previously [36]. In brief, blastocysts were incubated at room temperature for 40–60 sec in 0.2% (v/v) Triton-X100 with 0.1 mg/ml propidium iodide (P4864; Sigma-Aldrich). Blastocysts were then stained with 25 µg/ml of Hoechst reagent (Sigma-Aldrich) in 100% (w/v) EtOH at 4°C for 3 h. The stained blastocysts were rinsed in glycerol, mounted onto a glass slide, and observed with a fluorescence microscope (Nikon, Tokyo, Japan). The nuclei of the inner cell mass (ICM) were stained in blue by Hoechst reagent and the nuclei of trophoectoderm (TE) cells were stained in pink by both Hoechst reagent and propidium iodide.

2.7. Apoptosis analysis

A Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay kit was used to assess the presence of apoptotic cells (In Situ Cell Death Detection Kit; Roche) in day 8 blastocysts, as described previously [10] with some modifications. In brief, blastocysts were fixed in 4% (w/v) paraformaldehyde solution for 15–30 min. After rinsing 4 times in PBS, blastocysts were permeabilized in PBS with 0.2% Triton-X and 0.2% polyvinylalcohol (PVA) for 20 min. Blastocysts were then washed thrice in PBS with 0.1% Triton-X and 0.3% BSA for 10 min. The fragmented DNA ends were labeled with fluorescein-dUTP for 60 min at 37°C. After incubation, the blastocysts were washed thrice in PBS with 0.2% PVA for 10 min each, followed by mounting onto glass slides using mounting solution (Vectashield with DAPI, Vector Laboratories, Burlingame, CA, USA). The fluorescence of fragmented DNA was detected by using a fluorescence microscope BZ-9000 Biorevo (Keyence) and 450-500-nm excitation filter.

2.*8. Experimental design*

Experiment 1: Effect of pyridoxine during IVM on CTSB activity of COCs and oocytes

To investigate the inhibitory effect of Pyridoxine HCL (Sigma–Aldrich) on CTSB, CTSB activity was examined in bovine COCs and oocytes matured in TCM-199 with or without 250 μM (prepared in PBS) pyridoxine for 24 h. The concentration of pyridoxine was selected based on a previous study [24]. After maturation, both COCs and denuded oocytes were evaluated for

CTSB activity using Magic Red Detection Kit. Denuded oocytes were obtained by mechanical removal of cumulus cells in PBS supplemented with 0.1% (W/V) hyaluronidase (H3506; Sigma-Aldrich).

Experiment 2: Effect of pyridoxine supplementation during IVM on developmental competence of bovine oocytes

Pyridoxine (250 µM) was added to the maturation medium to assess its effect on the subsequent developmental competence of oocytes. After 24 h of maturation, matured COCs were fertilized and cultured at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Developmental competence was assessed by calculating the cleavage and blastocyst formation rates on days 2 and 8, respectively. In addition, the hatched rate was also calculated for day 8 blastocysts.

Experiment 3: Effect of pyridoxine supplementation during IVM on the quality of day 8 blastocysts

After COCs maturation with or without 250 µM pyridoxine, COCs were fertilized and cultured for 8 days. The quality of day 8 blastocysts was evaluated using total cell number, differential staining, TUNEL staining, and *CSTB* gene expression.

2.9. Statistical analysis

Each experiment was performed at least three times, and the data are expressed as the means ± standard error of the mean (SEM). The statistical significance was analyzed by Student's *t*-tests. Non-parametric Mann-Whitney's test was also employed to confirm the significance. Data for cleavage and blastocyst rates were analyzed by one-way ANOVA with Fisher protected least significant difference using the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA); $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of pyridoxine on CTSB activity in IVM COCs and oocytes

To confirm the inhibitory effect of pyridoxine on CTSB activity in bovine oocytes and cumulus cells, COCs were cultured with or without 250 µM pyridoxine for 24 h followed by detection of CTSB activity. CTSB activity was clearly decreased in the cumulus cells of IVM COCs matured with 250 µM pyridoxine (Fig. 1B,D) compared with those matured without pyridoxine (Fig. 1A,C). Quantification of the fluorescence intensity corresponding to CTSB activity indicated a significant decrease in pyridoxine-treated COCs when compared with that of control COCs (Fig. 1E). In addition, CTSB activity was significantly lower in denuded oocytes matured with 250 µM pyridoxine (Fig. 1G,H) than denuded oocytes matured in pyridoxine-free medium (Fig. 1F, H).

