# Betaine Ameliorates Hyperosmotic Stress-Induced Apoptosis and Autophagy of Porcine Intestinal Epithelium In Vivo and Vitro.

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**Research**

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# Abstract

**Background:** Hyperosmotic stress resulting from lumen contents is a big challenge to the normal function of the intestinal epithelium. Betaine is a potent organic osmolyte, but it is mostly studied in kidney. The purpose of this study was to gain insight into the osmoprotectant role of betaine in intestinal epithelium of piglets and intestinal porcine epithelial cells (IPEC-J2 cells) under hyperosmotic condition.

**Results:** The result showed that the osmolarity of intestinal chyme was much higher than that of plasma ($P < 0.05$), indicating a natural hyperosmotic environment of intestinal lumen and subsequently leading to hyperosmotic stress to intestinal epithelium. Meanwhile, hyperosmolarity corresponding to intestinal environment was simulated by 150 mmol/L NaCl *in vitro* and caused a significant decrease of cell viability ($P < 0.05$). It was found that betaine could remarkably decrease hyperosmolarity-induced reactive oxygen species (ROS) of intestinal epithelium *in vivo and vitro* ($P < 0.05$) with the significant restoration of cell shrinkage ($P < 0.05$). Furthermore, since hyperosmolarity caused mitochondrial-related apoptosis with the remarkable increase of cleaved Caspase3, cleaved PARP-1, cytoplasm cytochrome *c* as well as obvious decrease of Bcl-2 in protein level ($P < 0.05$), betaine prevented mitochondria from membrane collapse and alleviated apoptosis ($P < 0.05$) *in vivo and vitro*. Meanwhile, it was also confirmed that betaine reduced hyperosmotic stress-induced apoptotic incidence in IPEC-J2 cells via fluorescence microscope and flow cytometry ($P < 0.05$). In addition, betaine supplementation significantly suppressed hyperosmotic stress-induced elevated expression of LC3 II and reduced expression of p62 ($P < 0.05$). indicating that betaine ameliorated autophagy of porcine intestinal epithelium caused by hyperosmolarity *in vivo and vitro*. Autophagic flux determined by mRFP-GFP-LC3B system in IPEC-J2 cells was in agreement with the result of western blotting as well ($P < 0.05$).

**Conclusions:** Betaine could alleviate hyperosmotic stress-induced cell shrinkage, ROS accumulation as well as ameliorate subsequently apoptosis and autophagy in small intestinal epithelium of piglets and IPEC-J2 cells.


# Background

Betaine, known as trimethyl glycine, is a nontoxic and stable natural compound which is widespread in animals, plants and microorganism[1]. Because of its three methyl groups, betaine is a potent methyl donor participating in methionine-homocysteine cycle and consequently influencing the lipid metabolism[2]. Thus,betaine is widely used in livestock production to decrease fat accumulation in broilers and pigs[3]. Besides, with the neutral zwitterionic structure, betaine acts as a vital osmolyte in organisms including bacteria, plants, marines and mammals[4]. Exposed to a hyperosmotic environment, cell suffers hyperosmotic stress as it loses water rapidly leading to cell shrinkage and accumulation of inorganic ion, which results in production of ROS, macromolecular crowding and cytoskeletal arrangement, proteins damaging and aggregation, depolarize mitochondria and even apoptosis[5; 6; 8; 7; 9]. Betaine exerts an osmoprotective effect by helping to minimize water loss and maintain cell volume as well as protect proteins and membrane from instability by inorganic ions in hyperosmotic

environment[10; 11]. Recently, there were studies reporting that hyperosmotic stress could induce autophagy during which cells degraded damaged proteins or cytoplasmic organelles within membrane-bound organelles (autophagosomes) fusing with lysosomes[12–14]. But the effect of betaine on hyperosmotic stress-induced autophagy is still unclear.

The gut epithelium is not only one of the most important barriers for animals[15], but also the place where nutrients are digested and absorbed, which plays an important role in animal health and growth. However, due to the dramatically constant change of the lumen contents which is hyperosmotic in relation to plasma[16; 17], the intestinal epithelial cells have to undergo the variable osmolarity and are especially prone to change in cell volume[10; 18]. For piglets, the underdeveloped intestine is more likely to suffer hyperosmotic stress and be damaged by the fluctuating contents which can be one of the reasons of its weakened absorbing function and the decline of growth performance[4]. Thus, it is of great necessity to explore how to alleviate the harmful effects of hyperosmolarity on intestinal epithelium. However, most studies of betaine as an organic osmolyte were documented in kidney[19; 20], and few attentions were focus on osmoregulatory function of betaine in intestine. Kettunen et al. reported that betaine might act as osmolyte to affect the movement of water across the intestinal epithelium in broiler chicks[21]. It was further confirmed by Abdulaziz et al. that betaine could modulate water channel activity to regulate water deprivation of the intestine[22]. In our previous study, it was found that betaine could enhance intestinal function in hyperosmotic stressed rats and improve intestinal structure of piglets, which was likely to relate with its osmoregulatory function [4; 23]. Nevertheless, what the protective mechanism of betaine in the small intestine against hyperosmolarity is remains poor understood. Hence, in this study, we aim to investigate the hyperosmotic damage to the porcine intestinal epithelium and the osmoprotective function of betaine counteracting the deleterious effect *in vivo and vitro*.

## Methods

# Animal experiments and sample collection

The experiment processes were conducted strictly according to the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). A total of 39d 150 crossbred weaning piglets (Duroc× Landrace× Yorkshire, weaned at 25d, n = 5/treatment; 8.52 ± 0.26 kg) were randomly allocated into three groups with different dietary treatment which were basal diet supplemented with 0, 1250, 2500 mg/kg betaine, respectively. All pigs could get water and feed freely. The basal diets were formulated in accordance with NRC 2012. The betaine in this trial was provided by the Healthy Husbandry Sci-tech Co., Ltd (Hangzhou, China) with the purity of 994 g/kg. Different segment of small intestine and intestinal chyme were collected separately after 30 days feeding and were stored in -80℃ for further analysis. Plasma was isolated from blood by centrifuging at 3000×g for 15 min at 4°C and then frozen at − 20°C until analysis.

# Cell culture and treatment

Isosmotic and hyperosmotic medium were used to further explore the osmoprotective function of betaine against deleterious effect induced by hyperosmolarity. It was set four treatments in which cells were cultured for 12 h: isosmotic medium (C), isosmotic medium with 20 mmol/L betaine (CB), hyperosmotic medium (H), hyperosmotic medium with 20 mmol/L betaine (HB). For hyperosmolarity, hyperosmotic medium (600mOsM) was made by adding 150 mmol/L NaCl into the isosmotic medium. Betaine used in cell experiments was purchased from Sigma-Aldrich (St. Louis, MO, USA) and it was dissolved directly into the medium. Before treatment, IPEC-J2 cells were cultured in complete medium, which is fresh DMEM/F12 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Sigma, USA), in the incubator with 5% $CO_2$ at 37℃.

# Measurement of osmotic pressure

The intestinal chyme was diluted with deionized water according to the weight-volume ratio of 1:9 (w/v = 1:9). The osmotic pressure of plasma, chyme and medium was directly measured by the freezing point osmometer (Loser, German).

# Cell viability assays

IPEC-J2 cells were seeded on a 96-well plate so that each well of cells were allowed to reach 80% confluence after 24 h. Then the original medium was replaced by the conditional medium mentioned above and cells were treated for 2 h, 6 h, 12 h, 24 h, respectively. Each treatment replicated for 6 times. After that, cells were incubated with fresh medium containing 10 uL CCK-8 reagent (Biosharp, China) for 2 h. Absorbance at 450nm was measured by a microplate reader (Bio-Rad, USA).

