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Imperatorin improves in vitro porcine embryo development by reducing oxidative stress and autophagy

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Abstract

Imperatorin (IMP), a furanocoumarin derivative with many biological properties and pharmacological activities, is widely used as an antibacterial, anti-inflammatory, antiviral, anticancer, cardiovascular and neuroprotective agent. The purpose of this study was to explore the effects of IMP on early embryo development in pigs as well as the potential mechanisms. Our results showed that IMP can enhance the developmental competence of porcine early embryos. Supplementation of in vitro culture medium with 40 μM IMP significantly increased the blastocyst rate and total cell number. At the same time, apoptosis of blastocysts was also significantly decreased in the supplemented group compared with the control group, in accordance with the subsequent results of FAS and CASP3 gene expression analysis. Furthermore, IMP attenuated intracellular reactive oxygen species (ROS) generation, increased fluorescein diacetate (FDA) and glutathione (GSH) levels. Importantly, IMP not only improved the activity of mitochondria but also inhibited the occurrence of autophagy. In addition, pluripotency-related genes (OCT4, NANOG, and SOX2) and a growth and metabolism regulatory gene (mTOR) were upregulated after IMP supplementation on Day 7. These results demonstrate that IMP exerts a beneficial effect on preimplantation embryo development by reducing oxidative stress and autophagy.

Key words: Imperatorin; Porcine; Embryo development; Oxidative stress; Autophagy

Introduction

In vitro culture (IVC) of embryos is an important technique that is widely used in animal embryo in vitro production (IVP) and artificially assisted reproduction[1, 2]. In the past few decades, scientists have made many attempts to improve the efficiency of IVP. Despite some progress, the efficiency and quality of IVP are still far from those of in vivo production because embryos are very sensitive to the environment during growth and development, especially porcine embryos[3, 4]. One of the important factors affecting embryo development is oxidative stress, which is caused by excess reactive oxygen species (ROS)[5]. ROS include oxyanions, free radicals (such as superoxides and hydroxyl radicals) and peroxides (such as hydrogen peroxide), which can be produced by a variety of metabolic pathways, such as oxidative phosphorylation, and enzymes, including NADPH oxidase and xanthine oxidase[6]; ROS can cause lipid oxidation[7], DNA damage[8], protein denaturation[9], and mitochondrial damage[10].

During the growth and development of embryos in vitro and in vivo, embryonic ROS remains in a relatively balanced state due to the presence of various antioxidants in the body[11]. To improve the production efficiency and quality of porcine embryos, inhibiting the production of excessive ROS is a feasible strategy. Previous studies have shown that cysteine[12], melatonin[13], resveratrol[14], linoleic acid[15], and laminarin[16] can improve the oxidation resistance of embryos. However, the current IVC environment does not replicate the in vivo environment. Therefore, elucidating the mechanisms of antioxidative damage and exploring effective antioxidant substances for use in culture medium are effective ways to improve the developmental abilities of embryos in vitro[17].

Imperatorin (IMP), a furanocoumarin derivative also known as 9-[(3-methyl-2-buten-1-yl)oxy]-7H-furo[3,2-g]chromen-7-one, is mainly distributed among plants, such as *Angelica dahurica*[18], *Clausena anisata*[19] and *Aegle marmelos Correa*[20]. IMP, an effective component extracted from traditional

Chinese medicines, has many biological properties and pharmacological activities. IMP not only has good broad-spectrum antibacterial activity but also has definite value as an antiviral, anticancer and cardioprotective agent[21, 22]. Moreover, IMP also has inflammation-inhibiting[23], vasodilatory, antihypertension[24], memory-enhancing[25], antianxiety[26], and antiepilepsy[27] effects and is therefore a potentially valuable resource for the prevention and treatment of certain central nervous system diseases. IMP has become a potential drug candidate due to its multiple pharmacological properties. Some of the mechanisms by which IMP exerts its pharmacological effects are closely related to the antioxidant effects of IMP[28]. However, the effect of IMP on reproduction has not been clearly identified.

In this study, we hypothesized that IMP can enhance the growth and development of porcine embryos. Therefore, IMP was added to IVC medium. **Esterase activity**, intracellular glutathione (GSH) and ROS levels, mitochondrial membrane potential (MMP) and autophagy levels were evaluated to gain insights into how IMP affects embryo development.

2. Materials and methods

All chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.1. Oocyte collection and in vitro maturation (IVM)

Porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory within 2 h in a sterile saline solution supplemented with 100 μg/mL penicillin G and 100 μg/mL streptomycin sulfate at 30-35°C. Next, the cumulus–oocyte complex (COC) was aspirated from a 3-8 mm antral follicle on each ovary using a 10 mL syringe with an 18-gauge needle. Only oocytes with a minimum of three layers of cumulus cells were selected. Approximately 100 oocytes were transferred into each well of four-well embryo culture plates (Nunc, Roskilde,

Denmark) containing 500 μL of mineral oil-covered IVM medium (M199 with 10 ng/mL epidermal growth factor, 1 μg/mL insulin, 75 μg/mL kanamycin, 0.91 mM sodium pyruvate, 10% porcine follicular fluid, 0.5 μg/mL stimulating hormone, and 0.5 μg/mL luteinizing hormone). The selected COCs were incubated at 38.5℃ in an atmosphere of 5% CO_2 and 100% humidity for 44 h.