3.2. Effect of pyridoxine supplementation during IVM on the developmental competence of bovine oocytes

Different concentrations (0, 100, 250, and 500 µM) of pyridoxine were added to the IVM medium and both cleavage and blastocyst formation rates were evaluated on days 2 and 8, respectively. Although cleavage rate did not differ significantly among treated groups, the highest blastocyst formation rate was observed in the presence of 250 µM of pyridoxine (Fig. S1). Therefore, pyridoxine concentration of 250 µM was selected for further evaluation.

Interestingly, addition of 250 µM pyridoxine to the maturation medium increased the blastocyst formation rate and the percentage of hatched blastocysts significantly (Table 2).

3.3. Effect of pyridoxine supplementation during IVM on the quality of blastocysts

The quality of preimplantation embryos could be evaluated using many parameters including the total cell number, the number of trophoectoderm cells and the apoptotic rate. The results of differential staining showed that pyridoxine significantly increased the number of both total cells and trophectoderm cells of day 8 blastocysts when compared with the control group (Table 3). Importantly, the percentage of TUNEL-positive cells in blastocysts derived from COCs matured with pyridoxine was significantly lower than that in the control group (Fig. 2A,C). Consistent with this result, *CTSB* expression was decreased significantly in blastocysts obtained from pyridoxine-supplemented COCs than that of the control group (Fig. 2B,D).

4. Discussion

Our results showed that pyridoxine is an efficient tool to inhibit CTSB activity in bovine COCs and oocytes. Moreover, supplementation of pyridoxine during IVM improved the developmental competence and quality of the produced blastocysts. These results suggest a promising role of pyridoxine in improving the efficiency of *in vitro* embryo production.

Pyridoxine inhibits CTSB activity in helper T lymphocyte type-2 [27]. This finding is in agreement with our results showing that pyridoxine inhibits CTSB activity in bovine oocytes. This inhibitory effect of pyridoxine on CTSB might be attributed to the presence of active aldehydes at position 4 of the pyridine ring. Such unique structure is critical to inhibit CTSB activity by its affinity for the active SH-site of CTSB forming irreversible thiosemiacetal bonds [24]. In addition, vitamin B_6 has also antioxidant activities [25]. *SOR 1* showed that pyridoxine quenches singlet oxygen at a rate comparable to those of vitamin C and E, two of the most highly efficient biological antioxidants [26]. However, the possible effect of pyridoxine on the developmental competence of mammalian oocytes was unknown.

CTSB inhibition emerged as a new approach to improve the developmental competence of bovine oocytes and preimplantation embryos [3, 10, 37]. Although the developmental competence of control oocytes was relatively low, most likely due to the unavoidable delay of ovary retrieval from the slaughterhouse, addition of pyridoxine to the IVM medium significantly improved the developmental competence of bovine oocytes by increasing the blastocyst rate. The average total cell number and the percentage of TUNEL positive cells can be used as markers for evaluating the quality of preimplantation embryos [36]. Addition of pyridoxine to the IVM medium increased the number of trophectoderm cells and the total cell number of day 8 blastocysts and decreased the apoptotic rate. We previously demonstrated that CTSB activity is inversely correlated with the quality of bovine embryos and, thus, can be used as an indicator of embryo quality [3, 38]. Our data showed that addition of pyridoxine to the IVM medium significantly decreased *CTSB* mRNA expression in the produced blastocysts. Collectively, these results indicate that pyridoxine is a novel component to enhance the developmental competence of bovine oocytes and the quality of their blastocysts.

Our observation of improved developmental competence of pyridoxine-treated oocytes is consistent with the results obtained with E-64, another CTSB inhibitor. Addition of E-64 to the IVM medium significantly improved the developmental competence of bovine oocytes [10, 11] and the quality of the produced embryos [3]. Although E-64 has beneficial effect on developmental competence of bovine oocytes, it has been known to inhibit several types of cysteine proteases including CTSL papain, calpain, and trypsin [20]. On the other hand, to our knowledge, pyridoxine does not have the ability to inhibit these proteases. Taken together, this observation suggests that both inhibitors improved the developmental competence of bovine oocytes by inhibiting CTSB activity, but not through inhibiting other proteases. Furthermore, the similar effects of pyridoxine and E-64 on oocyte developmental competence and subsequent quality supports the hypothesis that pyridoxine is an effective inhibitor of CTSB activity and provides further evidence that inhibiting CTSB activity is a promising approach to improve the developmental competence of bovine oocytes.