# Cell volume and apoptosis detection

Cells were seeded on the 6-well plate at the density of $1\times10^5$ per well. After 24h, cells were treated with different medium for 12 h. Then, cells were collected and washed with cold sterile phosphate buffer saline (PBS) for 2 times. After cells were suspended with 500 uL 1X binding buffer, mix 5 uL FITC Annexin V and 5 uL PI (KeyGen Biotech, China) with cell suspension in order. Cells were incubated in the dark at room temperature for 10 minutes. As soon as possible, cells were viewed under a fluorescence microscope (OLYMPUS, Japan) and analyzed by flow cytometry (BD FACSCalibur, USA).

# Measurement of ROS and mitochondrial membrane potential(△Ψm) of cells

Cells were seeded on the 6-well plate at the density of $1\times10^5$ per well. After 24 h, cells were treated with different medium for 12 h after which they were collected and washed with cold PBS.

As for ROS measurement, cells were suspended with 500 uL PBS containing 5 uM CM-H2DCFDA dye (Invitrogen, USA) for cell ROS detection or 5 uM MitoSOX Red dye (Invitrogen, USA) for mitochondrial ROS detection. As for mitochondrial membrane potential(△Ψm) measurement, cells were added with 100nM TMEM cell staining solution (Invitrogen, USA).

After reaction in the dark at the cell incubator for 10 minutes, resuspend the cells with fresh PBS and analyze by flow cytometry (BD FACSCalibur, USA).

# Measurement of cell autophagosomes and autolysosomes

Cells were seeded on the 24-well plate at the density of $6\times10^4$ per well. After 12 h, cell confluence reached 50% and then cells were transfected with mRFP-GFP-LC3 adenoviral vectors (MOI = 60) (HanBio Technology, China). When the transfection efficiency reached 80%, the transfected cells were reseeded into the confocal dish at the density of $1\times10^5$ per well. After 12 h, cells were treated as indicated and were visualized by confocal microscopy. Autophagy and autophagic flux were determined by evaluating the number of GFP and RFP puncta respectively (puncta/cell were counted).

# Frozen section immunofluorescence for intestinal ROS

Intestinal samples were made frozen sections by Cryotome E (Thermo, USA). Frozen sections were rewarmed at room temperature and drew a circle with the histochemical pen. Then add autofluorescence quencher (ServiceBio, China) to the circle for 5 minutes and rinse with running water for 10 minutes. The tissue was incubated with ROS dye (Sigma, USA) in the dark at 37℃ incubator for 30 minutes followed by incubating with DAPI dye (ServiceBio, China) in the same environment for 10 minutes. Slides were mounted with antifade solution (ServiceBio, China) and were observed by fluorescence microscope (Nikon, Japan). The images were quantified by Image J program.

# Western blot analysis

Cells were treated as indicated and were lysed by RIPA Lysis Buffer (Beyotime Tech, China) with 1% PMSF (BosterBio, China) while intestinal samples were lysed by Subcellular Structure Nuclear and Cytoplasmic Protein Extraction Kit (BosterBio, China), which gathered the total cellular proteins. To separate mitochondria and cytoplasmic proteins, cells and intestinal samples were extracted by Subcellular Structure Mitochondrial Extraction Kit (BosterBio, China). The concentration of cell proteins was determined by BCA assay (KeyGen Biotech, China). 20 μg of protein samples were electrophoresed through SDS polyacrylamide gels and transferred electronically onto PVDF membranes. The membranes were blocked with 5% fat-free milk for 2 h and then incubated with primary antibody at 4℃ overnight. After washing the membranes with TBST buffer for three times, the relevant secondary antibodies were applied at room temperature for 2 h. Do the washing matters again and detected the chemiluminescence signals by ECL Chemiluminescence solution (Biosharp, China) and visualized using Chemiluminescence imager (Bio-Rad, USA). The information of primary antibodies was listed in **Supplemental Table 1**.

Subsequently, the images were analyzed by Image Lab software. All expression levels were normalized to β-actin.

# Statistical analysis

All experiments were independently repeated for at least three times. All data were assessed by one-way ANOVA using IBM SPSS Statistics 26 following LSD posthoc test. In this study, $P < 0.05$ was regarded as

statistically significant.

## Results

# The osmotic pressure of small intestine in piglets

As shown in Table 1, regardless of the treatment, the osmotic pressure of intestinal chyme was all significantly far higher than plasma, indicating a natural hyperosmotic environment in intestinal lumen ($P < 0.05$). Betaine supplementation did not have remarkable influence on the osmotic pressure of plasma and intestinal chyme among different treatments ($P > 0.05$).

Table 1
Osmotic pressure of different parts of piglets (mOsM/kg)

| Project | Betaine, mg/kg | | |
|---|---|---|---|
| | 0 | 1250 | 2500 |
| Plasma | 325 | 332 | 339 |
| Chyme | | | |
| Duodenum | 585 | 615 | 625 |
| Jejunum | 575 | 620 | 615 |
| Ileum | 545 | 580 | 585 |
| Compared to plasma, the osmolarity of different segment of intestine chyme were all significantly higher ($P < 0.05$). | | | |

# Betaine reduced ROS deposition in intestinal epithelium in piglets

Hyperosmotic stress triggers harmful effects on cells including the elevation of ROS. Figure 1 showed the effect of betaine on ROS of small intestine that dietary betaine could significantly reduce ROS production in intestinal epithelium of piglets ($P < 0.05$). But there was no significant difference between the two groups with different dose of betaine ($P > 0.05$).

# Betaine alleviated apoptosis of intestinal epithelium in piglets

It has been reported hyperosmotic stress could cause apoptosis in other type of cells[5], which may influence the morphology of tissues. Based on the morphology of small intestine previously studied[4], the key proteins involved in apoptosis were measured by western blotting to determine whether apoptosis was responsible for hyperosmotic stress-induced poor intestinal morphology. Cleaved Caspase3 is a marker for apoptosis and it is active enough to cut target protein including PARP-1(ADP-ribose polymerase). As shown in Fig. 2A, dietary betaine could significantly decrease the expression of cleaved

Caspase3 and cleaved PARP-1 as well as increase the expression of PARP-1($P < 0.05$), indicating the alleviation of apoptosis with betaine supplementation.

Apoptosis is accompanied by a sequence of characteristic biochemical changes, including mitochondrial outer membrane permeabilization (MOMP) resulting in the release of cytochrome *c* from the mitochondrial intermembrane space, which can be depressed by Bcl-2. Mitochondria is also the main target of ROS to be attacked. Thus, related proteins were determined to further confirm the apoptotic incidence. As shown in Fig. 2B, betaine supplementation remarkably decreased the expression of cytoplasm cytochrome *c* ($P < 0.05$) while it was obvious to increase the expression of Bcl-2 ($P < 0.05$), which indicated that betaine supplementation ameliorated apoptosis, possibly related to mitochondrial dysfunction, in small intestine of piglets.

# Betaine attenuated autophagy of intestinal epithelium in piglets

Microtubule-associated protein light chain 3 (LC3) is an essential component of autophagy. During the process of autophagy, LC3 I can be converted to LC3 II and the latter binds to autophagosomal membrane. Therefore, the ratio of LC3 II to LC3 I is a signal of autophagy. p62(SQSTM1, Sequestosome 1) is a substrate of autophagy and it is decreased when lysosomes do degradation work, so that its protein level is opposite to the level of autophagy.

In Fig. 3, it was shown that dietary betaine decreased the ratio of LC3II to LC3I ($P < 0.05$) and increased the protein level of p62 ($P < 0.05$). But there was no remarkable difference on autophagy-related protein expression between the groups with different doses of betaine ($P > 0.05$). The results suggested that betaine supplementation could alleviate autophagy of small intestine in piglets.

### Betaine attenuated the decrease of cell viability and restored the cell shrinkage induced by hyperosmotic stress in IPEC-J2 cells

IPEC-J2 cells were used to further confirm the effects of hyperosmolarity and betaine on porcine small intestine *in vitro*. According to the preliminary research of our own, 150 mmol/L NaCl was added to create a hyperosmotic model *in vitro* which was closed to the osmotic pressure of intestinal environment *in vivo*. The osmotic pressure of four treating mediums was listed in Table 2. As shown in Fig. 4, compared to the control, hyperosmolarity significantly reduced the cell viability ($P < 0.05$) since treated 2 h. Betaine could attenuate the hyperosmotic-induced reduction of cell viability after treating 12 h ($P < 0.05$). Interestingly, under isosmotic condition, cell viability increased or had the increasing trend in the presence of betaine.

Table 2
The osmotic pressure of different treating medium

| Treating medium | C | CB | H | HB |
|---|---|---|---|---|
| Osmotic pressure (mOsm/kg $H_2O$) | 292 ± 2.25 | 308.5 ± 2.05 | 598 ± 6.57 | 609 ± 4.22 |
| C-complete medium, CB- complete medium with 20 mmol/L betaine, H- hyperosmotic medium, complete medium with 150 mmol/L NaCl, HB- hyperosmotic medium with 20 mmol/L betaine. | | | | |
| Osmotic pressure of four groups was all significantly different with each other ($P < 0.05$). | | | | |

Based on the result of cell viability above, morphology of IPEC-J2 cells with different treatment for 12 h were observed. Light microscopy (Fig. 5A) illustrated that a considerable part of cells in H group went through cell shrinkage and floated. Betaine supplementation visually decreased the number of non-adherent and shrunken cells under hyperosmotic condition while there was no visible difference of cells under isosmotic condition with or without betaine. Flow cytometry was used to quantify the cells with normal volume and shrunken cells under different treatment (Fig. 5B). It was shown that hyperosmolarity induced cell shrinkage ($P < 0.05$) and betaine could attenuate the reduction of cell volume ($P < 0.05$).

# Betaine reduced hyperosmotic stress-induced cellular and mitochondrial ROS in IPEC-J2 cells

90% intercellular ROS is generated by oxidative respiratory chain in mitochondria[24]. Thus, based on the result of ROS *in vivo*, cellular and mitochondrial ROS were monitored in IPEC-J2 cells. As shown in Fig. 6A&B, compared to the control, hyperosmolarity obviously increased the average fluorescence intensity of ROS and mitochondrial ROS ($P < 0.05$) which could significantly blocked by betaine supplementation ($P < 0.05$). Notably, the extent of decrease of mitochondrial ROS is more than cellular ROS ($P < 0.05$) suggesting the potential protective role of betaine on mitochondria under hyperosmotic condition.

# Betaine alleviated hyperosmotic stress-induced apoptosis in IPEC-J2 cells

Based on the result above, mitochondria is possible to have relation with hyperosmotic stress-induced apoptosis. Hence, MOMP, a key indicator of mitochondrial function, and relevant proteins were measured in this trial. It was shown in Fig. 7A that hyperosmolarity decreased the level of MOMP significantly ($P < 0.05$) and betaine could visibly recover the loss ($P < 0.05$) in IPEC-J2 cells. Consistently, as shown in Fig. 7B, the protein expression of Bcl-2 was reduced and the protein expression of cytoplasm cytochrome *c* was improved in H group compared to control ($P < 0.05$), which was blocked in the presence of betaine ($P < 0.05$). What's more, downstream relevant proteins of apoptosis were also monitored corresponding to internal experiments. A significant elevation on the protein expression of cleaved Caspase3 and cleaved RARP-1 ($P < 0.05$) as well as an obvious reduction on the protein expression of RARP-1($P < 0.05$) in H group compared to control was shown in Fig. 7B, which was all reversed in HB group ($P < 0.05$). And there

was no obvious difference between the C group and CB group ($P > 0.05$). These results confirmed the suppose that betaine could ameliorate the hyperosmotic stress-induced apoptosis related to mitochondria.

To further determine whether betaine could exert protective function on apoptosis of the whole cell, apoptotic incidence was estimated by Annexin V, PI staining. Together the result in Fig. 7C&D, hyperosmolarity induced apoptotic incidence significantly ($P < 0.05$) which could be suppressed by betaine supplementation ($P < 0.05$).

Taken together, it is concluded that betaine ameliorates cell apoptosis induced by hyperosmolarity.

**Betaine attenuated hyperosmotic stress-induced autophagy in IPEC-J2 cells.**

LC3 and p62 were conducted to detect the autophagic activity of IPEC-J2 cells with different treatment due to their important roles in autophagy. As shown in Fig. 8A, cells treated with hyperosmotic medium exhibited higher LC3 II/LC3 I ratio and lower p62 protein level than that in control ($P < 0.05$). Cotreated with betaine in hyperosmotic medium, the protein expression of LC3 II decreased ($P < 0.05$) and the protein expression of p62 increased ($P < 0.05$) compared to cells treated with hyperosmotic medium only. To further verify the effect of betaine against hyperosmolarity on autophagy in IPEC-J2 cells, mRFP-GFP-tagged version of LC3B was employed and directly visualized by confocal microscopy. The yellow and red puncta can represent autophagosome and autolysosome separately based on the principle of different pH stability of green and red fluorescent proteins. The fluorescent signal of GFP is green which could be quenched under the acidic condition (pH below 5) inside the lysosome while the mRFP fluorescent signal is not influenced. Thus, the progression of autophagy can be monitored by the exhibited yellow puncta and the red puncta. Consistent with the western blotting results, much more yellow and red puncta were found (Fig. 8B) in hyperosmolarity group than control group ($P < 0.05$) suggesting hyperosmolarity induced autophagy in IPEC-J2 cells. Interestingly, when treated with betaine under hyperosmotic condition, although both puncta decreased significantly ($P < 0.05$), the yellow puncta seemed to have a sharper decline than the red one. These finding indicated that betaine could ameliorate autophagy induced by hyperosmolarity which was likely to function in preventing the formation of autophagosomes rather than the fusion of autophagosomes and lysosomes.

# Discussion

In this study, we confirmed the hyperosmotic environment of intestinal lumen related to plasma as previously reported[16; 17], and it is a big challenge for intestinal epithelium. As for cells, hyperosmolarity shrinks cells by osmosis leading to the decrease of cell volume, the elevation of ROS and macromolecular crowding which are all candidates to be sensors triggering cell responses[25]. With the underdeveloped structure, small intestine of piglets is more susceptible to hyperosmolarity so as to be damaged. In the present study, similar to other tissues or cells experiencing dramatic osmotic changes[26–28], betaine facilitates the adaption of porcine intestinal epithelial cells to environmental osmotic strength *in vivo and vitro* by counteracting the deleterious effect induced by hyperosmolarity.

Increased formation of ROS occurs when cells receive stress signaling[29]. There were several researches reporting that hyperosmolarity caused oxidative stress with elevation of ROS[30–32], which was in agreement with our result. Meanwhile, we found that betaine decreased ROS accumulation in porcine intestinal epithelium *in vivo and vitro*, which was a good evidence for betaine to ameliorate hyperosmotic stress in porcine intestinal epithelium. Importantly, betaine functions as an osmoprotectant and helps to maintain cell volume, contributing to the decrease of hyperosmotic stress-induced ROS formation. Meanwhile, the methyl donor function of betaine may also contribute to the decrease of ROS. Betaine is capable of entering the SAAs (sulfur-containing amino acids) metabolism cycle via transmethylation with betaine-homocysteine methyltransferase (BHMT) (**Supplemental Fig. 1**) and consequently convert to SAAs including methionine, SAM and cysteine, which can relieve oxidative stress by scavenging ROS and increasing the production of GSH[33–37]. Sun et.al found that betaine prevented liver from oxidative injury by modulating the SAAs metabolism in which it increased the level of methionine, SAM and GSH[38]. Moreover, the same result was also confirmed by Zhang et.al *in vitro*[36]. In this study, it was worth noting that betaine supplementation significantly improved the expression of BHMT in intestinal epithelium of piglets (**Supplemental Fig. 2**) indicating the enhancement of transmethylation and SAAs metabolism in this trial. Thus, it is the promising next step to evaluate the antioxidative effect of betaine against hyperosmotic stress via SAAs metabolism with its three methyl groups.

Furthermore, we supported findings of others that hyperosmotic stress could lead to apoptosis[39–41], and betaine ameliorated hyperosmotic stress-induced mitochondrial dysfunction followed by apoptosis. Mitochondrial dysfunction is always an early event in hyperosmolarity-induced apoptosis, which can be caused directly by changes in matrix volume, secondary to the increased osmolality out of cell and hence in cytoplasm[42]. Characterized with the permeabilization and depolarization of the outer membrane, mitochondrial membrane potential collapse is the first index of mitochondrial dysfunction and consequently results in the release of cytochrome *c* initiating caspases cascade reaction with the terminal of activated Caspase3[43]. Bcl-2 belongs to Bcl-2 family and functions as a repressor to apoptosis by controlling the release of cytochrome *c*[43]. Meanwhile, it is reported that Bcl-2 contributes to regulate excessive mitochondrial ROS-triggered promotion of apoptosis in a AMPK(AMP-activated protein kinase)-dependent way or by inducing the release of cytochrome *c*[44–46]. Consistent with researches in other tissue or cells[47; 48], our results demonstrated that hyperosmotic stress increased mitochondrial ROS and decreased mitochondrial membrane potential, which proved the hyperosmotic damage to mitochondria and the initiation of apoptosis. At the same time, the concentration of cytochrome *c* in cytoplasm and following cleaved Caspase3 rose accompanied with the decrease of the protein level of Bcl-2 under hyperosmotic condition, indicating the enhancement of mitochondrial-dependent apoptotic process in IPEC-J2 cells. However, betaine reversed the devastating effect of hyperosmolarity on mitochondria in this study so as to inhibit following apoptosis, at least, partly resulting from its osmoprotective role for maintaining mitochondrial homeostasis and its integrity.

Betaine alleviated the autophagy of porcine intestinal epithelium caused by hyperosmolarity. Upon hypertonic exposure, accumulation of ROS and molecular crowding together damage and aggregate proteins, which can activate cellular degradation mechanisms including autophagy[13]. Autophagy, a

type of programmed cell death, is a catabolic pathway involving self-degradation of damaged or aggregated protein or organelles and serves a critical role in cellular survival during pathophysiological processes[49; 50]. Since the autophagy is initiated, the cargo is sequestered into autophagosomes, the double-membrane vesicles tagged with lipid-conjugated microtubule-associated protein 1 light chain 3(LC3 II), and subsequently fuses with lysosomes forming autolysosomes to accomplish degradation[51]. Sequestosome-1(SQSTM1/p62) is a ubiquitin-binding protein that promotes protein aggregate formation prior to their incorporation into autophagosomes[52]. Thus, it serves as a key role in eliminating protein aggregate and it is negatively correlated with autophagy activation[53]. Quite a few studies evaluating the role of hyperosmotic stress in autophagy induction in different species or tissues includes yeast[54], cardiomyocytes[50], rat notochordal cells[55], porcine renal proximal tubule-like(LLC-PK) cell[12]. Here, we investigated the effect of hyperosmolarity on autophagy whose results were according with previous reports, as the increase expression of LC3 II and the decrease expression of p62 compared to the control in IPEC-J2 cells. Augment of autophagosomes and autolysosomes in hyperosmotic group was also a visible evidence of the enhancing autophagic flux. Milena Veskovic et al. found that betaine supplementation benefits MCD(methionine-choline deficient)-induced NAFLD (nonalcoholic fatty liver disease) mice by promoting autophagy[56]. However, present studies showed that betaine alleviated hyperosmotic stress-induced autophagy with the decreased expression of LC3 II and increased expression of p62 *in vivo and vitro*. It can be on account of the compatible osmoregulatory properties of betaine that it not only protects cells from water loss but also stabilizes the structure of proteins under hyperosmotic condition[57]. Instead of binding to the protein, betaine is excluded from a protein's hydration layer so that protein tends to fold up more compactly[57]. Therefore, exposed to hyperosmolarity, betaine decreases the damaged proteins and inhibits the initiation of autophagy. Furthermore, we found that the decrease of autophagosomes was more dramatic than that of autolysosomes by betaine under hyperosmotic condition. It was suggested that betaine could reduce stimulus inducing autophagy such as protein damage and aggregate caused by hyperosmotic stress in cells without influencing the elimination of impaired content. Interestingly, although not achieving statistical significance, there was a decline of autophagy when cotreated with betaine in isosmotic medium *in vitro* as the lower level of LC3 II and higher level of p62. Based on the above result, we speculated that the effect of betaine on autophagy might be tissue-related, or other signaling pathway existed for inhibiting autophagy by betaine in addition to osmoprotective mechanism, which needed more exploration.

# Conclusions

In conclusion, betaine could ameliorate hyperosmotic stress-induced cell shrinkage and ROS accumulation as well as alleviate apoptosis and autophagy of porcine intestinal epithelium. This study is a good evidence and support for betaine to widely utilize in animal production with its osmoprotective property, especially in young livestock who has the underdeveloped intestine, contributing to maintain intestinal homeostasis and subsequently improve growth performance.

## Abbreviations

| | |
|---|---|
| **AMPK** | AMP-activated protein kinase |
| **BHMT** | Betaine-homocysteine methyltransferase |
| **FBS** | Fetal bovine serum |
| **GSH** | Glutathione |
| **LC3** | Microtubule-associated protein light chain 3 |
| **LLC-PK** | Porcine renal proximal tubule-like |
| **MCD** | Methionine-choline deficient |
| **MOMP** | Mitochondrial outer membrane permeabilization |
| **NAFLD** | Nonalcoholic fatty liver disease |
| **p62** | Sequestosome-1 |
| **PARP-1** | ADP-ribose polymerase |
| **qPCR** | Quantitative real-time polymerase chain reaction |
| **ROS** | Reactive oxygen species |
| **SAAs** | Sulfur-containing amino acids |
| **SAM** | S-adenosylmethionine |

## Declarations

# Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

# Ethics approval and consent to participate

The experiment processes were conducted strictly according to the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

# Consent for publication

Not applicable.

# Competing interests

The authors declare that they have no conflict of interest.

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# Authors' contributions

JF conceived and supervised the study; JF and SX designed the experiments; SX, SL, HW and SL were involved in performing the experiment; SX analyzed the data; SX drafted the manuscript and JF revised the manuscript. All authors read and approved the final manuscript.

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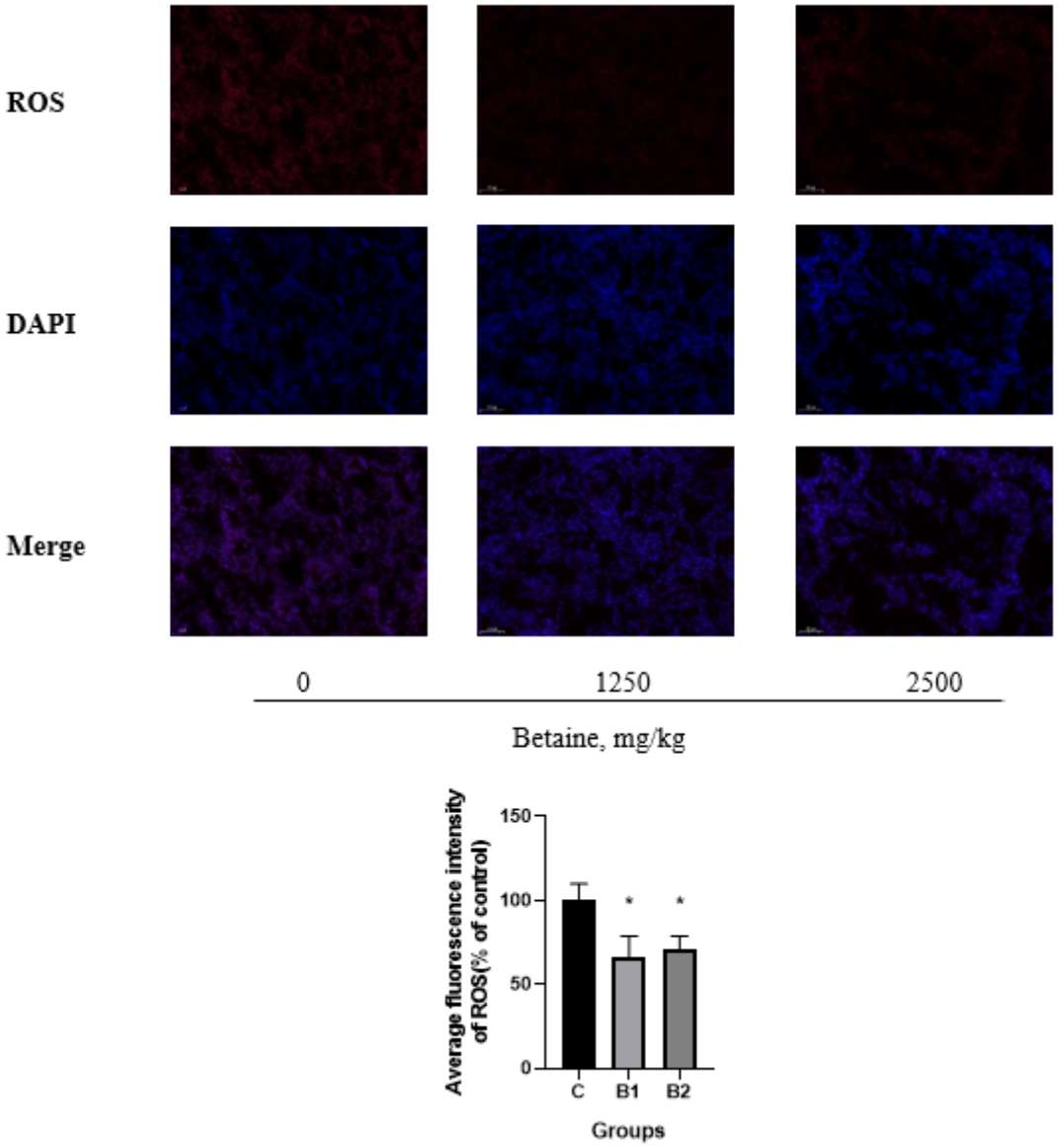
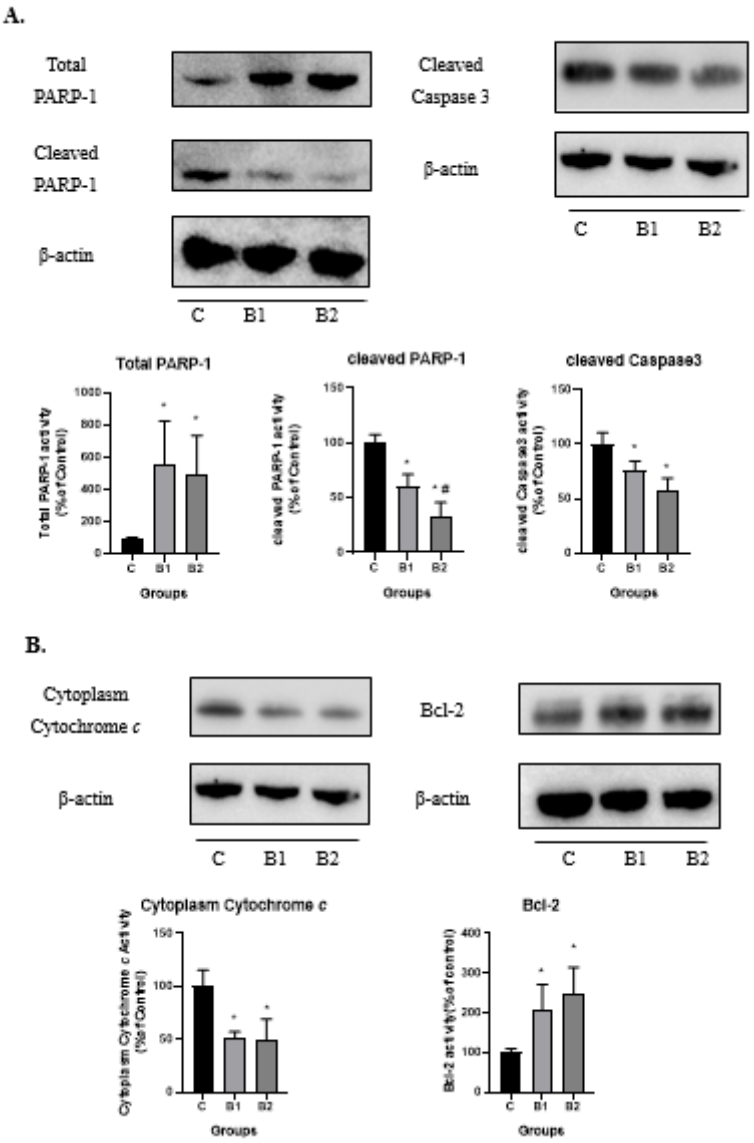
# Figures



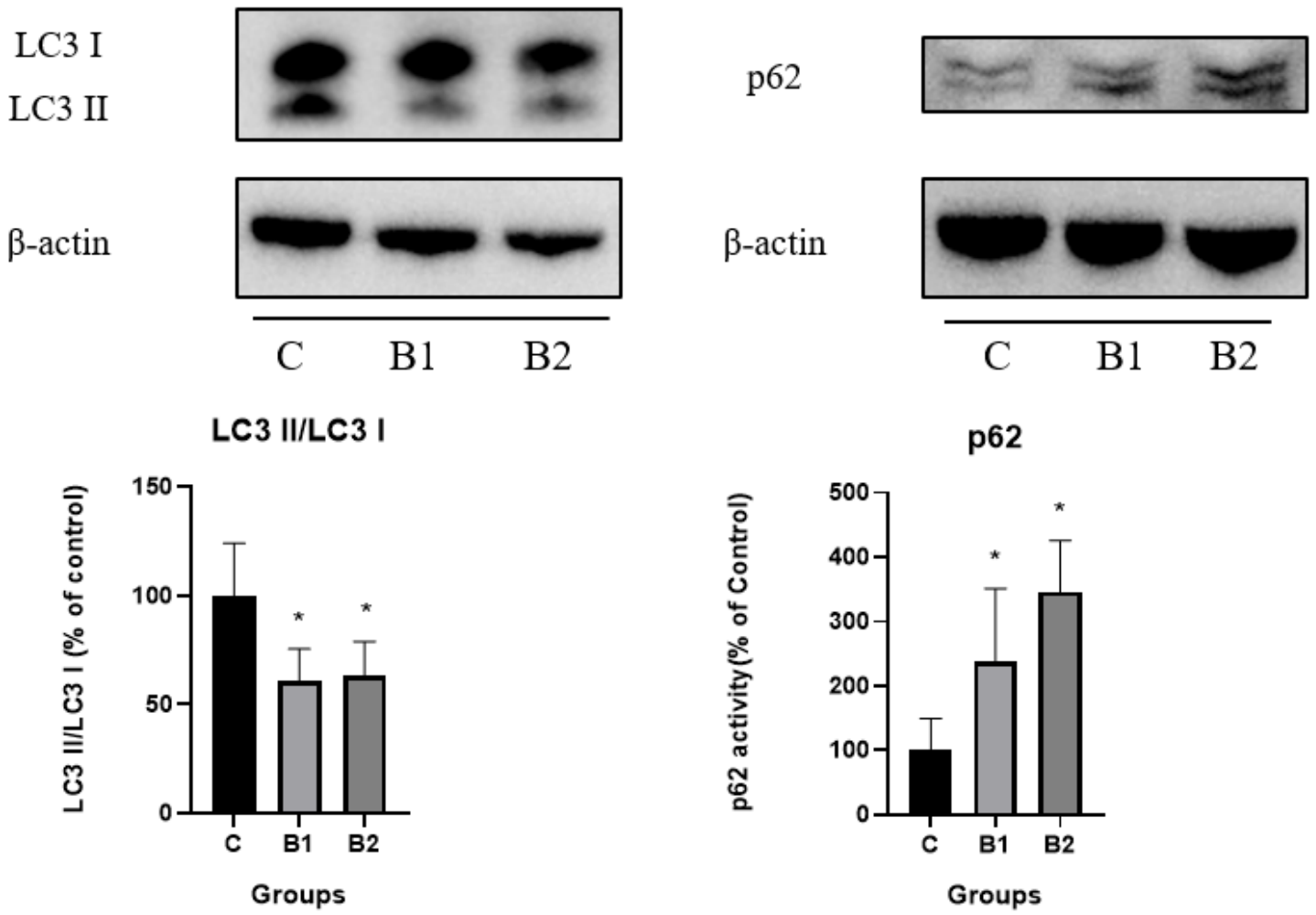

## Figure 1

ROS staining of small intestine and quantification. Scale bar, 50μm. C-basal diet, B1-basal diet supplemented with 1250 mg/kg betaine, B2- basal diet supplemented with 2500 mg/kg betaine. * denotes significant difference compared to C group ($P < 0.05$).



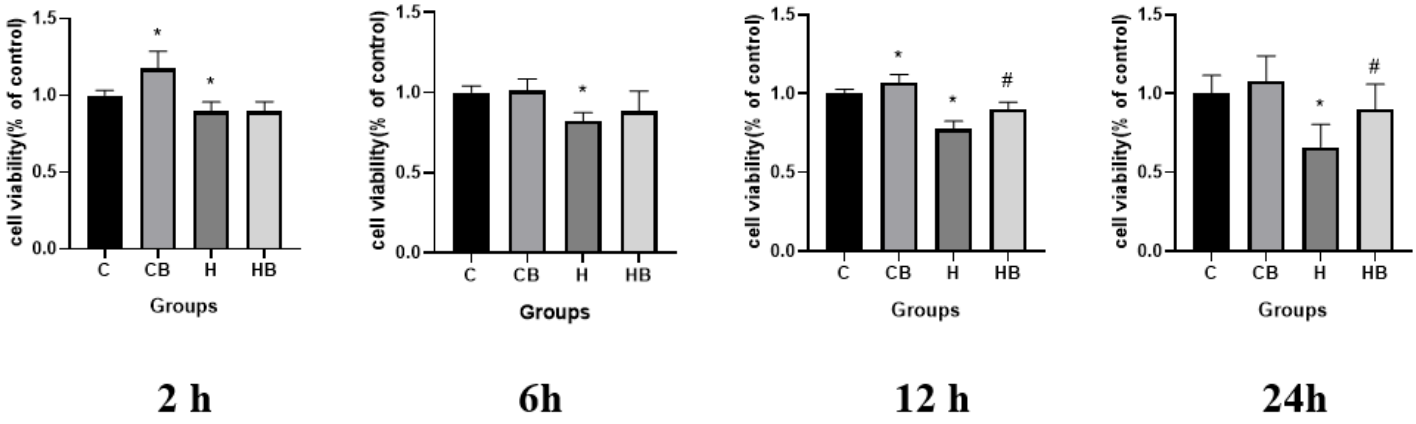

## Figure 2

Effects of dietary betaine on apoptosis of the intestinal epithelium of piglets. A. Protein expression and quantitation of PARP-1, cleaved PARP-1 and cleaved Caspase3 were determined by western blotting in small intestine of piglets. B. Protein expression and quantitation of cytoplasm Cytochrome c and Bcl-2 were determined by western blotting in small intestine of piglets. C-basal diet, B1-basal diet supplemented with 1250 mg/kg betaine, B2- basal diet supplemented with 2500 mg/kg betaine. * denotes significant difference compared to C group ($P < 0.05$). # denotes significant difference compared to B1 group ($P < 0.05$).



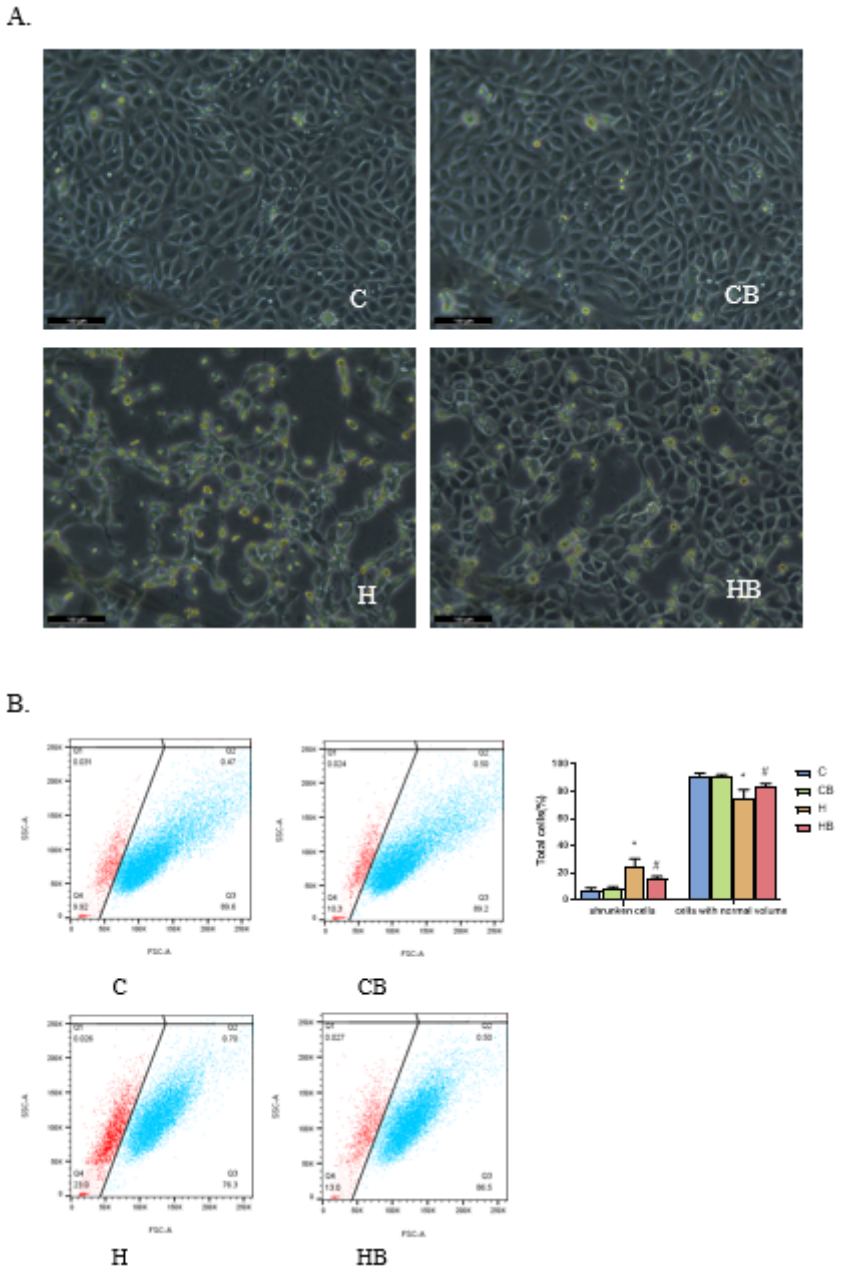

## Figure 3

Effects of dietary betaine on autophagy of the intestinal epithelium of piglets. Protein expression and quantitation of LC3 II, LC3 I, p62 were determined by western blotting in small intestine of piglets. C-basal diet, B1-basal diet supplemented with 1250 mg/kg betaine, B2- basal diet supplemented with 2500 mg/kg betaine. * denotes significant difference compared to C group (P < 0.05).



## Figure 4

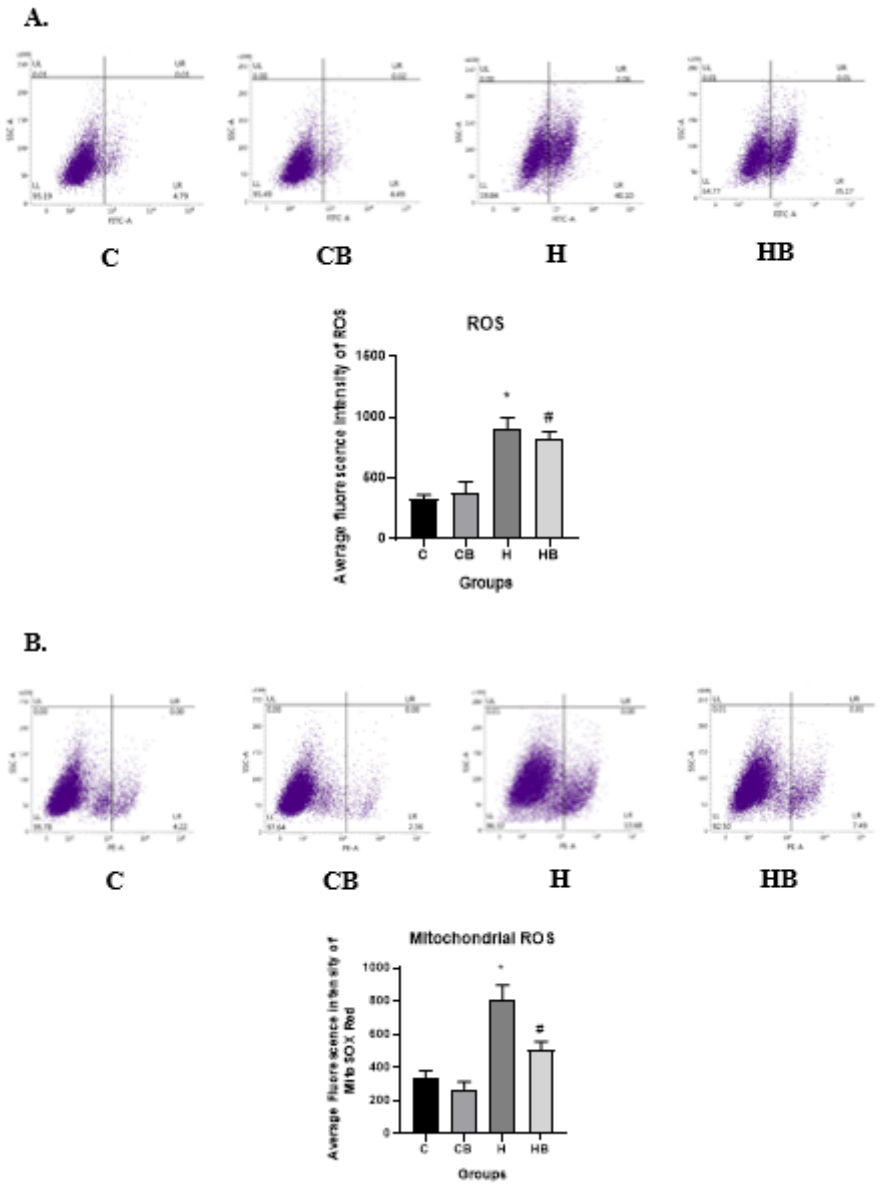
The influence of high osmotic pressure and betaine on cell viability in IPEC-J2 cells. C-complete medium, CB- complete medium with 20 mmol/L betaine, H- hyperosmotic medium, complete medium with 150 mmol/L NaCl, HB- hyperosmotic medium with 20 mmol/L betaine. * denotes significant difference compared to C group ($P < 0.05$). # denotes significant difference compared to H group ($P < 0.05$).




## Figure 5

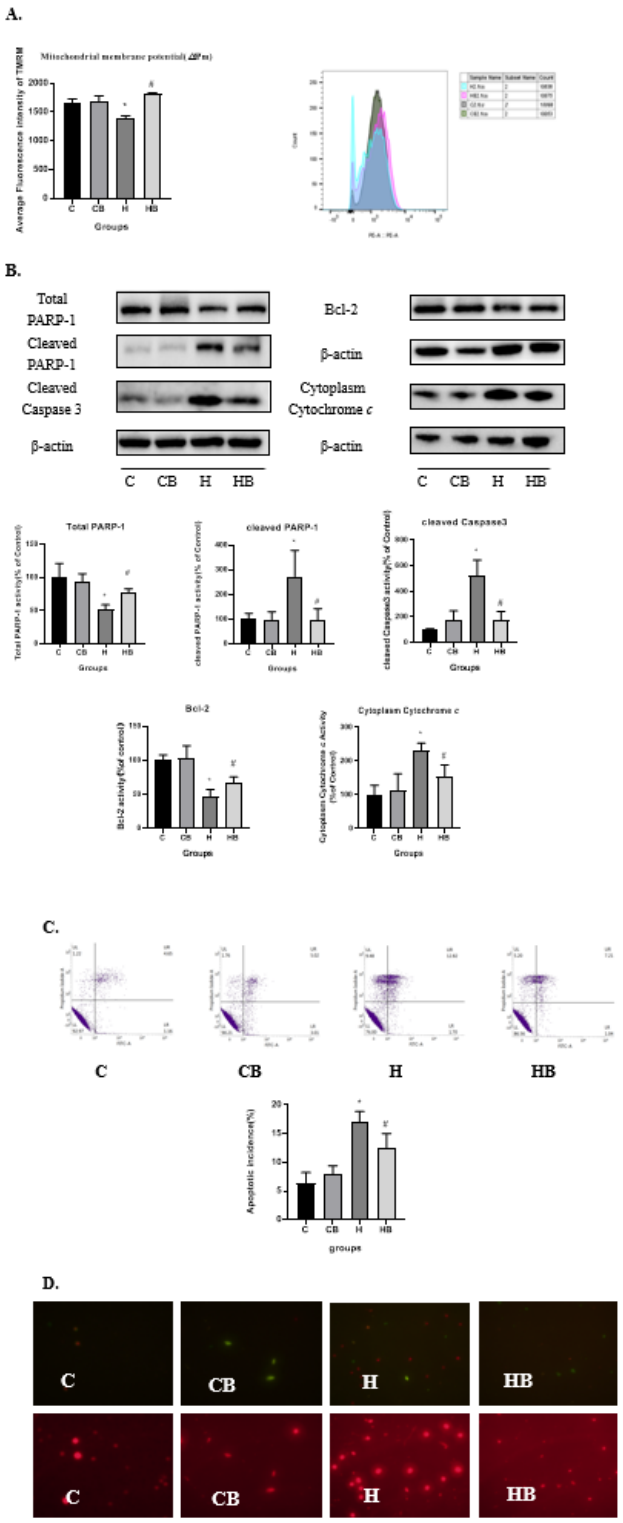
Effects of betaine and hyperosmolarity on morphology of IPEC-J2 cells after 12 h treatment. A. The morphology of IPEC-J2 cells under light microscopy. Scale bar, 100 μm. B. The cell volume determined by flow cytometry. The red and blue part represents shrunken cells and cells with normal volume, respectively. * denotes significant difference compared to C group ($P < 0.05$). # denotes significant difference compared to H group ($P < 0.05$). C-complete medium, CB- complete medium with 20 mmol/L

betaine, H- hyperosmotic medium, complete medium with 150 mmol/L NaCl, HB- hyperosmotic medium with 20 mmol/L betaine.




## Figure 6

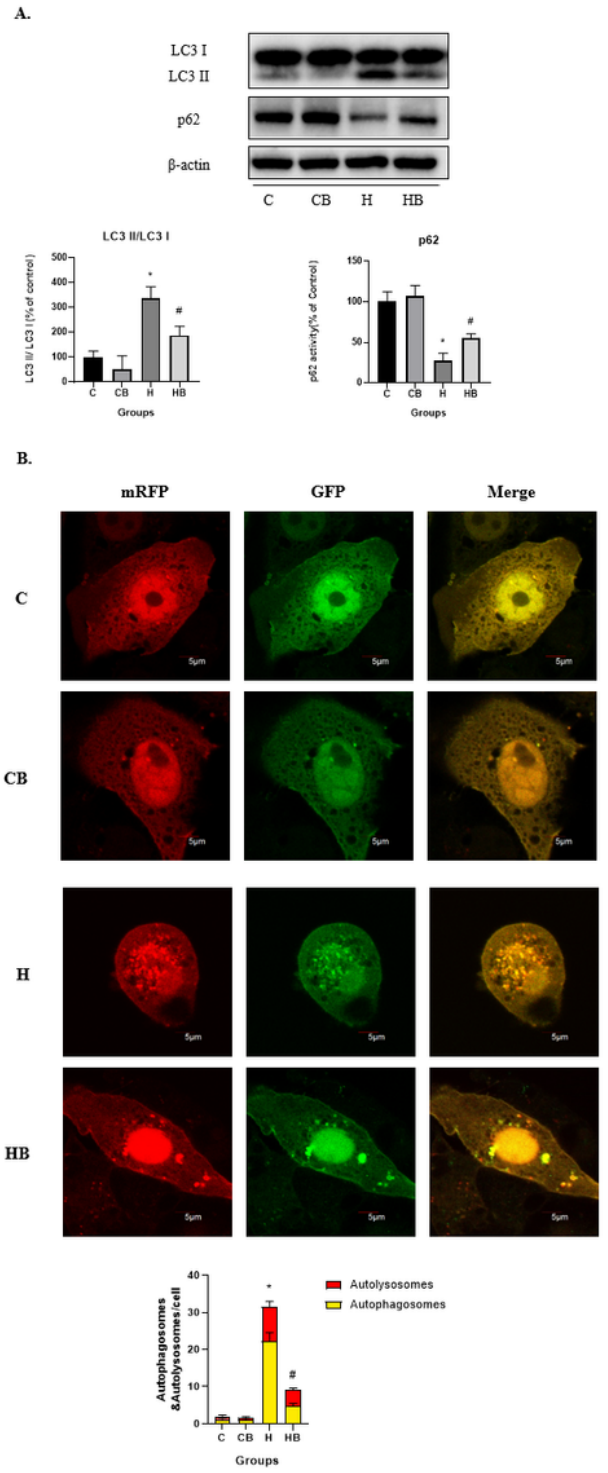
Effects of hyperosmolarity and betaine on ROS and mitochondria after 12 h treatment. A. Flow cytometry was used to determine cellular reactive oxygen species (ROS) level and quantification. B. Flow cytometry was used to determine mitochondrial reactive oxygen species (Mito-ROS) level and quantification. C- complete medium, CB- complete medium with 20 mmol/L betaine, H- hyperosmotic medium, complete medium with 150 mmol/L NaCl, HB- hyperosmotic medium with 20 mmol/L betaine. * denotes significant difference compared to C group ($P < 0.05$). # denotes significant difference compared to H group ($P < 0.05$).




## Figure 7

Effects of high osmotic pressure and betaine on apoptosis in IPEC-J2 cells after 12 h treatment. A. Flow cytometry was used to determine mitochondrial membrane potential (MMP) level and quantification. B. Protein expression and quantitation of PARP-1, cleaved PARP-1, cleaved Caspase3, cytoplasm cytochrome c, Bcl-2 were determined by western blotting in IPEC-J2 cells. C. Annexin V and PI dye were stained to measure the ratio of apoptotic cells by flow cytometry. The result was quantified. D. Annexin V

and PI dye were stained and the result was observed under fluorescence microscope (100x). C-complete medium, CB- complete medium with 20 mmol/L betaine, H- hyperosmotic medium, complete medium with 150 mmol/L NaCl, HB- hyperosmotic medium with 20 mmol/L betaine. * denotes significant difference compared to C group ($P < 0.05$). # denotes significant difference compared to H group ($P < 0.05$).




## Figure 8

Effects of high osmotic pressure and betaine on autophagy in IPEC-J2 cells. A. Protein expression and quantitation of LC3 II, LC3 I, p62 were determined by western blotting in IPEC-J2 cells. B. Autolysosomes and autophagosomes were determined by transfecting mRFP-GFP-LC3 labelled adenoviruses and the result was viewed under confocal fluorescence microscope and quantified. Scale bar, 5 μm. C-complete medium, CB- complete medium with 20 mmol/L betaine, H- hyperosmotic medium, complete medium with 150 mmol/L NaCl, HB- hyperosmotic medium with 20 mmol/L betaine. * denotes significant difference compared to C group ($P < 0.05$). # denotes significant difference compared to H group ($P < 0.05$).

## Supplementary Files

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