2.2. Parthenogenetic activation and embryo IVC

After 44 h of incubation for IVM, the COCs were denuded by gently pipetting with 0.1% hyaluronidase. The denuded oocytes were parthenogenetically activated using two direct-current pulses of 120 V for 60 μs in 297 mM mannitol containing 0.5 mM HEPES, 0.1 mM $CaCl_2$, 0.05 mM $MgSO_4$, and 0.01% polyvinyl alcohol (PVA). Then, the activated oocytes were cultured in bicarbonate-buffered PZM-5[29] containing 4 mg/mL BSA (IVC medium) and 7.5 mg/mL cytochalasin B for 3 h. After carefully washing the oocytes with PZM-5 3 times, approximately 50/well parthenogenetically activated oocytes were transferred into four-well plates, each well of which contained 500 μL of IVC medium with or without (negative control group, NC) 40 μM IMP (Selleck, Shanghai, China), and then cultured at 38.5 ℃ and in 5% CO_2 without changing the medium. The blastocyst rates were detected on days 5, 6, and 7.

2.3. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and cell counting

Apoptosis was analyzed using a TUNEL detection kit (Roche Diagnostics, IN, USA) following the manufacturer's instructions. Briefly, blastocysts were collected on Day 7 and carefully washed three times with phosphate-buffered saline with 0.1% polyvinyl alcohol (PBS-PVA) and fixed in PBS containing 3.7% paraformaldehyde for 30 min at room temperature. Then, the embryos were permeabilized by incubation in 0.1% Triton X-100 for 30 min at room temperature. After 1 h of incubation in a

blocking buffer (1% BSA in PBS), the embryos were incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (Roche) in the dark for 1 h at 37℃. Next, the embryos were incubated with 10 μg/mL Hoechst 33342 for 5 min at 37℃ to label the nuclei. Finally, a fluorescence microscope (Nikon, Tokyo, Japan) and ImageJ software (NIH, Bethesda, MD, USA) were used to analyze the fluorescence intensities, the numbers of apoptotic nuclei and the total numbers of nuclei. Apoptosis was evaluated based on the percentage of apoptotic nuclei in blastocysts.

2.4. Esterase activity, intracellular ROS and GSH level assays

To detect Esterase activity**,** intracellular ROS and GSH levels, the 4-cell stage embryos were incubated in PBS-PVA medium containing 2.5μg/L fluorescein diacetate (FDA, Solarbio, Beijing, China), 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCFH, Invitrogen, Grand Island, NY, USA), or 10 μM 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF_2HC, Invitrogen) for 10min, 15 min and 30 min, respectively. After washing the embryos three times in PBS-PVA, a fluorescence microscope (Nikon) and ImageJ software were used to analyze the fluorescence intensities.

2.5. Mitochondrial membrane potential (MMP, ΔΨ) assay

Four-cell-stage embryos were incubated in PBS-PVA containing 2 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanineiodide (JC-1; Invitrogen) dye for 30 min. After washing the embryos three times with PBS-PVA, a fluorescence microscope (Nikon) was used to capture the red/green fluorescence signals. ImageJ software was used to analyze the fluorescence intensities, and the average MMPs of entire 4-cell-stage embryos were calculated as the ratios of red fluorescence intensity (J-aggregates; corresponding to high MMP) to green fluorescence intensity (J-monomers; corresponding to decreased MMP).

2.6. Immunofluorescence staining

Blastocysts were collected on Day 7 and carefully washed three times with PBS-PVA. Then, the embryos were fixed in PBS-PVA containing 3.7% paraformaldehyde for 30 min at room temperature. Next, the embryos were washed with PBS-PVA three times and permeabilized by incubation in 0.1% Triton X-100 for 30 min at room temperature. After being blocked with PBS-PVA containing 1% BSA for 1 h, the embryos were incubated with a primary LC3 antibody (diluted at 1:200, Abcam, Cambridge, MA, USA, #48394) overnight at 4°C. After extensive washes with PBS-PVA, the embryos were incubated with a secondary antibody (diluted at 1:1000, Abcam, #150073) for 1 h at room temperature. Finally, the embryos were washed three times with PBS-PVA and incubated with 10 μg/mL Hoechst 33342 in PBS-PVA for 10 min. Finally, the embryos were mounted onto glass slides and examined using a confocal laser scanning microscope (Zeiss, Oberkochen, Germany). The levels of autophagy in the embryos were measured by the number of LC3 dots.