The beneficial effect of pyridoxine on the developmental competence of oocytes might be attributed to its effect on inhibiting CTSB-induced apoptosis pathway. Apoptosis has been shown to be induced by proteases such as CTSB that leaked from lysosomes partially damaged by moderate stress [9, 39]. CTSB leakage can initiate apoptosis by activating initiator caspases and executioner caspases [40]. The significant decrease the rate of TUNEL-positive cells and *CTSB* transcript abundance in blastocysts produced from pyridoxine-treated oocytes, suggests that pyridoxine improved the developmental competence of bovine oocytes by inhibiting CTSB, which in turn, perturbed the apoptotic pathway. However, further investigations are required to elucidate whether pyridoxine has another pathway to promote the developmental competence of bovine oocytes.

Pyridoxine has been used to treat diseases including adult-onset clinical conditions [29] and carpal tunnel syndrome [41]. Importantly, although no data for developmental competence and quality of oocytes, pyridoxine was used as a maternal dietary supplement to affect gene expression patterns of *in vivo* derived porcine blastocysts [42]. In addition, pyridoxine could be used to treat nausea and vomiting during pregnancy in women [29, 43]. Taken together, the natural and nontoxic properties of pyridoxine make it an important candidate as a natural drug to improve the developmental competence and the quality of embryos by inhibiting CTSB *in vivo*.

In conclusion, our results show that 1) pyridoxine can inhibit CTSB activity during oocyte maturation and 2) addition of pyridoxine to the IVM medium is a promising tool to enhance the developmental competence of bovine oocytes and the quality of their embryos.

5. Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

6. Submission declaration

The authors declare that the work described above has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

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Figure legends

Figure 1: Pyridoxine inhibits CTSB activity in bovine oocytes.

CTSB activity was higher in IVM COCs matured without 250 µM pyridoxine (A) than that matured with pyridoxine (B). The corresponding DNA was labelled by Hoechst 33342 (C and D). Quantification of CTSB activity was evaluated by measuring the fluorescence intensity (Fig. 1E). Total number of COCs used is 59. CTSB activity was higher in IVM oocytes matured without pyridoxine (F) than those matured with pyridoxine (G). Corresponding quantification of CTSB activity (H). PN, pyridoxine. Total number of oocytes used is 63. The experiments were replicated 3 times. Original magnification is 100X. The data are expressed as the means ± SEM.; * $P < 0.05$, *** $P < 0.001$.

Figure 2 Effect of pyridoxine treatment during IVM on the percentage of TUNNEL positive cells and *CTSB* expression in day 8 blastocyst.

Number of TUNEL positive cells was higher in the blastocysts from oocytes matured without 250 µM pyridoxine (A) than those matured with pyridoxine (B). The corresponding DNA was labelled (C and D). Corresponding quantification of the percentage of TUNEL positive cells (E). This experiment was replicated 3 times and total number of embryos used is 43. *CTSB* expression was significantly lower in the resultant blastocysts from oocytes matured with pyridoxine than control group (B). PN, pyridoxine. This experiment was replicated 3 times. The data are expressed as the means ± SEM.; * $P < 0.05$.

Figure S1 Effect of different concentrations of pyridoxine during IVM on cleavage and blastocyst rates.

Bovine COCs were matured for 24 h in maturation medium supplemented with different concentrations (0, 100, 250 and 500 µM) of pyridoxine to evaluate the dose-dependent effect. IVM COCs were fertilized prior to IVC for 8 days. Highest blastocyst rate was achieved by using 250 µM pyridoxine (PN). However, cleavage rates showed no significant difference among the concentrations. This experiment was replicated 4 times and the total number of oocytes was 613. The data are expressed as the means ± SEM.; * $P < 0.05$.

Table 1: List of oligonucleotide primers used for RT-PCR

Target gene	Gene bank accession number	Primer sequence (5' -3')	Product length (bp)
CTSB	NM_174031.2	CACTTGGAAGGCTGGACACA	141
		GCATCGAAGCTTTCAGGCAG	
H2AFZ	NM_174809.2	AGAGCCGGTTTGCAGTTCCCG	116
		TACTCCAGGATGGCTGCGCTGT	

Table 2: Effect of pyridoxine on the developmental competence of bovine oocytes

Treatment	No. of replicates	Cleavage rate (%)	Blastocyst rate (%)	Hatched Blastocysts (%)
Control	6	50.9 ± 7.0[a]	17.6 ± 3.1[a]	8.3 ± 2.3[a]
250 μM PN	6	69.3 ± 5.4[a]	32.4 ± 4.5[b]	21.4 ± 4.2[b]

Total number of putative zygotes used was 299. PN: pyridoxine. Values with different letters across treatments differ significantly. The data are expressed as the means ± SEM.; $P < 0.05$.

