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—Original Article—

Carnosic acid improves porcine early embryonic development by inhibiting the accumulation of reactive oxygen species

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Abstract. Carnosic acid (CA), a natural catechol rosin diterpene, is used as an additive in animal feeds and human foods. However, the effects of CA on mammalian reproductive processes, especially early embryonic development, are unclear. In this study, we added CA to parthenogenetically activated porcine embryos in an *in vitro* culture medium to explore the influence of CA on apoptosis, proliferation, blastocyst formation, reactive oxygen species (ROS) levels, glutathione (GSH) levels, mitochondrial membrane potential, and embryonic development-related gene expression. The results showed that supplementation with 10 μM CA during *in vitro* culture significantly improved the cleavage rates, blastocyst formation rates, hatching rates, and total numbers of cells of parthenogenetically activated porcine embryos compared with no supplementation. More importantly, supplementation with CA also improved GSH levels and mitochondrial membrane potential, reduced natural ROS levels in blastomeres, upregulated *Nanog*, *Sox2*, *Gata4*, *Cox2*, *Itga5,* and *Rictor* expression, and downregulated *Birc5* and *Caspase3* expression. These results suggest that CA can improve early porcine embryonic development by regulating oxidative stress. This study elucidates the effects of CA on early embryonic development and their potential mechanisms, and provides new applications for improving the quality of *in vitro*-developed embryos.
Key words: Antioxidant, Carnosic acid, Embryo development, Oxidative stress, Porcine

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There are significant differences between the *in vivo* microenvironment and *in vitro* culture (IVC) systems. Increased oxygen partial pressure may be one of the main reasons for the increased oxidative stress in IVC environments [1, 2]. *In vitro* embryo production (IVP) procedures, including oocyte *in vitro* maturation (IVM) and early embryo IVC, have attracted attention due to their potential applications in agricultural production and scientific research [3–5]. Compared to embryos of other species, such as cattle and mice, porcine embryos are more sensitive to environmental culture conditions, such as nutrient content, ambient temperature and humidity, ROS levels, and osmotic pressure [6, 7]. Therefore, optimization of the embryo production process is key to improving the production of porcine embryos [8].

Studies have shown that adding antioxidants to the IVC medium can effectively reduce ROS accumulation during aerobic metabolism of the embryo [9]. Currently, ferulic acid [10], lycopene [11], and melatonin [12] have been shown to improve the quality of early embryonic development. However, the use of additives has certain side effects. For example, melatonin is associated with seasonal reproductive capacity and sexual maturation [13], and is not recommended for use in breast-feeding women [14]. Therefore, it is critical to develop more additives that can improve the quality of early embryonic development.

Carnosic acid (CA) is a phenolic diterpenoid that is abundant in the leaves of rosemary [15] and is used to protect chloroplasts from oxidative damage [16]. CA is widely used as a food preservative and protective agent, and it is used in the nutritional healthcare and cosmetic industries due to its fragrance and antibacterial activity. In addition, studies have shown that CA can reduce lipid oxidation, and exhibits great potential for the protection of nerves and the treatment of obesity-related diseases [17–19]. However, its effects on mammalian reproductive processes, especially in female ovarian development and early embryonic development, are still unclear.

Proper addition of antioxidants can reduce oocyte and embryo damage caused by oxidative stress [20, 21]. Studies have shown that CA can improve antioxidant activity in cells, inhibiting oxidative stress caused by H_2O_2 or other toxic substances *in vivo* to reduce cell damage. More importantly, CA supplementation improves the quality of thawed sperm [22]. However, whether CA has the potential to improve early embryonic development by lowering ROS levels is still unclear.

In this study, we identified the effects of CA supplementation on anti-ROS and mitochondrial functions during early parthenogenetic activation in the context of embryonic development. Our findings provide a new theoretical basis for improving the quality of *in vitro*-

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developed embryos and enhance understanding of the biological effects of CA.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless explicitly stated otherwise. CA (Selleck Chemicals, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) and then diluted to specific concentrations used for the experiment.

Oocyte collection and IVM

Briefly, porcine ovaries were collected from a local slaughterhouse and transferred to the laboratory within 1 h in normal saline at 30–35ºC. A 10 ml syringe was used to aspirate the cumulus-oocyte complexes (COCs) from 3–6 mm follicles. Oocytes with at least three layers of cumulus cells were washed thrice in Tyrode's Lactate HEPES (TL-HEPES) and selected for further experiments. No more than 100 oocytes were transferred to 500 µl of mineral oil-covered IVM medium (medium 199 supplemented with 10% porcine follicular fluid, 1 µg/ml insulin, 75 µg/ml kanamycin, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 10 ng/ml epidermal growth factor, 0.5 µg/ml porcine follicle-stimulating hormone, and 0.5 µg/ml sheep luteinizing hormone) and cultured at 38.5ºC in an atmosphere of 5% CO_2 and 100% humidity for 44 h.

All experimental procedures performed in this study have been approved by the Animal Protection and Use Committee of Jilin University (201802070).

Parthenogenetic activation and early embryo IVC

Denuded mature oocytes were parthenogenetically activated using two direct-current pulses of 120 V for 60 µsec in 297 mM mannitol containing 0.5 mM HEPES, 0.05 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.01% polyvinyl alcohol (PVA). Next, the oocytes were cultured in bicarbonate-buffered porcine zygote medium 5 (PZM-5) [23] containing 4 mg/mL BSA and 7.5 µg/ml cytochalasin B for 3 h to suppress the extrusion of the pseudo-second polar body. Finally, the oocytes were washed and cultured in 500 µl of bicarbonate-buffered PZM-5 containing 4 mg/ml BSA with or without CA, covered with mineral oil at 38.5°C in 5% CO_2 without changing the medium. Embryos cultured in IVC medium with 0.1% DMSO served as negative control (NC) groups. To determine the dose-dependent effects of CA on early porcine embryos cultured *in vitro*, activated embryos were cultured with different concentrations (0 µM, 5 µM, 10 µM, 25 µM, and 50 µM) of CA (the final concentration of DMSO was 0.1%). Blastocyst formation rates were detected on days 5 and 6.

Cell counting and TUNEL assays

Apoptosis was analyzed using a TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) assay kit (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. In brief, blastocysts were washed three times with phosphate-buffered saline containing 0.1% PVA (PBS-PVA), fixed with 3.7% paraformaldehyde, and permeabilized by incubation in 0.1% Triton X-100 for 30 min at room temperature. Next, the embryos were washed three times with PBS-PVA and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (Roche Diagnostics, Indianapolis, IN, USA) in the dark for 1 h at 37°C. After incubation with 1 mg/ml Hoechst 33342 for 5 min at 37°C to label the nuclei, a fluorescence microscope (Olympus, Tokyo, Japan) and ImageJ software (NIH, Bethesda, MD, USA) were used to analyze the fluorescence intensities, the numbers of apoptotic nuclei, and the total number of nuclei observed. Apoptosis was evaluated based on the percentages of apoptotic nuclei in the blastocysts.

ROS and glutathione (GSH) level assays

After culture for 48 h, intracellular ROS and GSH levels in the blastomeres were determined by incubating the 4-cell-stage embryos in PBS-PVA medium containing 10 mM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen) and 10 mM 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF_2HC, Invitrogen) for 15 min and 30 min at 37°C, respectively. After washing three times in PBS-PVA, a fluorescence microscope (Olympus) and ImageJ software were used to analyze the fluorescence intensities.

Determination of mitochondrial membrane potential (MMP, ΔΨm)

Mitochondrial function was detected by measuring the ΔΨm. Briefly, 4-cell-stage embryos were washed three times with PBS-PVA and incubated in PBS-PVA containing 2 µM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine-iodide dye (JC-1; Invitrogen) at 37°C for 30 min. After washing with PBS-PVA three times, the red and green fluorescence intensities were captured using a fluorescence microscope. The average ΔΨm values of entire 4-cell-stage embryos were then calculated as the ratios of red fluorescence intensity (corresponding to active mitochondria) to green fluorescence intensity (corresponding to inactive mitochondria) using ImageJ software.

RNA extraction and qRT-PCR assay

Total mRNA was extracted from approximately 30 blastocysts using a Dynabeads mRNA DIRECT Purification Kit (Invitrogen). cDNA was synthesized from the extracted mRNA using a reverse transcription kit (Tiangen Biotech, Beijing, China). Each 20 µl qRT-PCR reaction mixture included 8 µl of deionized water, 10 µl of SuperReal PreMix Plus (Tiangen), 1 µl of cDNA, 0.5 µl of the forward primer (10 mM), and 0.5 µl of the reverse primer (10 mM). The qRT-PCR conditions included denaturation at 95°C for 300 sec followed by 40 cycles of 95°C for 20 sec, 60°C for 20 sec, and 72°C for 20 sec. Gene expression was quantified using a Mastercycler ep realplex (Eppendorf, Hamburg, Germany) and the $2^{-\Delta\Delta ct}$ method with *18S rRNA* as the standard. The primer sequences are shown in Supplementary Table 1 (online only).

Statistical analysis

The statistical results are presented as means ± standard deviation (SD). The total number of embryos used (n) and the number of independent repeats (*R*) for each experiment are shown in the figure notes. Data for two groups were compared using Student's *t*-test. Three or more means were analyzed using one-way ANOVA (Tukey-Kramer). All statistical analyses were performed using SPSS version 22.0 (IBM, Chicago, IL, USA) software. Significant differences are

represented with * (P < 0.05) and ** (P < 0.01).

Results

Different CA concentrations affect porcine early embryonic development

In this study, four concentrations were selected. As shown in Fig. 1, the blastocyst rates in the 0 μM, 5 μM, 10 μM, 25 μM, and 50 μM concentration groups on Day 5 were 23.06 ± 1.50, 27.30 ± 4.22, 38.91 ± 2.80, 17.71 ± 2.31, and 4.14 ± 0.75%, respectively, while those on Day 6 were 31.88 ± 0.71, 36.04 ± 2.37, 45.59 ± 4.01, 21.89 ± 2.16, and 7.74 ± 2.05%, respectively. On the basis of these results, the 10 μM concentration was selected for subsequent experiments in this study.

CA improves early embryonic development

As shown in Fig. 2, the cleavage rates (86.06 ± 2.42 *vs*. 93.73 ± 1.31%), blastocyst formation rates (22.08 ± 2.29 *vs*. 31.70 ± 4.16% on Day 5, 32.07 ± 2.11 *vs*. 45.59 ± 3.97% on Day 6, and 41.61 ± 2.94 *vs*. 51.38 ± 3.56% on Day 7), hatching rates on Day 7 (10.92 ± 1.25 *vs*. 13.76 ± 1.05%), and blastocyst diameters on Day 7 (179.92 ± 37.38 *vs*. 205.66 ± 40.89 μm) were significantly higher in parthenogenetic embryos cultured with CA in the IVC medium than in those cultured without CA in the medium.

CA improves total cell numbers and reduces apoptosis in blastocysts

Subsequently, Hoechst 33342 staining and TUNEL staining (Fig. 3A) were used to assess changes in total cell numbers and apoptosis in early embryos, respectively. CA increased the total number of blastocyst cells from 59.25 ± 21.36 in the control group to 72.34 ± 23.05 in the treated group (Fig. 3B, P < 0.01). The apoptosis rate of blastocysts decreased from 6.71 ± 2.85% in the control group to 3.10 ± 1.71% in the CA-treated group (Fig. 3C, P < 0.05).

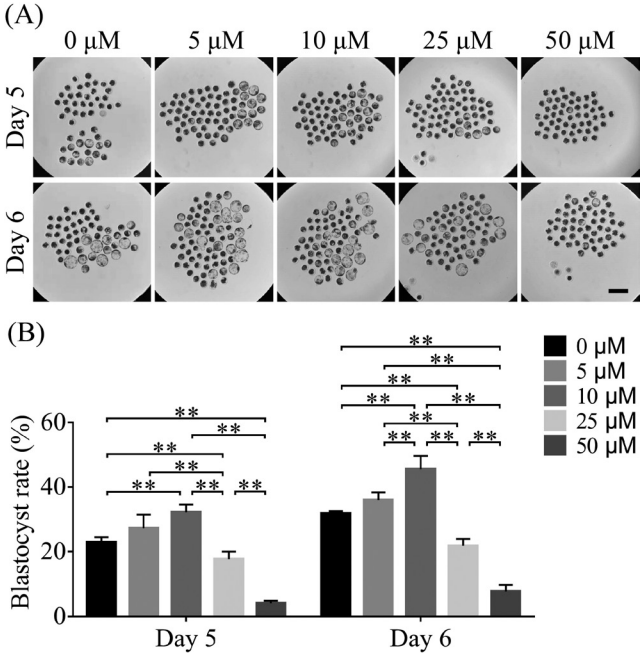

Fig. 1. Blastocyst rates on Day 5 and Day 6 after treatment with different carnosic acid (CA) concentrations. (A) Embryonic development on Day 5 and Day 6 after treatment with different CA concentrations. Scale bar = 400 μm. (B) Blastocyst rates in the groups treated with 0 μM (*n* = 160), 5 μM (*n* = 173), 10 μM (*n* = 180), 25 μM (*n* = 182), and 50 μM (*n* = 168) CA. *R* = 3. Significant differences are represented with ** (P < 0.01).

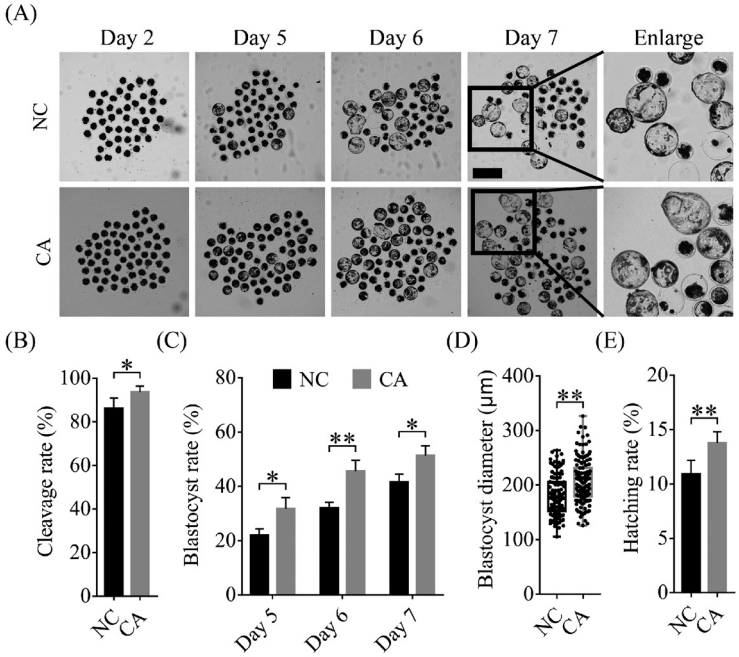

Fig. 2. Effects of carnosic acid (CA) on the development of parthenogenetic embryos. (A) Embryonic development in the negative control (NC) and CA-treated groups on different days. Scale bar = 400 μm. (B) Cleavage rates of embryos in the NC group (*n* = 214) and the CA-treated group (*n* = 217). *R* = 4. (C) Blastocyst formation rates in the NC group (*n* = 254) and the CA-treated group (*n* = 298) on different days. *R* = 5. (D) Average diameters of blastocysts on Day 6 in the NC group (*n* = 107) and the CA-treated group (*n* = 133). *R* = 4. Plot depicting the distributions of embryo diameter for black dots in the NC and CA-treated groups. (E) Hatching rates in the NC group (*n* = 254) and the CA-treated group (*n* = 298). *R* = 5. Significant differences are represented with * (P < 0.05) and ** (P < 0.01).

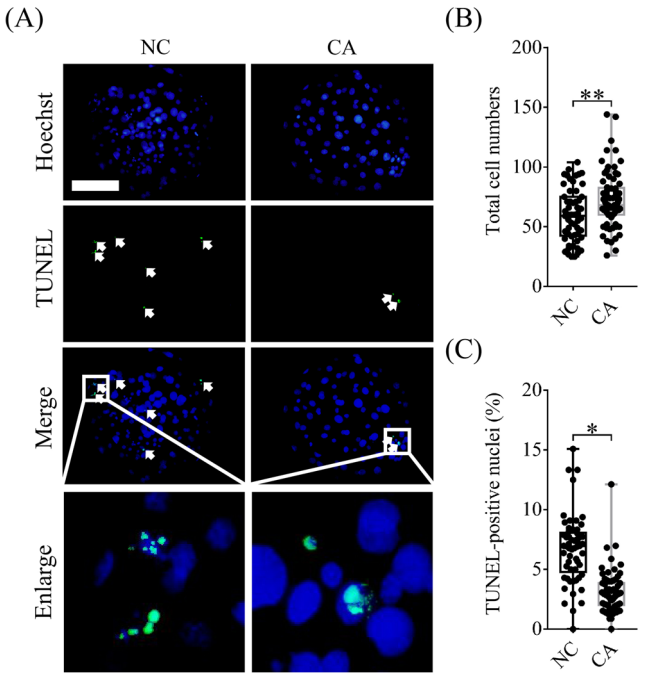

Fig. 3. Total cell numbers and apoptotic nuclei in blastocysts with or without carnosic acid (CA) treatment. (A) Apoptotic nuclei in the negative control (NC) and CA-treated groups. The white arrows indicate nuclei positively stained for apoptosis. Scale bar = 100 μm. (B) and (C) Total cell numbers and apoptosis rates in blastocysts with (n = 57) or without (n = 73) CA treatment, respectively. R = 3. Black dots represent the tested values. Significant differences are represented with * ($P < 0.05$) and ** ($P < 0.01$).

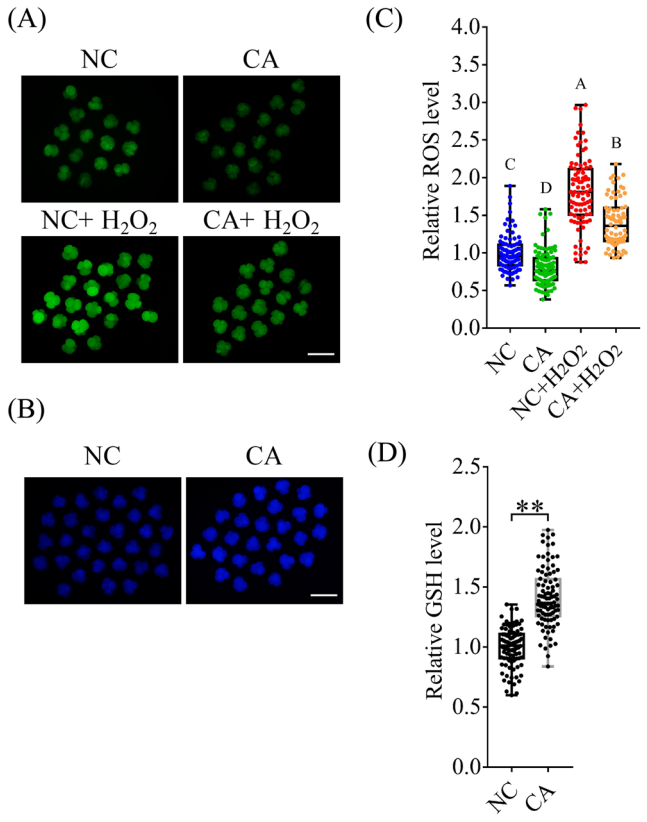

Fig. 4. Effects of carnosic acid (CA) on oxidation resistance in 4-cell-stage embryos. (A) Fluorescence intensity of H_2DCFDA staining in different groups. Scale bar = 200 μm. (B) Fluorescence intensity of CMF_2HC staining in the negative control (NC) and CA-treated groups. Scale bar = 200 μm. (C) Relative reactive oxygen species (ROS) levels in 4-cell-stage embryos. Plot depicting the distributions of relative embryo ROS levels for blue (n = 86), green (n = 104), red (n = 90), and orange (n = 74)-colored dots in different groups. Significant differences are represented with different capital letters ($P < 0.01$). R = 5. (D) Relative GSH levels of 4-cell-stage embryos in the NC (n = 91) and CA-treated (n = 93) groups. R = 3. Significant differences are represented with different capital letters ($P < 0.01$) and ** ($P < 0.01$).

CA reduces ROS accumulation and improves GSH levels in porcine embryos

Excessive ROS levels *in vivo* cause oxidative stress, which greatly reduces early embryonic developmental competence. Therefore, we first analyzed whether supplementation with CA could reduce ROS levels during early embryonic development. As shown in Figs. 4A

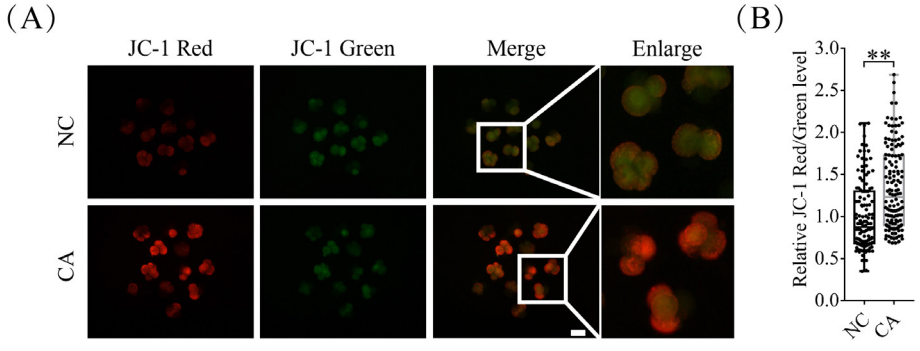

Fig. 5. Carnosic acid (CA) enhances mitochondrial activity. (A) JC-1 staining in 4-cell-stage embryos with or without CA treatment. (B) Relative fluorescence levels of JC-1 in the negative control (NC) (n = 126) and CA-treated (n = 140) groups. R = 5. Black dots represent the tested values. Scale bar = 100 μm. Significant differences are represented with ** ($P < 0.01$).

and 4C, the ROS levels in embryos treated with CA were lower (0.81 ± 0.23-fold) than those in the negative control (NC) embryos. To further confirm the effect of CA on oxidation resistance, we treated parthenogenetic embryos with H_2O_2 for 30 min before IVC with CA. At the 4-cell stage, we found that CA-treated embryos exposed to H_2O_2 showed significantly lower ROS production than non-CA-treated embryos exposed to H_2O_2, although the levels in the CA-treated H_2O_2-exposed group were still higher than those in the NC group. In addition, the relative GSH fluorescence intensity was significantly higher (1.41 ± 0.24-fold, $P < 0.01$) in CA-treated embryos than in NC embryos (Figs. 4B and 4D).

CA enhances mitochondrial activity during early porcine embryonic development

Mitochondrial activity is related to oocyte quality and embryonic developmental potential. We subsequently tested mitochondrial activity in early porcine embryos (Fig. 5). Our results showed that the ΔΨm values of embryos in the CA treatment group were significantly higher (1.34 ± 0.48-fold, $P < 0.01$) than those of NC embryos. Thus, ΔΨm increased significantly after CA treatment, indicating that supplementation with CA enhanced mitochondrial activity.

Differential gene expression in blastocysts with or without CA

To analyze the potential mechanism by which CA influenced embryonic development, the expression of pluripotency-related genes (*Nanog* and *Sox2*), hatching-related genes (*Cox2*, *Gata4*, and *Itga5*) and apoptosis-related genes (*Rictor*, *Caspase3*, and *Birc5*) were detected by qRT-PCR (Fig. 6). The results showed that the expression levels of *Nanog* ($P < 0.05$), *Sox2* ($P < 0.05$), *Gata4* ($P < 0.05$), *Cox2* ($P < 0.05$), *Itga5* ($P < 0.05$), and *Rictor* ($P < 0.01$) were higher, while those of *Birc5* ($P < 0.05$) and *Caspase3* ($P < 0.01$) were lower, in the CA-supplemented group compared with the NC group.

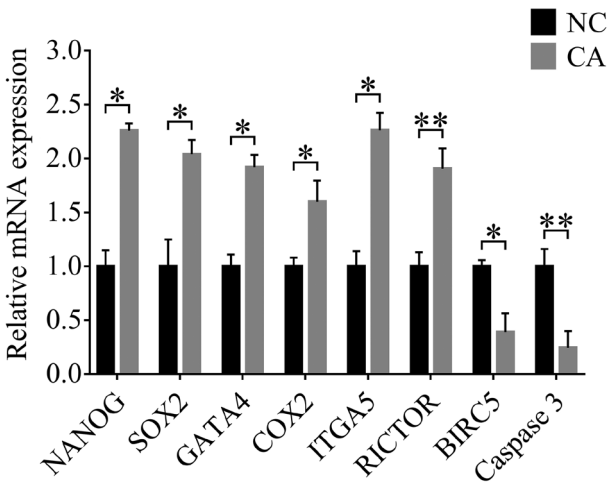

Fig. 6. Differential gene expression in blastocysts. Gene expression levels were analyzed in porcine blastocysts with or without carnosic acid (CA) treatment on Day 7. Significant differences are represented with * ($P < 0.05$) and ** ($P < 0.01$).

Discussion

At present, CA is mainly used as an anti-inflammatory, antibacterial, and anticancer agent, as well as for cardiovascular disease therapy. However, studies on its effects on mammalian reproductive processes, especially its antioxidant effects during early embryonic development, are limited. This study found that 10 μM CA significantly increased the blastocyst rates and hatching rates of early embryos (Fig. 1 and Fig. 2). More importantly, the cleavage rates and the developmental competency of embryos at the 4-cell stage were significantly improved with CA supplementation. The cleavage stage and the 4-cell stage are key stages in porcine embryonic development and zygotic genome activation [24]. Appropriate additives can be beneficial for overcoming blocks in embryonic development and for improving embryonic developmental potential [11, 25, 26]. Therefore, we also investigated the effects of CA on 4-cell-stage embryonic development.

Many factors, such as visible light, ambient air, and the composition of the culture medium, can accelerate the production of ROS in embryos in IVC [27–30], affecting the growth and development of early stage embryos or even causing their death [30, 31]. In this study, CA supplementation significantly decreased ROS levels and significantly increased GSH levels in 4-cell-stage embryos (Fig. 4). Studies have shown that the regulatory effects of CA on HO-1, NQO-1, γ-GCS, and Nrf2 indicate the potential of CA in regulating GSH [32–34]. GSH is a major non-enzymatic member of the antioxidant stress system and plays a central role as an endogenous antioxidant scavenger [35]. GSH is involved in various cellular processes and regulates intracellular redox reactions to protect cells from oxidative damage and maintain normal cell states [36]. Therefore, we hypothesized that CA could not only reduce ROS levels in cells but also remove ROS from porcine embryos cultured in IVC medium. We used H_2O_2 to induce intracellular production of large amounts of ROS and found that CA supplementation effectively inhibited H_2O_2-induced increases in ROS levels. Thus, consistent with other research results, our results indicated that CA could effectively reduce ROS levels during early embryonic development (Fig. 4A). The main mechanism may involve regulation of ERK, Keap1/Nrf2, PI3K/Akt, and the *c-fos* signaling pathway [37–40].

Mitochondria are important energy supply units during embryonic development [41]. Whether or not mitochondrial function is normal directly affects embryonic development competency [42, 43]. Most of the adenosine triphosphate (ATP) required for cell growth is produced by mitochondrial oxidative phosphorylation, and ATP insufficiency in cells leads to cell aging and apoptosis [44–46]. Cells and embryos show better growth, proliferation, and growth potential when ΔΨm is high [47–49]. In this study, CA supplementation effectively improved porcine early embryo ΔΨm values (Fig. 5), which suggested that CA can strengthen mitochondrial function in all blastomeres. Studies have shown that high levels of ROS usually cause mitochondrial dysfunction, resulting in DNA, protein, and lipid oxidation and ultimately lead to cell aging and death [50–52]. However, supplementation with CA increased intracellular GSH levels and decreased ROS levels, which meant that CA can stabilize or enhance mitochondrial function by inhibiting ROS production, consistent with the results of other studies [53, 54]. This mechanism may be an important way in which CA effectively improves embryonic developmental capacity.

Furthermore, our TUNEL results showed that supplementation with CA effectively reduced apoptosis while increasing the total viable cell numbers of blastocysts (Fig. 3). This finding is related to the ability of CA to increase GSH levels and reduce ROS accumulation, because high levels of ROS can damage cell membranes and DNA, causing apoptosis [55, 56]. The downregulation of the apoptotic genes *Birc5* and *Caspase3* and the upregulation of the anti-apoptotic gene *Rictor* in the CA-supplemented group also support the anti-apoptotic effect of CA [57–59]. In addition, CA is known to alleviate endoplasmic reticulum stress [60], which reduces apoptosis through inhibition of the JNK, p38, MAPK, and NF-κB signaling pathways [61–63]. However, we also found that the blastocyst rate in the 25 μM CA group was lower than that in the NC group; this result is similar to the findings of some studies indicating that CA also affects the degradation of CDK [64] and induces apoptosis by regulating Akt, NF-κB, mTOR, and the Src/STAT3 signaling pathways, depending on the concentration and cell line [65–68].

Finally, we examined the expression of the pluripotency-related genes *Nanog* and *Sox2* and the blastocyst formation-related genes *Gata4*, *Cox2*, and *Itga5* to explore the effects of CA on early embryo pluripotency (Fig. 6). The gene expression of *Sox2* and *Nanog* were upregulated by supplementation with CA, indicating that CA can maintain pluripotency and self-renewal as well as the development of pre-implantation embryos [69, 70]. The higher expression of *Gata4*, *Cox2*, and *Itga5* genes in the CA group compared to the NC group was consistent with the higher blastocyst rate and better blastocyst quality in the CA supplementation group [71–73]. This finding also indicated that CA can affect blastocyst hatching and embryonic stem cell differentiation into mature functional cells by regulating Gata4 and Itga5 [74, 75], and that it can affect embryo implantation and spacing by regulating Cox2 [76, 77].

In this study, we tested the effects of CA on early parthenogenetic embryo development, because we can easily obtain a larger number of embryos with similar biological properties. Parthenogenetic embryos have been widely accepted as a model for embryo development mechanism research as well as drug screening, toxicity research, and human-assisted reproduction [78, 79]. However, gene expression and certain biological characteristics of parthenogenetic embryos still have some differences compared with IVF embryos [80, 81]. Therefore, before applying CA as an antioxidant to IVP or a functional health care drug, more in-depth research on its safety and biological effects is still needed. Nevertheless, our results suggest that supplementation of IVC medium with appropriate concentrations of CA can enhance early embryonic development by reducing ROS levels and apoptosis, as well as enhancing mitochondrial function. More importantly, CA-supplemented feeds or diets may not only have benefits on improving the plasma lipid profile [82], detoxifying the body [83], and delaying aging [84], but also have potential on stabilizing the microenvironment and physiological functions of female mammals by reducing the content of ROS accumulation in the ovary and reproductive tract, as well as in the fertilization of eggs and implantation [85, 86].

In summary, this study suggests that supplementation of the IVC medium with 10 μM CA can improve the quality of early embryos, enhancing parameters such as cleavage rates, blastocyst rates, blastocyst diameters, hatching rates, and total cell numbers in blastocysts. More importantly, we confirmed that CA can improve GSH levels, inhibit ROS accumulation, enhance mitochondrial function, upregulate embryo pluripotency, and suppress early embryo apoptosis. These results will aid in the development of antioxidants and in the improvement of IVC systems in the future.

Conflict of interests: The authors have no conflicts of interest regarding the contents of this article.

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