2.7. Quantitative RT-PCR (qRT-PCR) analysis

Blastocysts were collected on Day 7, and mRNA was extracted using a Dynabeads™ mRNA DIRECT™ Purification Kit (Invitrogen) according to the manufacturer's instructions. Gene expression was quantified with a Mastercycler ep realplex system (Eppendorf, Hamburg, Germany) and the $2^{-\Delta\Delta Ct}$ method with 18S rRNA as the standard using the following protocol: 95°C for 3 min; 40 cycles at 95°C for 15 sec, 60°C for 30 sec, and 72°C for 20 sec; and a final extension at 72°C for 10 min. All primers used are listed in Supplementary Table S1.

2.8. Statistical analysis

Total numbers of embryos (n) used in each group and independent repeat times (*R*) of experiments are shown in figure notes. Data obtained from two groups were

compared using the Student's t-test. Tests with three or more means were analyzed using a one-way ANOVA (Tukey-Kramer). All statistical analyses were performed using SPSS version 22.0 (IBM, IL, USA) software.

3. Results

3.1. IMP enhanced embryo developmental competence and reduced apoptosis

As shown in Fig. 1, the blastocyst rate in the IMP-treated group was significantly higher than that in the NC group (40.16% ± 6.41% versus 31.54% ± 5.10% on Day 5, 45.35% ± 6.51% versus 35.76% ± 5.34% on Day 6, and 49.40% ± 4.76% versus 38.25% ± 5.24% on Day 7). At the same time, the blastocyst total cell number in the IMP-treated group (59.05% ± 12.81%) was significantly higher than that in the NC group (49.51% ± 11.54%; Fig. 2B). We also measured apoptosis using a TUNEL assay, and the proportion of apoptotic cells in the IMP-treated group was significantly lower than that in the NC group (6.61% ± 3.51% versus 8.38% ± 4.22%, respectively, $P<0.05$; Fig. 2A and C).

3.2. IMP enhanced the oxidation resistance of porcine early-stage embryos

To explore whether IMP improves the resistance of embryos to oxidative stress, we first detected the esterase activity and ROS level by FDA and DCFH staining, respectively. As shown in Fig. 3, the relative ROS level in the IMP-treated group was much lower ($P < 0.01$) while esterase activity is much higher ($P < 0.01$) than that in NC group. At the same time, the GSH fluorescence intensity in IMP-treated embryos were also significantly higher than that in NC embryos ($P < 0.01$).

3.3. IMP enhanced mitochondrial function during early-stage porcine embryo development

To explore the potential mechanism by which IMP supplementation enhances the proliferation and developmental potential of porcine embryos, we examined the MMP

at the four-cell-stage. Representative images of the MMP in the NC and IMP-treated groups are shown in Fig. 4A-4F. The results showed that the average relative MMP in the IMP-treated group was 1.5 times as high as that in the NC group (Fig. 4G).

3.4. IMP reduced autophagy occurrence during early-stage porcine embryo development

To assess whether IMP regulates autophagy, we measured the amount of LC3, which correlates well with the number of autophagosomes and is widely used to monitor autophagic activity. As shown in Fig. 5, the number of LC3 dots in blastocysts was significantly lower in the IMP-treated group (200.82 ± 29.45) than in the NC group (220.81 ± 35.04) on Day 6. Therefore, the IMP addition group had a significantly lower level of autophagy than the NC group ($P<0.05$).

3.5. Differential gene expression in 4-cell embryos and blastocysts with or without IMP

To identify the potential molecular pathway and mechanism of IMP influence on embryo development, the expression of pluripotency-, blastocyst formation-, apoptosis- and autophagy-related genes was examined (Fig. 6). We found that the expression levels of OCT4, NANOG, SOX2 and mTOR were significantly increased in the IMP supplementation group. At the same time, the expression levels of CASP3 in the IMP supplementation group were decreased during the IVC period ($P < 0.05$). No significant difference in FAS gene expression was observed between the NC and IMP-treated groups ($P>0.05$).

4. Discussion

During the process of IVP, embryos are more sensitive to oxidative stress caused by ROS in IVC environments than in vivo [30], and oxidative stress can induce various impairments in early stage embryos resulting in developmental blocking [31].

Therefore, inhibition of ROS generation by antioxidant supplementation is an effective strategy for improving the developmental competence of embryos. Our results indicate that IMP can enhance embryo growth and development by reducing the production of endogenous ROS. Importantly, IMP not only improved the activity of mitochondria but also inhibited the occurrence of autophagy. These results indicate that IMP has a beneficial effect on preimplantation embryo development.

In this study, the blastocyst rate increased significantly upon supplementation of the IVC medium with 40 μM IMP, and the blastocyst total cell numbers also significantly increased. Early embryo growth and development is mainly composed of a series of cleavage steps, and the rate of proliferation is positively correlated with the developmental potential of the embryo[32]. In addition to improved proliferation of blastomeres in the blastocysts, reduced apoptosis was observed in the IMP-treated group compared with the NC group, which suggested that IMP enhanced the potential of early embryo development by enhancing cell proliferation and preventing blastomeres from undergoing apoptosis[33-35]. However, several studies have shown that IMP inhibits proliferation and induces apoptosis in cancer cell lines[36-40], which may be related to the fact that additives can exert different effects in cancer cells and noncancer cells through different pathways; such conflicting effects have also been found for other additives believed to make beneficial contributions to embryo development[41-43]. Therefore, the effects of IMP and related mechanisms require further study.

To explore the possible mechanism by which IMP enhances early embryo development, we first measured esterase activity, ROS and GSH levels in embryos during the four-cell-stage. The four-cell-stage is the zygotic genome activation period for porcine embryos. The embryos are more sensitive during this period than in other periods, and oxidative stress can induce various impairments, resulting in developmental inhibition. In this study, the levels of ROS were significantly lower while esterase activities were significantly higher in the group with IMP in the culture

medium than in the NC group. This may have been due to the fact that IMP regulates superoxide dismutase, xanthine oxidase, nicotinamide adenine dinucleotide phosphate oxidase, glucose oxidase, cyclooxygenase, nitric oxide synthase, and myeloperoxidase as well as the normal respiration of cells [44-46]. At the same time, addition of IMP increased the levels of GSH and reduced intracellular ROS levels, which is also consistent with some previous studies[47, 48]. High levels of GSH help clear ROS and increase mitochondrial activity, thereby improving early embryo quality and development[49, 50]. The significantly increased GSH levels in the IMP supplementation group indicate that IMP has beneficial effects on metabolic and biochemical reactions such as DNA synthesis and repair and protein synthesis, thereby affecting apoptosis and proliferation by decreasing cellular ROS accumulation[51] during early embryo development.

Mitochondria are necessary organelles in embryos. Excessive ROS disrupt the functioning of mitochondria[52, 53]. Disorder of mitochondrial function not only affects the development of embryos but also leads to abnormal autophagy, apoptosis and even death[54, 55]. We found that IMP supplementation can effectively enhance the mitochondrial function of 4-cell-stage embryos. Combined with the corresponding results regarding blastocyst total cell numbers and blastocyst rates, these findings suggest that IMP can promote embryo development by reducing ROS content, stabilize or even activate mitochondrial function[56], and prevent embryos from intrinsic apoptosis induced by the explosion of ROS and mitochondrial energy metabolism disorder, consistent with the effects of antioxidants in other studies[57-59].

Autophagy is the process by which lysosomes degrade proteins or organelles in cells to achieve self-renewal of cells[60]. In the early embryo development process, autophagy is necessary, and defective autophagy leads to developmental stagnation[61, 62]. Moreover, autophagy plays an important role in protecting cells or organisms from severe environmental effects[63] and is capable of inducing cell

death[64]. However, autophagy is also related to oxidative stress, crosstalk during endoplasmic reticulum stress and cell death versus survival [65, 66], thereby affecting oocyte maturation, ectoplasmic specialization assembly in sertoli cells, and maintenance of intestinal stem cells and intestinal regeneration [67-69], and promotes cell death through excessive self-digestion and degradation of essential cellular constituents[70]. Based on the current results, addition of IMP leads to changes in the levels of autophagy, and these changes are related to changes in ROS content and mitochondrial function in embryos. The reductions in ROS and the enhancement of mitochondrial activity caused by IMP supplementation are involved in the induction of autophagosomes and autophagic cell death[71]. Since the level of intracellular autophagy is often related to cellular physiological activity and self-regulation in vivo, we speculate that the reduction in autophagy may be related to a relatively improved physiological state and environment[72-75]. The mechanism may involve IMP acting as an antioxidant to reduce the release of ROS and cytokines, leading to inhibition of apoptosis and autophagy via downregulation of phosphorylation-mediated activation of related proteins in the MAPK family, such as P38 MAPK, JNK and ERK[76-78].

To explore the beneficial potential of IMP in early embryo development, we examined the expression of blastocyst pluripotency-related genes (OCT4, NANOG, and SOX2), apoptosis-related genes (FAS and CASP3), and a growth and metabolism regulatory gene (mTOR). OCT4, NANOG and SOX2 are key transcription factors that maintain pluripotency and regulate cell differentiation and fate determination[79]. Losses of or reductions in these factors can affect the normal development of early embryos[80-82]. We first observed significant upregulation of OCT4, NANOG, SOX2, and mTOR in the IMP-treated group. This effect may have been related to the role of IMP in regulating the cellular environment by regulating the PI3K, MAPK, Akt/NF-κB pathways[83-85], thereby indicating that addition of IMP improves embryo developmental competence and pluripotency. At the same time, significant decreases in the apoptosis-related genes FAS and CASP3 were found in the

IMP-treated group compared with the NC group. FAS and CASP3 are activated in apoptotic cells by both extrinsic (death ligand) and intrinsic (mitochondrial) pathways and signals[86-88]. These findings are consistent with other studies showing that IMP inhibits apoptosis by inhibiting the expression or activity of FAS and CASP3[33, 89, 90] and are supported by the results of the TUNEL assay. IMP supplementation will help improve embryo quality and enhance early embryo development.

In summary, IMP can improve embryo quality, prevent apoptosis, and enhance embryo development during the early stages by increasing GSH content, reducing ROS accumulation, increasing mitochondrial function, and reducing autophagy levels. These findings will contribute to improving the in vitro production of embryos. In future research, we will explore the effects of IMP on mitochondrial oxidative respiration, energy metabolism and its potential mechanism to provide more evidence for the use of IMP in IVP.

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Competing interests

The authors declare no conflict of interest.

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Figure legends

Fig. 1. Effects of IMP on the development of porcine parthenogenetic embryos. (A) Embryo development on different days in the NC and IMP-treated groups (40× magnification). (B) Blastocyst formation rate on different days in the NC group (n = 200) and the IMP-treated (n = 202) group. *R* = 5. Data are presented as the mean ± SD. Significant differences are represented with *(P < 0.05) and **(P < 0.01).

Fig. 2. Effects of IMP on cell proliferation and apoptosis in blastocysts. (A) Hoechst and TUNEL staining of blastocysts on Day 6 (200× magnification). (B) Total cell number of blastocysts on Day 7 in the NC (n=71) and IMP (n = 85) groups. Arrow head indicates TUNEL-positive nuclei. (C) The proportion of apoptotic cells in the NC (n=71) and IMP-treated (n = 85) groups. Data are presented as the mean ± SD. Significant differences are represented with **(P < 0.01).

Fig.3. Effects of IMP on Esterase activity, ROS level, and GSH level in 4-cell embryos. (A) Bright field and DCFH staining images of 4-cell-stage embryos. (B) Relative esterase activity change in 4-cell-stage embryos in the NC (n = 99) and IMP-treated (n = 104) groups. *R* = 3. (C) Bright field and FDA staining images of 4-cell-stage embryos. (D) Relative ROS levels in 4-cell-stage embryos in the NC (n = 72) and

IMP-treated (n = 71) groups. *R* = 3. (E) Bright field and CMF_2HC staining images of 4-cell-stage embryos. (F) Relative GSH levels of 4-cell-stage embryos in the NC (n = 92) and IMP-treated (n = 100) groups. *R* = 3. All images are captured at 40× magnification. Data are presented as the mean ± SD. Significant differences are represented with **(P < 0.01).

Fig. 4. IMP enhanced mitochondrial function in 4-cell embryos.

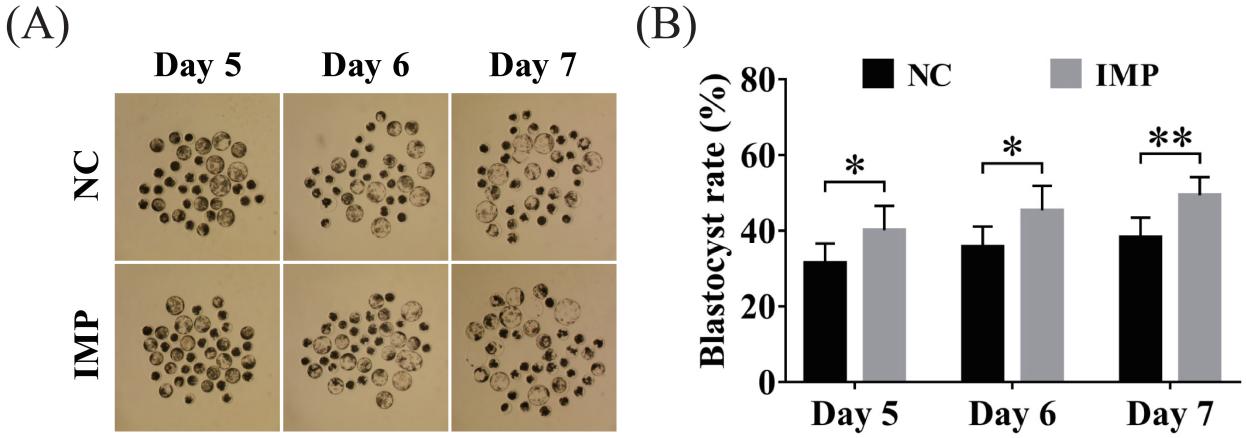
(A-F) JC-1 staining of 4-cell-stage embryos in the control and IMP groups (40× magnification). (G) Relative fluorescence levels of JC-1 in the NC (n = 66) and IMP-treated (n = 65) groups. R=4. Data are presented as the mean ± SD. Significant differences are represented with **(P < 0.01).

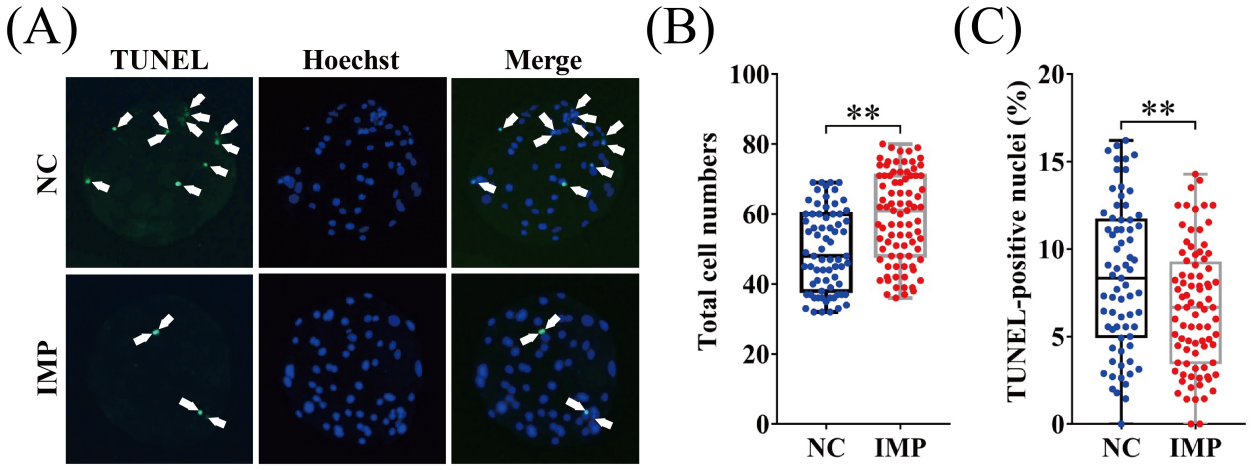
Fig. 5. LC3 dots in the NC and IMP-treated groups.

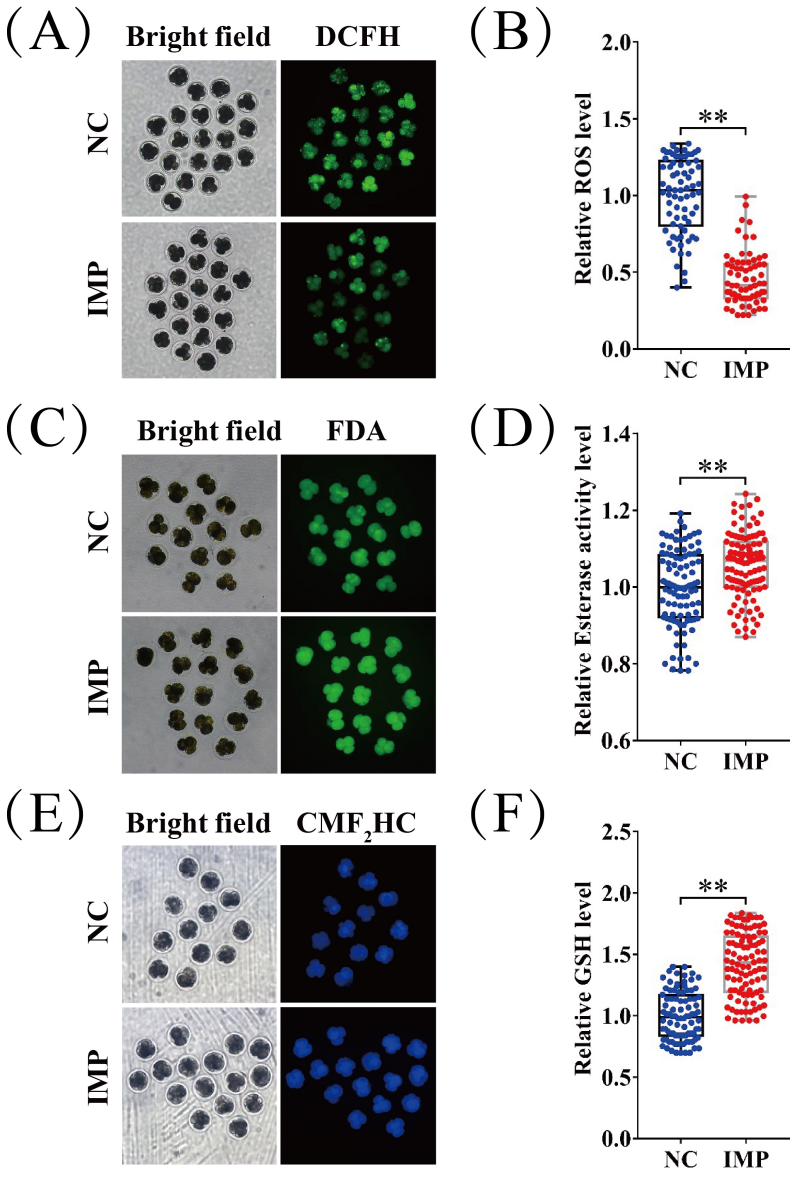
(A) Hoechst and LC3 staining at Day 6 (200× magnification). (B) Number of LC3 dots in the NC group (n = 33) and IMP-treated group (n = 34). Data are presented as the mean ± SD. Significant differences are represented with **(P < 0.01).

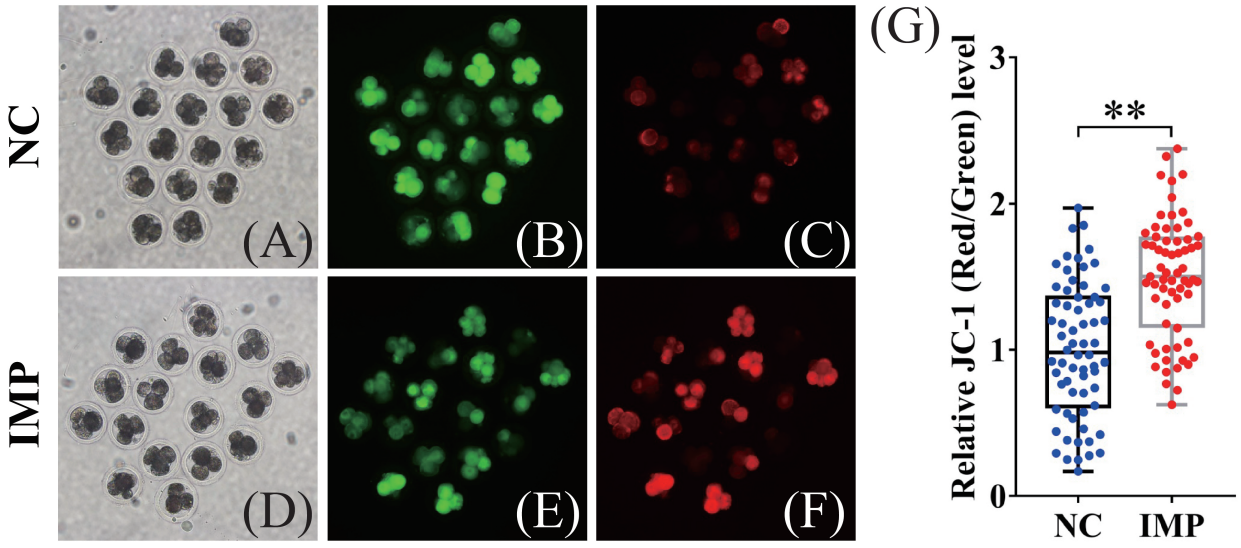
Fig.6. Differential gene expression in blastocysts.

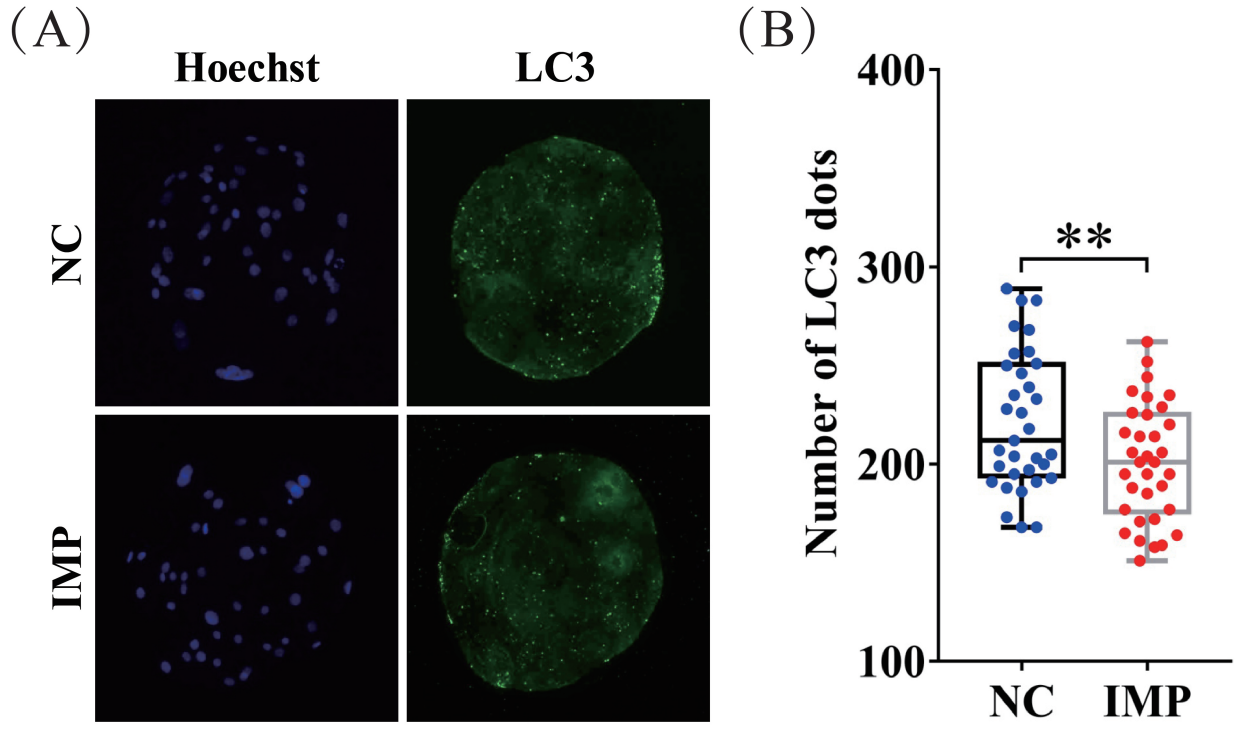
All genes were detected in blastocysts on Day 7. *R* = 3. Data are presented as the mean ± SD. Significant differences are represented with *(P < 0.05) and **(P < 0.01).

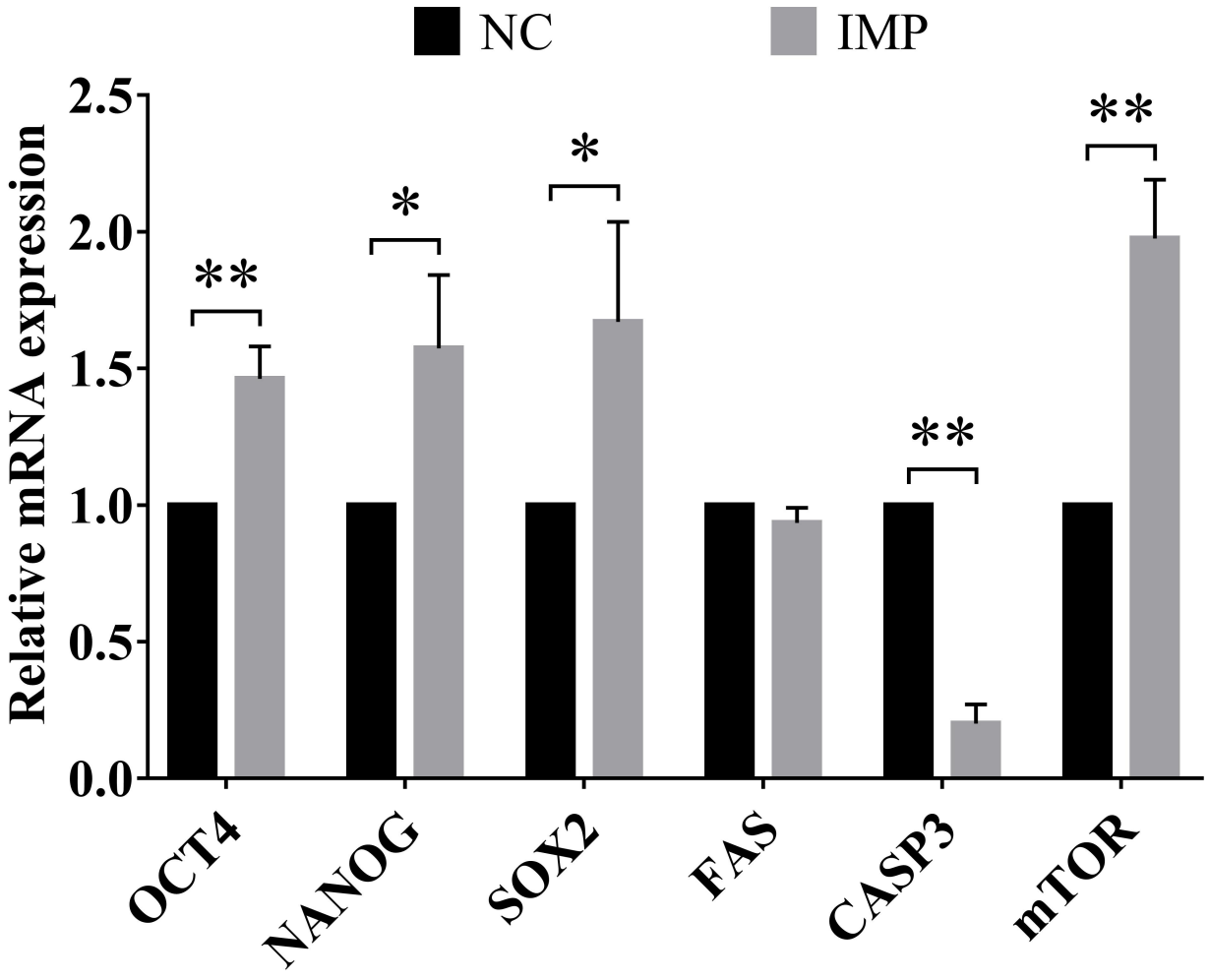





(A)
Bright field
DCFH
NC
IMP
(B)
Relative ROS level
2.0
1.5
1.0
0.5
0.0
**
NC
IMP
(C)
Bright field
FDA
NC
IMP
(D)
Relative Esterase activity level
1.4
1.2
1.0
0.8
0.6
**
NC
IMP
(E)
Bright field
CMF2HC
NC
IMP
(F)
Relative GSH level
2.5
2.0
1.5
1.0
0.5
0.0
**
NC
IMP






NC
IMP
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
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*
*
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OCT4
NANOG
SOX2
FAS
CASP3
mTOR

Highlights:

- Imperatorin (IMP) improves porcine embryo developmental competence and pluripotency
- IMP enhances the oxidation resistance and mitochondrial function
- IMP reduces autophagy and apoptosis