Table 3: Effect of pyridoxine on cell number and allocation of day 8 blastocysts

Treatment	No. of Blastocyst examined	No. of cells in		
		ICM	TE	Whole embryo
Control	20	33.0 ± 0.7[a]	86.9 ±1.8[a]	119.9 ± 2[a]
250 μM PN	21	31.7 ± 0.7[b]	106.1 ± 2.4[b]	137.8 ± 2.5[b]

PN: pyridoxine, ICM: inner cell mass, TE: trophectoderm and TCN: total cell number. Values with different letters across treatments differ significantly. The data are expressed as the means ± SEM.; $P < 0.05$.

Fig. 1:

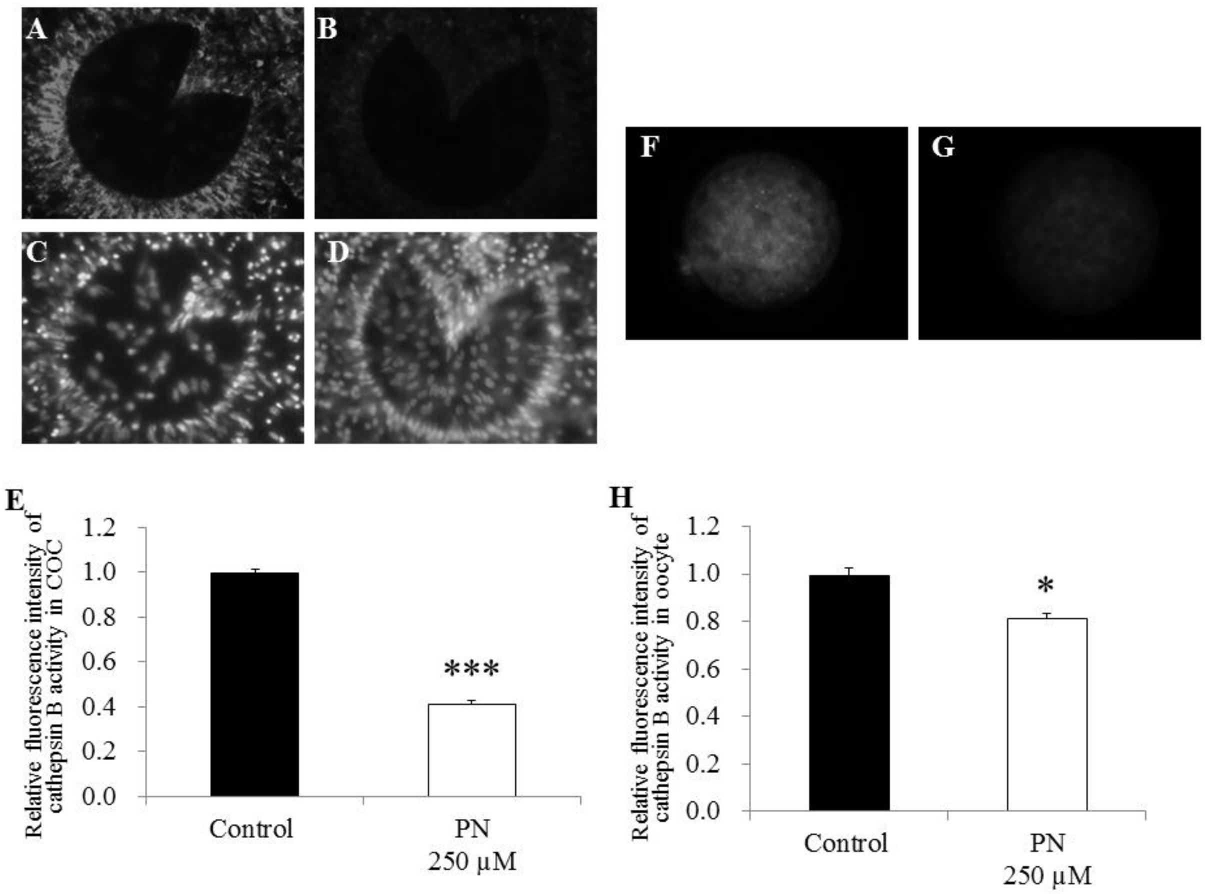
A
B
C
D
F
G
E
Relative fluorescence intensity of cathepsin B activity in COC
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
Control
PN
250 µM
H
Relative fluorescence intensity of cathepsin B activity in oocyte
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
PN
250 µM

Fig: 2

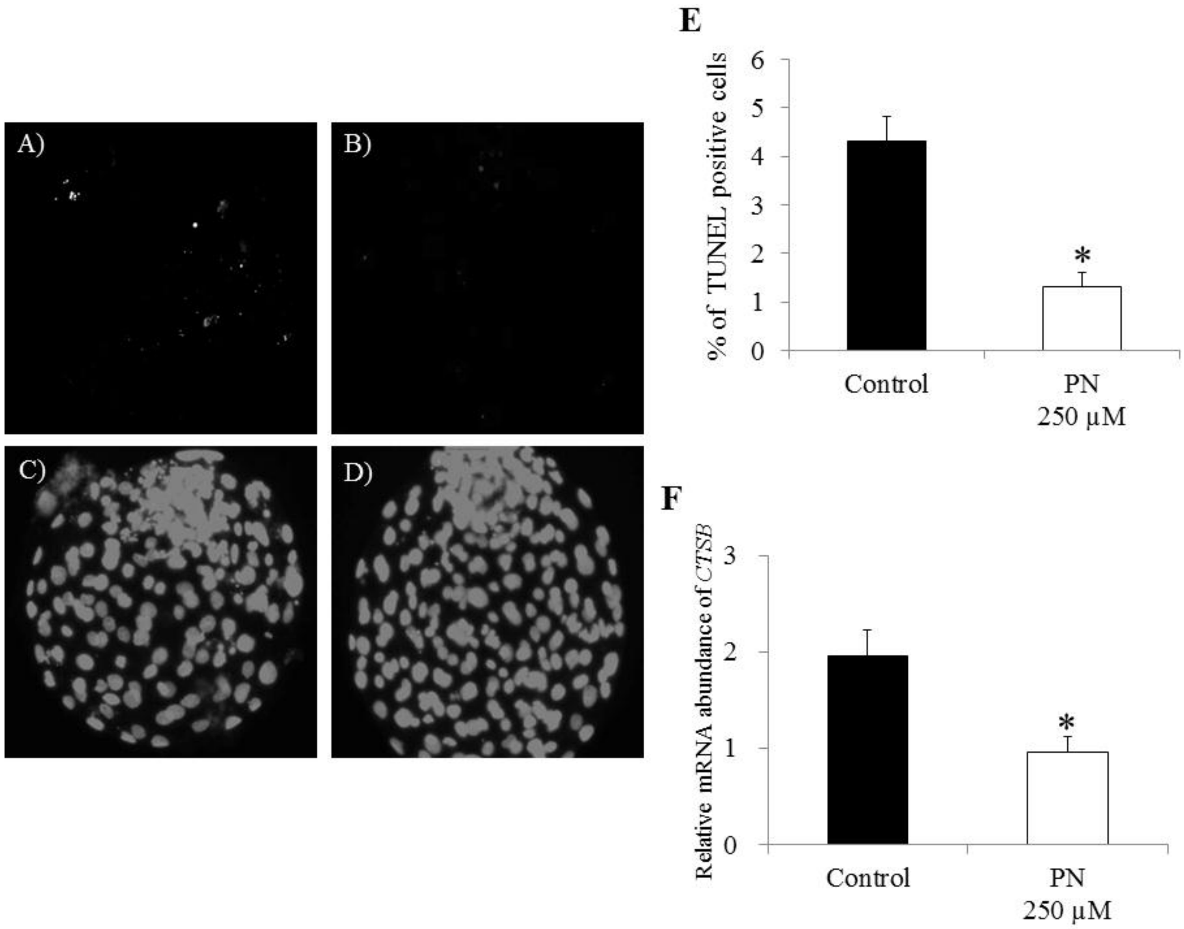
A)
B)
C)
D)
E
% of TUNEL positive cells
0
1
2
3
4
5
6
*
Control
PN
250 µM
F
Relative mRNA abundance of CTSB
0
1
2
3
*
Control
PN
250 µM

Fig. S1: