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Original Research Article

Plant-derived squalene supplementation improves growth performance and alleviates acute oxidative stress-induced growth retardation and intestinal damage in piglets

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ABSTRACT

Piglets are particularly susceptible to oxidative stress, which causes inferior growth performance and intestinal damage. Squalene (SQ), a natural bioactive substance enriched in shark liver oil, shows excellent antioxidant properties and can currently be obtained at a low cost from deodorizer distillate during the production of plant oil. This study aimed to evaluate the effects of plant-derived SQ supplementation on the growth performance of piglets and explore the beneficial roles of SQ against oxidative stress and intestinal injury in diquat-challenged piglets. Forty piglets were randomly divided into five groups and fed a basal diet supplemented with SQ at 0, 500, 1000, or 2000 mg/kg for 5 weeks. Acute oxidative stress was induced in the piglets with diquat (10 mg/kg BW) at the fourth week of the experiment, followed by a 7-d recovery period. Results showed that before the diquat challenge, SQ supplementation significantly improved growth performance (average daily gain and feed conversion ratio) and serum antioxidant status, and after the diquat challenge, SQ supplementation significantly mitigated diquat-induced growth arrest, intestinal villous atrophy, intestinal epithelial cell apoptosis, intestinal hyperpermeability, and deficiency of intestinal epithelial tight junction proteins (zonula occludens-1, occludin, and claudin-3). Under oxidative stress induced by diquat, SQ supplementation consistently improved the antioxidant status of the small intestine, liver, and muscle. In vitro, SQ was shown to alleviate hydrogen peroxide (H_2O_2)-induced increase of the levels of intracellular reactive oxygen species and apoptosis of porcine intestinal epithelial cells. Taken together, SQ supplementation improves growth performance and effectively alleviates acute oxidative stress-induced growth retardation and intestinal injury via improving antioxidant capacity in piglets. Our findings may provide an efficient strategy for alleviating oxidative stress-induced inferior growth performance and intestinal damage in piglets.

1. Introduction

Intensive farming in the modern pig industry boosts the productivity of pigs, but it also increases the risk of oxidative stress in pigs (Durand et al., 2022). Compared to pigs at other growth stages, post-weaning piglets are particularly vulnerable to oxidative stress arising from various factors, such as weaning stress, immune stress, and nutritional, environmental, and social challenges (Lapointe

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et al., 2020; Majumder et al., 2022). The occurrence of oxidative stress usually leads to inferior growth performance and a series of diseases, resulting in huge economic losses to the pig industry (Hao et al., 2021; Li et al., 2022). It has been proposed that the use of antioxidant feed additives is a nutritional strategy to improve the health status and growth performance of piglets.

Squalene (SQ), a natural polyunsaturated triterpene compound, can be found abundantly in shark liver oil and virgin olive oils (Dormont et al., 2020; Yarkent and Oncel, 2022). It is also an intermediate substance for the synthesis of cholesterol and is well tolerated by mammals (Relas et al., 2001; Tegenge et al., 2016). Notably, the supplementary SQ in the diet is easily absorbed and transported to various tissues along with low-density lipoprotein, and only a small amount of it is used for cholesterol synthesis in humans (Miettinen and Vanhanen, 1994). Studies have shown that SQ from plant oils and shark liver displays a wide range of biological activities, including antioxidant, anti-inflammatory, anticancer, and antiaging effects (Garcia-Bermudez et al., 2019; Lou-Bonafonte et al., 2018). Due to the key role of oxidative stress in disease development, the antioxidant properties of SQ have attracted great attention. A previous study has shown that dietary supplementation with SQ can improve the reproductive performance of boars (Zhang et al., 2008). Another study has reported that SQ supplementation can improve the antioxidant status, growth performance, and meat quality of broilers (Chen et al., 2020). Of particular note is that a recent study has demonstrated that SQ alleviates oxidative stress-induced growth retardation and liver injury in broilers (Chen et al., 2021). The increasing demand for SQ in human health has greatly promoted its large-scale production from plant oils, such as olive, palm, soybean, sunflower, and rice bran oil (Al-Darmaki et al., 2012; Cetinbas et al., 2022; Gunawan et al., 2008). Currently, SQ has gradually achieved low-cost and large-scale productions from the plants, which increases the potential of SQ as a livestock feed additive. However, the effects of SQ on the growth performance of piglets are still unknown.

The intestine is one of the most vulnerable organs to oxidative stress, and its health largely determines the growth performance of animals (Lauridsen, 2019; Wang et al., 2020). Growing evidence has demonstrated that oxidative stress causes damages to intestinal morphology and dysfunction, including the barrier, absorption, and immunity in piglets (Jin et al., 2021; Sun et al., 2021). The intestinal epithelium, which regenerates every 3 to 5 d, is considered a fundamental dynamic barrier against hostile factors from the intestinal lumen (Holloway et al., 2021). The balance between intestinal epithelial cell apoptosis and cell regeneration from crypts is easily disrupted by some negative factors, such as oxidative stress, infection, and malnutrition (Morris and Jasper, 2021). Several in vivo and in vitro studies have shown that oxidative stress triggers excessive apoptosis of intestinal epithelial cells, resulting in the dysfunction of the small intestinal mucosa (Jin et al., 2021; Liu et al., 2020). A previous in vitro study has shown that SQ protects human mammary epithelial cells against H_2O_2-induced DNA damage and efficiently scavenges intracellular reactive oxygen species (ROS) (Warleta et al., 2010). In vivo, studies have shown that dietary SQ supplementation alleviates DSS-induced acute colitis and LPS-induced intestinal hyperpermeability in mice (Sánchez-Fidalgo et al., 2015). However, little is known about the protective role of SQ against oxidative stress-induced intestinal damage.

Diquat is widely reported to induce acute oxidative stress in piglets, and hydrogen peroxide (H_2O_2) is commonly used to induce reversible oxidative stress in vitro (Koch and Hill, 2017). In terms of the action mode, diquat increases the production of superoxide anions and disrupts the cellular redox balance; however, it can hardly be metabolized and degraded in vitro (Magalhães et al., 2018). In contrast, H_2O_2, as an exogenous ROS, directly enters in vitro cells and gently induces reversible oxidative stress. In the present study, we applied intraperitoneal injection of diquat to induce oxidative stress in piglets and further explored the potential protective roles of SQ against oxidative stress. In vitro, the protective effects of SQ against oxidative damage in porcine intestinal epithelial cells were explored by inducing oxidative stress in the intestinal porcine epithelial cell line J2 (IPEC-J2) cells with H_2O_2. Our results indicate that SQ improves the growth performance of piglets and effectively alleviates oxidative stress-induced growth retardation and intestinal damage, which may provide an efficient solution for alleviating oxidative stress-induced inferior growth performance in piglets.

2. Materials and methods

2.1. Animal ethics statement

All animal experiments were performed in accordance with the guidelines of the Laboratory Animal Ethical Commission of the Institute of Subtropical Agriculture, the Chinese Academy of Sciences (Permit No. ISA 2021040).

2.2. Evaluation of SQ purity

The deodorized distillate of vegetable oils was subjected to emulsification, esterification, cooling crystallization, and pressure filtration to obtain the filtrate, following which SQ was obtained by multi-stage distillations of the filtrate. The content levels of SQ in olive oil, peanut oil, soybean oil, and rapeseed oil are 100–10,200 mg/kg, 41–1343 mg/kg, 16–184 mg/kg, and 10–125 mg/kg, respectively. To determine the purity of SQ used in this study, the samples were analyzed by gas chromatography (7890 Series, Agilent Technologies, Madrid, Spain) equipped with flame ionization (GC-FID). The SQ standard with a purity greater than 98% (Sigma) was used for determining the standard curve. The separation was performed on a non-polar BPX-5ms SGE column (30 m × 0.32 mm i.d.; 0.25 μm-film thickness). Chromatography conditions were set as follows: the column temperature was initially established at 160 °C, maintaining isothermal conditions for 5 min, increasing to 280 °C (15 °C/min), maintaining the isothermal conditions for 5 min, and then increasing to 300 °C (5 °C/min), and maintaining the isothermal conditions for 2 min. The temperature of the detector was 330 °C. The temperature of the injection port was 300 °C. Nitrogen with purity 99.99% was used as the carrier gas at a flow rate of 2.5 mL/min. The flow rate of makeup gas is 30 mL/min. The injection volume was 1.0 μL. The results of the purity evaluation of the SQ are shown in Fig. S1.

2.3. Animal care and experimental design

As shown in Fig. 1, a total of 40 Duroc × Landrace × Large Yorkshire crossbred piglets with the same paternal origin were randomly divided into five groups ($n = 8$/group, half male and half female) at 1 week after weaning (30-d-old). Piglets in both control and diquat groups were fed the basal diet, while piglets in SQ-supplement groups were fed SQ at 500 mg/kg diet (LSQ), 1000 mg/kg diet (MSQ), and 2000 mg/kg diet (HSQ). During the 35-d experiment, all piglets were individually housed in pens with hard plastic slatted flooring and had ad libitum access to the experimental diet and water.

The basal diet (Table 1) was formulated according to the recommended nutrient requirements of swine (NRC, 2012). The data of digestible energy, Met + Cys and available P were calculated using data provided by the China Feed Database (CFD, 2020). The

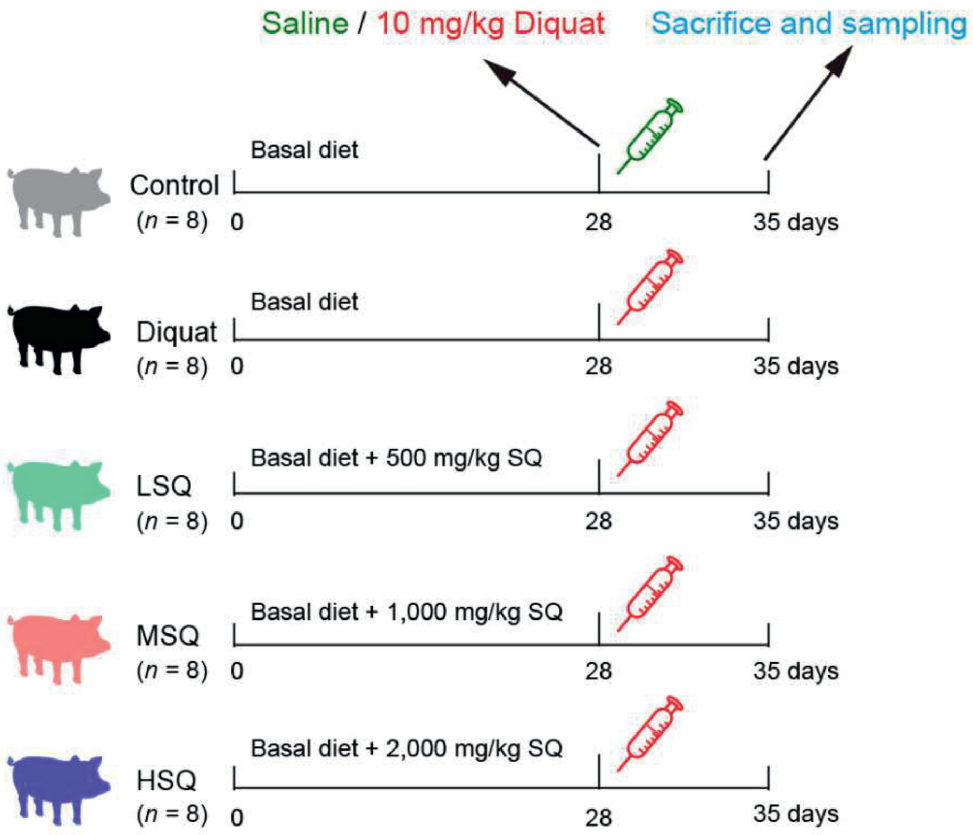

Fig. 1. The schematic diagram of experimental design. During the 35-d experiment, five groups of piglets were fed basal diet with or without squalene supplementation (500 mg/kg diet [LSQ], 1000 mg/kg diet [MSQ], 2000 mg/kg diet [HSQ]) and injected with saline or diquat at 10 mg/kg body wight at 28 d of the experiment.

levels of crude protein, calcium (Ca), and total phosphorus (P) were measured according to the national standards of China (GB/T6432-2018, GB/T 6436-2018, and GB/T 6437-2018, respectively) (SAC, 2018a,b,c). The levels of Lys, Met, and Thr were measured according to the national standards of China (GB/T18246-2000) and the level of Trp was measured according to the national standards of China (GB/T 15400-2018).

The dosages of diquat (Sigma–Aldrich, Inc., St. Louis, MO) and SQ were selected according to previous studies (Chen et al., 2020; Lv et al., 2012; Warleta et al., 2010). SQ (86% purity) used in this study was extracted and purified by Dahaigui Life Science Co., Ltd. (Yichun City, Jiangxi Province, China) from deodorizer distillate during the production of soybean, sunflower, camellia, and rape oil. To evenly mix the SQ in the basal diet, the SQ was pre-dissolved in soybean oil, which is one of the compositions of the basal diet. Diquat injections were prepared by dissolving liquid diquat into a phosphate buffer solution (PBS) solution to a concentration of 10 mg/mL.

The experiment lasted for 35 d, during which the piglets in each group were fed a basal diet or a basal diet supplemented with SQ. Feed intake and weight gain were monitored throughout the experiment. The average daily gain (ADG), average daily feed intake (ADFI), and the ratio of feed to BW gain (F:G ratio) of piglets during different stages of the experiment were recorded. On d 28, blood samples were taken from the piglets in each group via the anterior vena cava. Then, the piglets were intraperitoneally injected with PBS solution or diquat. On d 35, blood samples were taken from the anterior vena cava, following which the piglets were anesthetized with intravenous sodium pentobarbital (50 mg/kg BW) and bled by exsanguination. Jejunal mucosa, liver, and Longissimus dorsi muscle samples were collected, immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. Cell culture and SQ treatment

Cell culture was conducted according to our previous study (Zhao et al., 2022). IPEC-J2 purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) was used for in vitro testing. Cells were grown in DMEM/F-12 (Gibco, Life Technologies, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, California, USA) and 1% penicillin-streptomycin (Gibco, Life Technologies, Carlsbad, California, USA) solution and maintained in an atmosphere of 5% CO_2 at 37 °C. The H_2O_2 was purchased from Solarbio (Beijing, China). SQ (Sigma, St. Louis, Missouri, USA) was dissolved in alcohol and configured into a 100-fold concentrated stock ethanolic solution. The cells in the experimental groups were treated with SQ (1, 10, and 100 μmol/L), while the cells in the control group were exposed to ethanol at the same concentrations as the solutions used for the SQ treatment groups. The details of cell experiments and the treatments were described in the specific results section.

2.5. Cell viability and apoptosis assays

The cell viability was determined using the cell counting kit-8 (CCK-8; Dojindo, Kyushu, Japan), according to the manufacturer's instructions. Briefly, after specific treatments, cells in the 96-well plates were incubated with 10 μL CCK-8 working solution for 45 min, following which the OD value at 450 nm of each well was

Table 1
Ingredients and nutrition levels of basal diet for the piglets (as-fed basis, %).

Ingredients	Content
Corn	62.72
Soybean meal	18.51
Wheat bran	0.30
Whey powder	5.00
Fish meal	5.00
Soybean oil	2.50
Glucose	2.50
Limestone	0.60
$CaHPO_4$	0.60
Salt	0.40
Lys	0.54
Met	0.10
Thr	0.20
Try	0.06
Vitamin-mineral premix[1]	1.00
Total	100
Calculated nutrient levels[2]	
Digestible energy, MJ/kg	14.45
Met + Cys	0.74
Available P	0.40
Measured nutrient levels	
Crude protein	18.73
Lys	1.52
Met	0.43
Thr	0.85
Trp	0.26
Ca	0.79
Total P	0.61

[1] Provided the following per kilogram of diet: vitamin A, 8750 IU; vitamin D, 2500 IU; vitamin E, 25 IU; vitamin K_3, 2.5 mg; vitamin B_1, 2.5 mg; vitamin B_2, 6.25 mg; vitamin B_6, 2.5 mg; vitamin B_{12}, 25 μg; D-biotin, 0.10 mg; folic acid, 1.25 mg; nicotinamide, 30 mg; D-pantothenic acid, 25 mg; Zn, 100 mg; Fe, 100 mg; Cu, 20 mg; Mn, 20 mg; I, 0.3 mg; Se, 0.3 mg.
[2] Calculated from data provided by China Feed Database (CFD, 2020).

determined using a microplate reader (Infinite M200 PRO, Tecan, Swiss). To assess apoptosis, the pretreated cells were stained with PI/Annexin-V-FITC (KeyGEN, Nanjing, China) according to the manufacturer's instructions. The PI/Annexin-V-FITC stained cells were monitored by flow cytometry (BD, Franklin Lake, New Jersey, USA). According to the fluorescence intensities of cells in the negative control group without any staining, the normal cells, early apoptotic cells, late apoptotic cells, and mechanically damaged cells were gated using BD FACSDiva software (BD, Franklin Lake, New Jersey, USA).

2.6. ROS analysis by flow cytometry and fluorescence staining

Flow cytometry was employed for the determination of intracellular ROS levels, according to a previous study (Gao et al., 2022). Briefly, after the indicated treatments, IPEC-J2 cells were incubated with 20 μmol/L 2,7-dichlorodihydrofluorescein diacetate for 30 min at 37 °C in the dark and washed twice with PBS. Fluorescence intensities of 10,000 cells were analyzed using CELLQuest software on FACScalibur (Becton Dickinson, San Jose, CA). ROS fluorescence staining was conducted according to the manufacturer's instructions (Reactive Oxygen Species Assay Kit, Shanghai, China). The images of ROS fluorescence staining were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

2.7. Hematoxylin and eosin (H&E) staining

To analyze villus height and crypt depth, H&E staining was performed on the jejunal samples according to a previous study (Jiang et al., 2019). Briefly, approximately 3 cm of jejunum from piglets was collected, fixed in 4% paraformaldehyde, washed with PBS, dehydrated with alcohol, and embedded in paraffin blocks. Then, the sections were deparaffinized, hydrated, and stained with H&E staining. Villous heights and crypt depths were measured using ImageJ software (1.52P version).

2.8. Terminal deoxynucleotidyl transferase-mediated dUTP nick labeling (TUNEL) assay

For the apoptotic detection of the cells at the top of villi, TUNEL was performed by using TUNEL Kit (Keygen Biotech, Nanjing, China) according to the manufacturer's instructions. The pictures were taken using a confocal laser scanning microscope (Zeiss, Germany).

2.9. Scanning electron microscopy

The segments of the jejunum were fixed with cold 2.5% glutaraldehyde for 2 h and washed three times using PBS, with 3 min for each wash. Then, the tissues were postfixed in 1% osmium tetroxide for 12 h at 4 °C and then washed three times for 10 min each in PBS. After ethanol dehydration, tert-butyl alcohol storage, quick-drying silver paint installation, and gold-palladium coating, the tissues were examined by a scanning electron microscope. Specifically, after postfixation in 1% tetroxide, the segments were dehydrated, embedded, and cut into thin sections. Then, they were stained with uranyl acetate and lead citrate for 20 min before being scanned with the electron microscope.

2.10. Determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities, and malondialdehyde (MDA) levels in samples

After an overnight fast, blood samples were collected from the anterior vena cava and then centrifuged at 3000 × *g* for 10 min at 4 °C to obtain the serum for further assay. The jejunal mucosa, liver, and muscle samples were homogenized in ice-cold PBS. Then, the homogenates were centrifuged at 12000 × *g* for 10 min at 4 °C to collect supernatant for further assay. The activities of GSH-Px, SOD, and CAT, and the level of MDA in serum, tissues, and cells were determined following the manufacturer's instructions (Jiancheng Bioengineering Institute, China). To normalize these data, the protein concentrations of the tissue samples were measured using a bicinchoninic acid kit (Beyotime Biotechnology, Shanghai, China).

2.11. The Western blot

The Western blot analysis was conducted according to a previous study (Gao et al., 2022). Briefly, the total protein of the samples was extracted using a strong RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China). Protein concentrations were measured using a BCA kit (Beyotime Biotechnology, Shanghai, China) and then adjusted to a uniform concentration. The protein samples were mixed with a 5× SDS-PAGE Sample Loading Buffer (Beyotime Biotechnology, Shanghai, China), followed by boiling at 95 °C for 5 min. Then, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and blocked with 5% defatted milk in Tris-Tween saline buffer for 1 h, followed by incubating with corresponding primary antibodies zonula occludens-1 (ZO-1) (Rabbit, Thermal Scientific, 1:1000), occludin (Rabbit, Thermal Scientific, 1:1000), claudin-1 (Rabbit, Thermal Scientific, 1:1000), claudin-3 (Rabbit, Thermal Scientific, 1:1000), and β-actin (Rabbit, CST, 1:5000) for 12 h at 4 °C. Secondary antibodies were subsequently incubated for 1 h at 25 °C before the development of the blots using the Odyssey Infrared Imaging (Bio-Rad, Hercules, California, USA).

2.12. Statistical analysis

SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA) was used for the data analysis. Data are presented as the means ± standard error of the mean (SEM). Shapiro–Wilk was used to analyze whether the data conform to normal distribution. Data conforming to normal distribution were analyzed by one-way ANOVA and Duncan's multiple range test was used for multiple comparisons. If the data failed to meet normal distribution, statistical comparisons were analyzed using the Mann–Whitney test. $P < 0.05$ was considered significant.

3. Results

3.1. Squalene improves growth performance and the serum antioxidant status

To evaluate the effects of SQ supplementation on the growth performance and antioxidant capacity of piglets, both feed intake and weight gain were recorded during 0–28 d of the experiment. As shown in Table 2, compared to the control group, supplementing with 1000 or 2000 mg/kg SQ significantly increased the ADG and decreased the F:G ratio of piglets during 0–28 d. There is no difference in ADFI and BW among the five groups. Meanwhile, as shown in Table 3, supplementing with 1000 and 2000 mg/kg SQ decreased the concentration of MDA in serum. The serum SOD activity of the piglets in the HSQ group was higher than that in the control group. There is no difference in GSH-Px and CAT activities among the five groups. These results demonstrated that SQ supplementation improves the growth performance and serum antioxidant status of piglets under normal feeding conditions.

3.2. Squalene alleviates diquat-induced growth arrest and oxidative stress

To evaluate the soothing properties of SQ on diquat-induced growth retardation and oxidative stress, we recorded feed intake and BW gain and detected the antioxidant parameters in serum after diquat injection. As shown in Table 2 (28 to 35 d), results showed that the diquat challenge significantly decreased ADG, increased F:G ratio, and decreased ADFI. Compared to the diquat group, 1000 and 2000 mg/kg SQ supplementation increased the ADG and decreased F:G ratio. The ADFI and final weight of the piglets in the HSQ group were higher than those in the diquat group. As shown in Table 4, results further showed that the diquat challenge significantly increased the level of MDA, and decreased the activities of SOD and GSH-Px in serum. Neither the diquat nor the SQ affected the CAT activity in the serum. MSQ and HSQ supplementation effectively alleviated the increase in MDA and the decrease in SOD and GSH-Px activities induced by diquat.

Table 2
Growth performance of piglets.

Item	Dietary treatment groups[1]					SEM	*P*-value
	Control	Diquat	LSQ	MSQ	HSQ		
Day 0 to 14							
Day 1 BW, kg	9.30	9.31	9.31	9.32	9.31	0.057	1.000
ADFI, g	543.57	542.05	549.91	573.75	576.34	10.003	0.717
ADG, g	374.55	370.09	381.25	403.57	408.04	5.892	0.138
F:G ratio	1.45	1.46	1.44	1.42	1.41	0.008	0.315
Day 14 BW, kg	14.54	14.49	14.64	14.97	15.02	0.134	0.635
Day 14 to 28							
ADFI, g	1020.98	1021.79	1034.55	1118.04	1115.63	19.085	0.240
ADG, g	556.70^{b}	556.34^{b}	573.21^{ab}	642.41^{a}	631.70^{a}	11.482	0.022
F:G ratio	1.84^{a}	1.84^{a}	1.81^{abc}	1.74^{c}	1.77^{bc}	0.012	0.011
Day 28 BW, kg	22.34	22.28	22.67	23.96	23.86	0.277	0.123
Day 0 to 28							
ADFI, g	782.28	781.92	792.23	845.89	845.99	13.649	0.319
ADG, g	465.63^{b}	463.21^{b}	477.23^{ab}	522.99^{a}	519.87^{a}	8.203	0.027
F:G ratio	1.68^{a}	1.69^{a}	1.66^{ab}	1.61^{b}	1.63^{b}	0.009	0.013
Day 28 to 35							
ADFI, g	1210.36^{a}	922.50^{c}	930.54^{c}	934.46^{c}	1030.18^{b}	20.157	<0.01
ADG, g	643.75^{a}	392.68^{d}	405.36^{d}	437.50^{c}	525.89^{b}	15.586	<0.01
F:G ratio	1.88^{c}	2.35^{a}	2.29^{a}	2.13^{b}	1.96^{c}	0.032	<0.01
Day 35 BW, kg	26.84^{ab}	25.03^{b}	25.51^{b}	27.03^{ab}	27.54^{a}	0.316	0.048

BW = body weight; ADFI = average daily feed intake; ADG = average daily gain; F:G ratio = feed-to-gain ratio.
Data were presented as means and SEM ($n = 8$). Means without a common superscript differ at $P < 0.05$.
[1] Control, basal diet; Diquat, 10 mg/kg BW diquat; LSQ, 10 mg/kg BW diquat + 500 mg/kg squalene in basal diet; MSQ, 10 mg/kg BW diquat + 1000 mg/kg squalene in basal diet; HSQ, 10 mg/kg BW diquat + 2000 mg/kg squalene in basal diet.

Table 3
Serum redox status of piglets before diquat challenge.

Item	Dietary treatment groups[1]				SEM	*P*-value
	Control	LSQ	MSQ	HSQ		
MDA, nmol/mL	3.43^{a}	3.31^{ab}	3.14^{bc}	3.01^{c}	0.053	0.018
SOD activity, U/mL	129.40^{b}	137.06^{ab}	139.18^{ab}	147.24^{a}	2.237	0.035
GSH-Px activity, U/mL	732.69	775.57	778.36	791.51	11.336	0.292
CAT activity, U/mL	2.85	2.92	3.15	3.13	0.155	0.886

MDA = malondialdehyde; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; SQ = squalene.
Data were presented as means and SEM ($n = 8$). Means without a common superscript differ at $P < 0.05$.
[1] Control, basal diet; LSQ, diquat at 10 mg/kg BW + SQ at 500 mg/kg basal diet; MSQ, diquat at 10 mg/kg BW + SQ at 1000 mg/kg basal diet; HSQ, diquat at 10 mg/kg BW + SQ at 2000 mg/kg basal diet.

Table 4
SQ supplementation improved the serum redox status of piglets challenged with diquat.

Item	Dietary treatment groups[1]					SEM	*P*-value
	Control	Diquat	LSQ	MSQ	HSQ		
MDA, nmol/mL	3.13^{c}	4.39^{a}	4.16^{a}	3.77^{b}	3.44^{b}	0.086	<0.001
SOD activity, U/mL	179.19^{a}	143.10^{b}	153.60^{b}	154.03^{b}	172.81^{a}	3.313	0.001
GSH-Px activity, U/mL	1205.57^{a}	933.86^{c}	1018.29^{bc}	1022.71^{bc}	1102.29^{ab}	26.196	0.009
CAT activity, U/mL	4.17	3.48	3.45	3.65	3.80	0.173	0.682

MDA = malondialdehyde; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; SQ = squalene.
Data were presented as means and SEM ($n = 8$). Means without a common superscript differ at $P < 0.05$.
[1] Control, basal diet; Diquat, diquat at 10 mg/kg BW; LSQ, diquat at 10 mg/kg BW + SQ at 500 mg/kg basal diet; MSQ, diquat at 10 mg/kg BW + SQ at 1000 mg/kg basal diet; HSQ, diquat at 10 mg/kg BW + SQ at 2000 mg/kg basal diet.

Collectively, these results indicate that SQ supplementation alleviates diquat-induced growth retardation and oxidative stress.

3.3. Squalene alleviates diquat-induced oxidative stress in the liver and muscle

The activity of antioxidative enzymes and MDA levels in the experimental piglets were detected to elucidate the effects of SQ supplementation on the antioxidant status of the liver and muscle. As shown in Table 5, the diquat challenge significantly increased the MDA levels and decreased the activity of SOD and GSH-Px in the liver compared to the control group. Compared to the diquat group, both MSQ and HSQ diet supplementation significantly decreased the MDA levels and increased the activity of SOD in the liver. HSQ treatment reversed the diquat-induced adverse changes in the activity of liver GSH-Px. The diquat challenge significantly increased the MDA levels and decreased the SOD activity in the muscle. Compared to the diquat group, the muscle MDA levels of piglets in the HSQ group were significantly lower. There was no difference in muscle SOD activity between the HSQ group and the control group. These results suggest that SQ supplementation improves the antioxidant status of the liver and muscle against diquat-induced oxidative stress in piglets.

3.4. Squalene alleviates diquat-induced morphological damage in the jejunum

The scanning electron microscopy results showed that the diquat challenge induced more collapse and cell exfoliation of the jejunal epithelium, whereas MSQ and HSQ supplementation effectively ameliorated diquat-induced morphological damage of the jejunum (Fig. 2A). H&E staining results showed that the diquat challenge decreased villous height and the ratio of villous height to crypt depth, and increased crypt depth (Fig. 2B–E). MSQ and HSQ supplementation effectively alleviated the diquat-induced decrease in villous height and the ratio of villous height to crypt depth, as well as the increase in crypt depth (Fig. 2B–E). These results demonstrate that SQ supplementation alleviates diquat-induced morphological damage in the jejunum.

3.5. Squalene supplementation alleviates diquat-induced apoptosis of intestinal epithelial cells and disruption of intestinal epithelial integrity

Diquat challenge significantly increased the number of apoptotic jejunal epithelial cells (TUNEL-positive cells) compared to the control group (Fig. 3A and B). Compared to the diquat group, the number of apoptotic jejunal epithelial cells (TUNEL-positive cells) in piglets from the MSQ and HSQ groups was significantly decreased (Fig. 3A and B). Compared to the control group, the serum levels of D-lactate and diamine oxidase (DAO) were increased by the diquat challenge, but they were successfully alleviated by the HSQ supplementation (Fig. 3C and D). Compared to the control group, the levels of tight junction proteins ZO-1, occludin, and claudin-3 in the jejunum of piglets from the diquat group were significantly decreased (Fig. 3E–F, H). Compared to the diquat group, the protein levels of ZO-1, occludin, and claudin-3 in the jejunum of piglets were increased by SQ supplementation (Fig. 3E–F, H). These results suggest that SQ alleviates the malignant apoptosis of small intestinal epithelial cells, intestinal hyperpermeability, and the loss of tight junction proteins induced by diquat.

3.6. Squalene alleviates oxidative stress-induced apoptosis and loss of cell viability in IPEC-J2 cells

Oxidative-stressed IPEC-J2 cells were used to verify the protective role of SQ in porcine intestinal epithelial cells against oxidative damage. Considering that previous studies have shown that SQ does not affect cell viability between 0 and 100 μmol/L (Granados-Principal et al., 2012; Warleta et al., 2010), we choose 0,

Table 5
SQ supplementation improved the redox status of the liver and muscle in piglets challenged with diquat.

Item	Dietary treatment groups[1]					SEM	*P*-value
	Control	Diquat	LSQ	MSQ	HSQ		
Liver							
MDA, nmol/mg	0.32^{c}	0.43^{a}	0.41^{ab}	0.38^{b}	0.35^{c}	0.008	<0.001
SOD activity, U/mg	434.83^{a}	382.56^{c}	393.92^{bc}	403.02^{b}	421.85^{a}	4.032	<0.001
GSH-Px activity, U/mg	356.60^{a}	308.05^{b}	305.90^{b}	315.76^{b}	341.72^{ab}	6.134	0.021
Muscle							
MDA, nmol/mg	0.146^{c}	0.185^{a}	0.171^{a}	0.168^{ab}	0.150^{bc}	0.0037	0.001
SOD activity, U/mg	66.82^{a}	59.13^{bc}	56.80^{c}	61.15^{bc}	63.28^{ab}	0.937	0.004

MDA = malondialdehyde; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; SQ = squalene.
Data were presented as means and SEM ($n = 8$). Means without a common superscript differ at $P < 0.05$.
[1] Control, basal diet; Diquat, diquat at 10 mg/kg BW; LSQ, diquat at 10 mg/kg BW + SQ at 500 mg/kg basal diet; MSQ, diquat at 10 mg/kg BW + SQ at 1000 mg/kg basal diet; HSQ, diquat at 10 mg/kg BW + SQ at 2000 mg/kg basal diet.

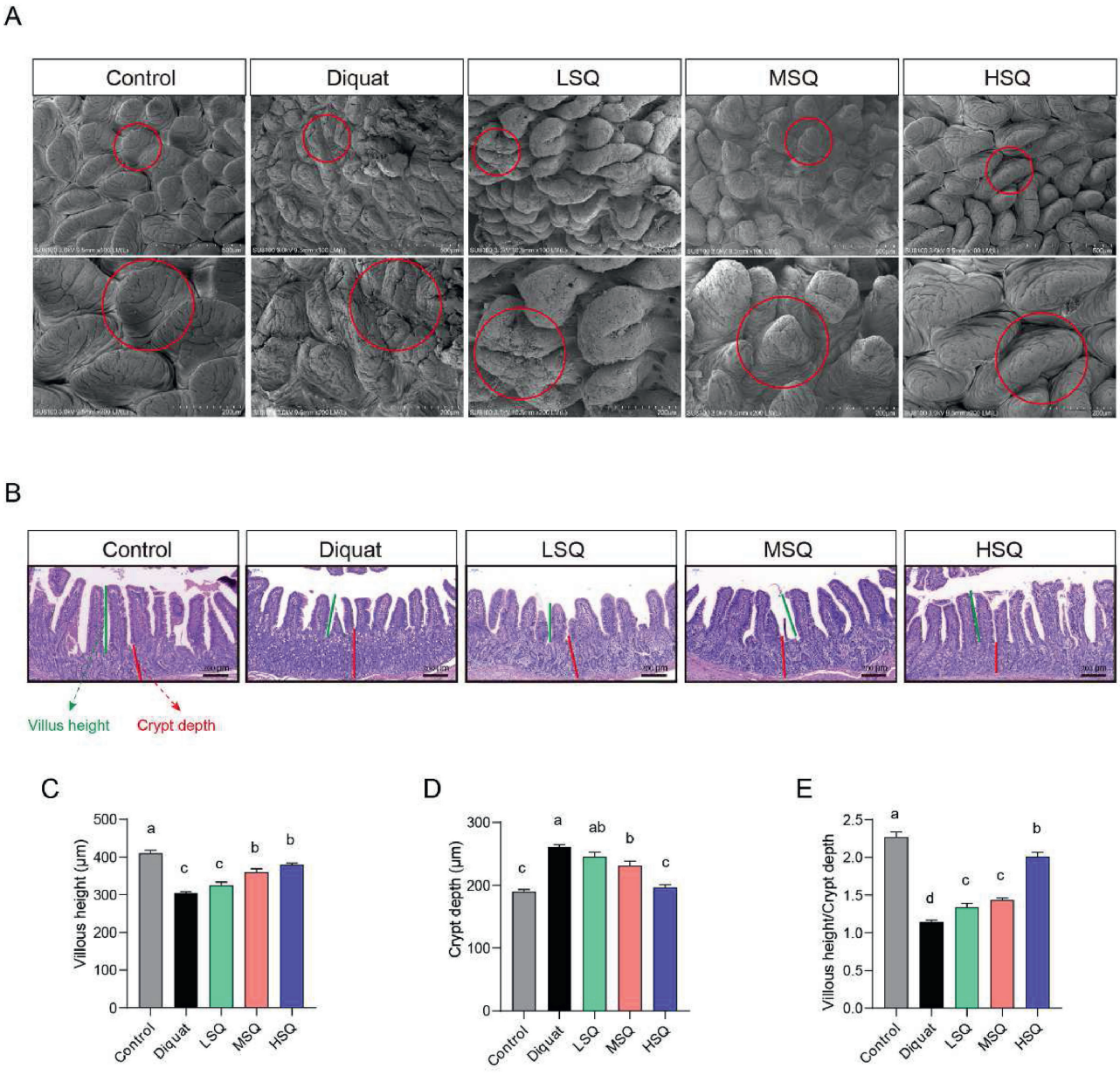

Fig. 2. Squalene (SQ) supplementation alleviates diquat-induced jejunal atrophy. (A) Scanning electron micrographs, (B) H&E staining, (C) villous height, (D) crypt depth, and (E) the ratio of villus height to crypt depth of the jejunum in the diquat-challenged piglets pretreated with LSQ, MSQ, and HSQ. Control, basal diet; Diquat, diquat at 10 mg/kg BW; LSQ, diquat at 10 mg/kg BW + SQ at 500 mg/kg basal diet; MSQ, diquat at 10 mg/kg BW + SQ at 1000 mg/kg basal diet; HSQ, diquat at 10 mg/kg BW + SQ at 2000 mg/kg basal diet. Data were presented as means ± SEM ($n = 8$). Means without a common superscript differ at $P < 0.05$.

1, 10, and 100 μmol/L as the concentrations for SQ pretreatment on IPEC-J2 cells. As shown in Fig. 4A, the cell viability was not negatively affected by the SQ (0, 1, 10, and 100 μmol/L) supplementation. To obtain the appropriate concentration of H_2O_2 to induce oxidative stress, cells were cultured in a serum-free medium supplemented with various concentrations of H_2O_2 (0, 50, 100, 200, 400, 800, 1600 μmol/L) for 4 h, and then returned to the normal medium for 24 h. As shown in Fig. 4B, the cell viability decreased in a dose-dependent manner when exposed to H_2O_2. We further chose the calculated half maximal inhibitory concentration (IC50) concentration of H_2O_2 (446 μmol/L) for further experiments. A previous study has demonstrated that SQ treatment for 3 h can significantly increase intracellular SQ content (Tatewaki et al., 2016). The treatment with SQ for 24 h can significantly enhance the antioxidant capacity of A549 cells and human mammary epithelial cells, and reduce the expression of CD36 scavenger receptor in macrophages (Granados-Principal et al., 2012; Tatewaki et al., 2016; Warleta et al., 2010). In the present experiment, cells were cultured with the medium supplemented with different concentrations of SQ (0, 1, 10, and 100 μmol/L) for 24 h and then incubated with serum-free medium supplemented with H_2O_2 and SQ for 4 h, before being returned to normal medium supplemented with different concentrations of SQ for 24 h. The results showed that SQ supplementation at levels of 10 and 100 μmol/L significantly alleviated the loss of cell viability induced by H_2O_2 (Fig. 4C and D). To determine whether SQ alleviates oxidative stress-induced intestinal epithelial apoptosis, cells were treated with the medium supplemented with 100 μmol/L SQ for 48 h, and then incubated with serum-free medium supplemented with H_2O_2 (446 μmol/L) and SQ (100 μmol/L) for 4 h. Compared to

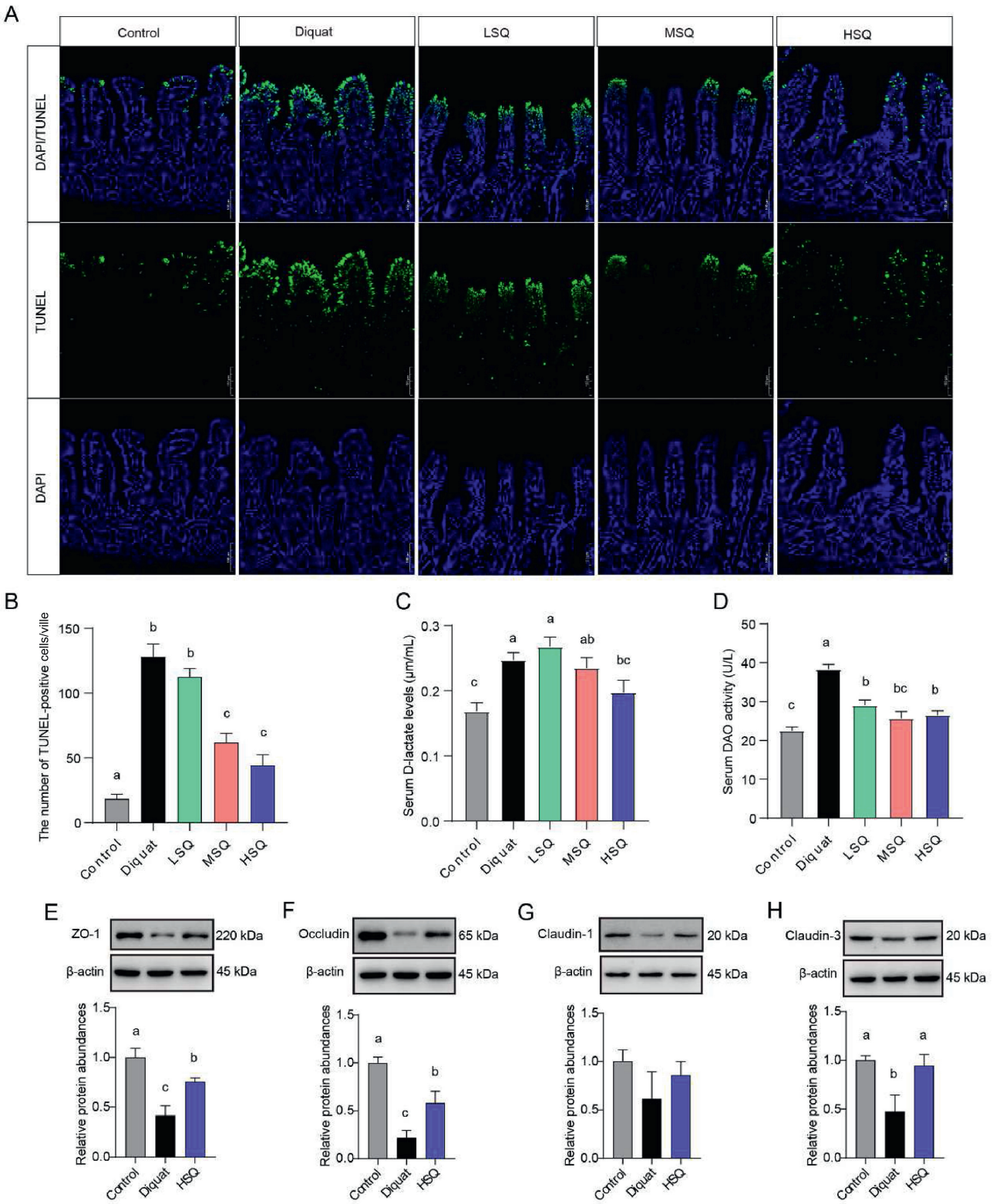

Fig. 3. Squalene (SQ) supplementation alleviates diquat-induced intestinal epithelial cell apoptosis and disruption of intestinal epithelial integrity. (A) Representative images of immunofluorescence for TUNEL and DAPI in the jejunum, (B) the number of TUNEL-positive cells in jejunal villi ($n = 8$), (C-D) serum D-lactate levels and DAO activity ($n = 8$), and (E-H) tight junction protein (ZO-1, occludin, claudin-1, and claudin-3) levels of jejunal mucosa ($n = 3$) in the diquat-challenged piglets pretreated with LSQ, MSQ, and HSQ. Control, basal diet; Diquat, diquat at 10 mg/kg BW; LSQ, diquat at 10 mg/kg BW + SQ at 500 mg/kg basal diet; MSQ, diquat at 10 mg/kg BW + SQ at 1000 mg/kg basal diet; HSQ, diquat at 10 mg/kg BW + SQ at 2000 mg/kg basal diet. Protein abundances for ZO-1, occludin-1, claudin-1, and claudin-3 were quantified by Image Lab 3.0. Beta-actin was used as the loading control. Data were presented as means ± SEM. Means without a common superscript differ at $P < 0.05$. TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick labeling; DAPI = 4′,6-diamidino-2-phenylindole; DAO = diamine oxidase; ZO-1 = zonula occludens-1.

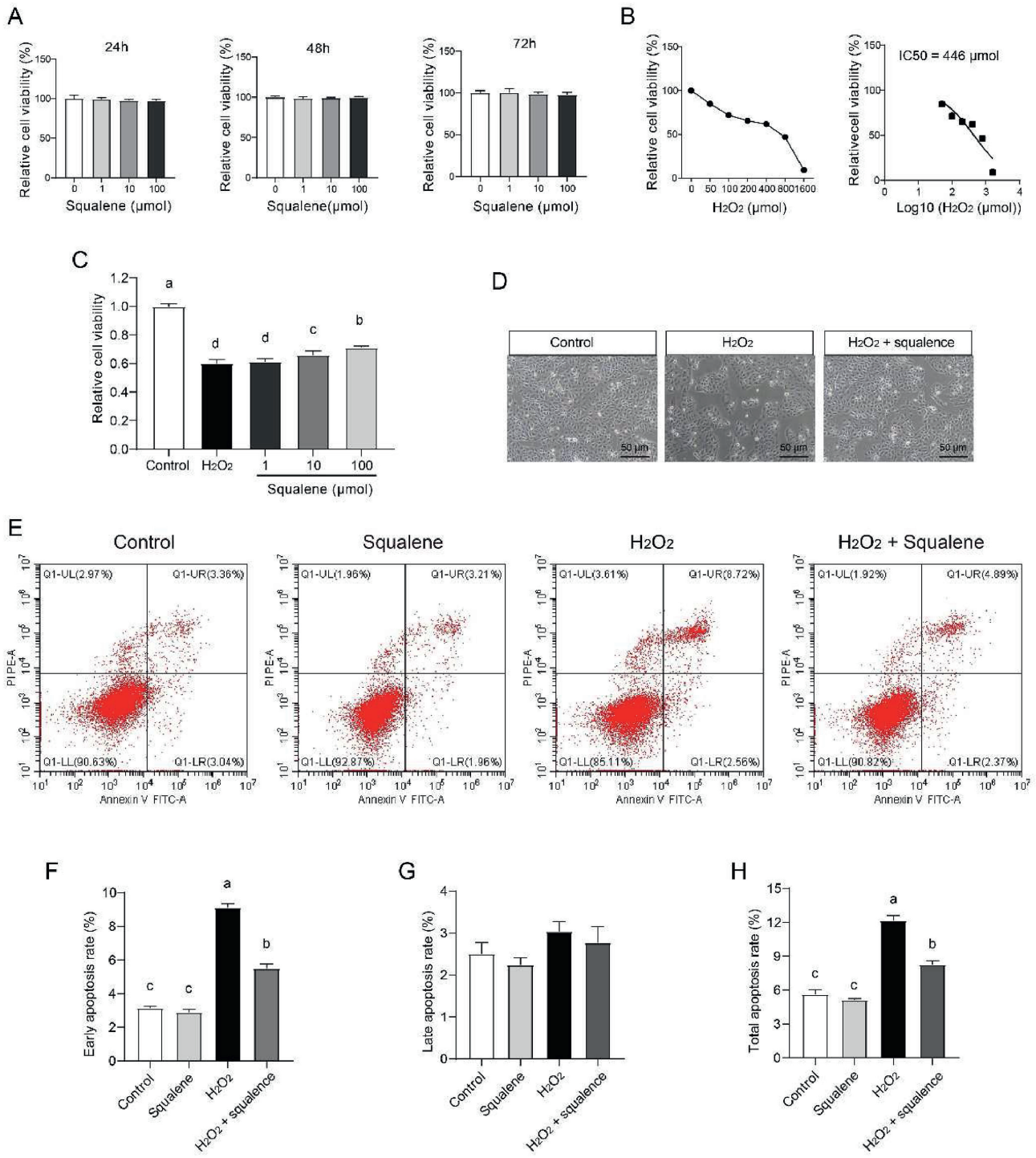

Fig. 4. Squalene (SQ) alleviates oxidative stress-induced excessive apoptosis and viability loss of IPEC-J2 cells. (A) Relative cell viability of IPEC-J2 after different concentrations of SQ treatment ($n = 6$). (B) Relative cell viability after different concentrations of H_2O_2 exposure ($n = 8$). (C) Relative cell viability after 446 μmol/L H_2O_2 exposure and different concentrations of SQ treatment ($n = 8$). (D) Representative morphology images, (E) representative flow cytometry images, (F) early apoptosis rate ($n = 4$), (G) late apoptosis rate ($n = 4$), and (H) total apoptosis rates ($n = 4$) of the cells. Data were presented as means ± SEM. Means without a common superscript differ at $P < 0.05$. IPEC-J2 = intestinal porcine jejunal epithelial cell line J2; IC50 = half maximal inhibitory concentration; PIPE = propidine iodide; FITC = fluorescein isothiocyanate.

the control group, the early apoptosis rate and total apoptosis rate increased when IPEC-J2 cells were exposed to H_2O_2, whereas SQ effectively decreased the early apoptosis rate and total apoptosis rate compared to the H_2O_2 group (Fig. 4E–H). These results demonstrate that SQ alleviates oxidative stress-induced apoptosis and the inhibition of viability in IPEC-J2 cells.

3.7. Squalene alleviates diquat-induced oxidative stress in jejunal mucosa and H_2O_2-increased intracellular ROS in IPEC-J2 cells

To elucidate the effects of SQ supplementation on the jejunal antioxidant status of the piglets during the diquat challenge, the activity of antioxidative enzymes and MDA levels in the jejunal

mucosa were detected. As shown in Fig. 5A–C, the diquat challenge significantly increased jejunal MDA level and decreased jejunal SOD and GSH-Px activity compared to the control group, whereas SQ supplementation, especially at high concentrations, effectively ameliorated diquat-induced adverse changes in jejunal mucosa. The CAT activity of the jejunal mucosa was not affected by diquat or SQ (Fig. 5D). To assess the effect of SQ supplementation on the level of intracellular ROS and the antioxidant status after exposure to H_2O_2, the IPEC-J2 cells were pretreated with 100 μmol/L SQ for 24 h and then challenged with 446 μmol/L H_2O_2 for 4 h, followed by a recovery incubation with 100 μmol/L SQ for 4 h. As shown in Fig. 5E–H, SQ pretreatment effectively decreased the intracellular

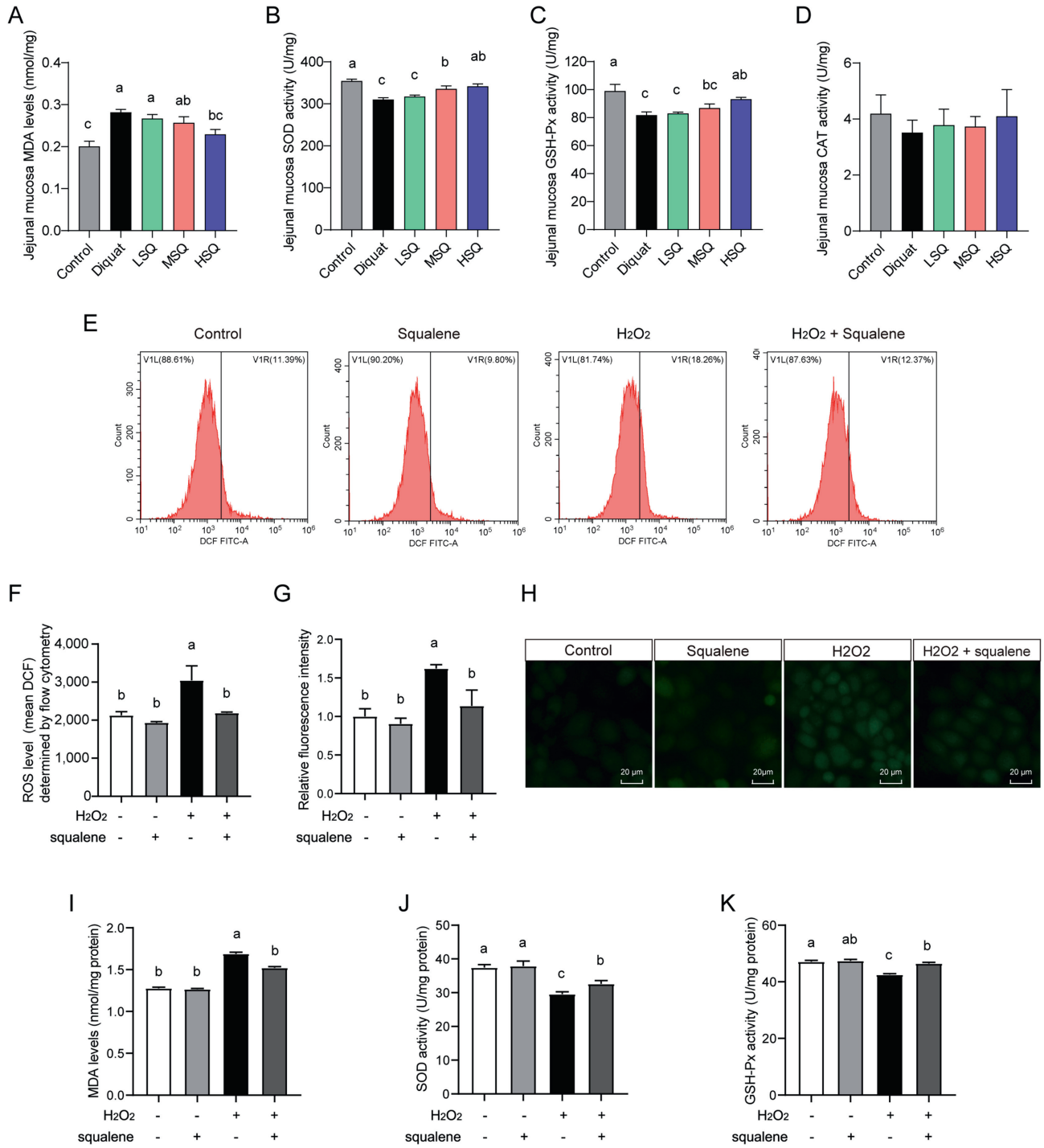

Fig. 5. Squalene (SQ) alleviates oxidative stress in the jejunal mucosa and IPEC-J2 cells. (A) MDA levels, (B) SOD activity, (C) GSH-Px activity, and (D) CAT activity of jejunal mucosa in the diquat-challenged piglets ($n = 8$) pretreated with LSQ, MSQ, and HSQ. (E) Graphical representation of ROS content in the IPEC-J2 cells analyzed by flow cytometry. (F) Relative ROS level, (G) relative ROS fluorescence intensity, and (H) representative ROS fluorescence images ($n = 4$) are shown. (I) MDA levels, (J) SOD activity, and (K) GSH-Px activity of cells after 446 μmol/L H_2O_2 exposure and 100 μmol/L SQ treatment. Control, basal diet; Diquat, diquat at 10 mg/kg BW; LSQ, diquat at 10 mg/kg BW + SQ at 500 mg/kg basal diet; MSQ, diquat at 10 mg/kg BW + SQ at 1000 mg/kg basal diet; HSQ, diquat at 10 mg/kg BW + SQ at 2000 mg/kg basal diet. Data were presented as means ± SEM. Means without a common superscript differ at $P < 0.05$. IPEC-J2 = intestinal porcine jejunal epithelial cell line J2; MDA = malondialdehyde; SOD = superoxide dismutase; ROS = reactive oxygen species; GSH-Px = glutathione peroxidase; CAT = catalase; DCF = 2′,7′-dichlorodihydrofluorescein; FITC = fluorescein isothiocyanate.

ROS levels in oxidative-stressed IPEC-J2 cells. As shown in Fig. 5I–K, SQ pretreatment alleviated the H_2O_2-induced increase in MDA levels and the decline in SOD and GSH-Px activities. These results suggest that SQ supplementation can effectively scavenge ROS and improve the antioxidant status in porcine intestinal epithelial cells, thereby contributing to alleviating diquat-induced acute oxidative stress in the jejunum of piglets.

4. Discussion

Piglets are particularly susceptible to oxidative stress, which results in inferior growth performance and intestinal damage. Squalene, can currently be obtained at a low cost from plants. This study examined the effects of plant-derived SQ on growth performance and the potential beneficial roles of SQ against diquat-induced acute oxidative stress and intestinal damage in piglets. We demonstrated that plant-derived SQ supplementation improves growth performance under normal feeding conditions and effectively alleviates oxidative stress-induced growth retardation and intestinal injury by improving antioxidant capacity in piglets.

In the modern pig industry, piglets are susceptible to oxidative stress induced by various stress factors (e.g., environmental, nutritional, and psychological) (Salami et al., 2016). Employing antioxidants in feed is an effective way to improve growth performance. To date, the effects of SQ supplementation on the growth performance of piglets are largely unknown. In the present study, we found that SQ supplementation, especially at levels of 1000 and 2000 mg/kg in feed, significantly improved the ADG and feed conversion efficiency of piglets under normal feeding conditions. This is similar to a recent report that dietary SQ supplementation from 250 to 2000 mg/kg linearly improved the growth performance of broilers (Chen et al., 2020). In addition, a previous study has shown that feeding a basal diet supplemented with SQ at 20 or 40 mg/kg can improve the reproductive performance of boars (Zhang et al., 2008). Generally, the beneficial effects of SQ on the growth performance of the animals may be attributed to its antioxidative, immune-regulatory, and nutritional effects (Kim and Karadeniz, 2012; Lou-Bonafonte et al., 2018; Yarkent and Oncel, 2022).

The growth performance of piglets is closely related to their health status, and piglets with oxidative stress usually show inferior growth performance (Hao et al., 2021; Lauridsen, 2019). Many studies have shown that diquat-induced oxidative stress causes growth retardation in piglets (Azad et al., 2021; Zheng et al., 2013). To further classify whether the antioxidative mechanism is involved in the health-promoting effects of SQ on post-weaning piglets, the piglets were further challenged with diquat to induce acute oxidative stress. As expected, in the present study, intraperitoneal injection of 10 mg/kg BW diquat decreased the ADG, the ADFI, and increased F:G ratio, and the oxidative stress model in piglets induced by diquat was successfully established.

Squalene, shows excellent antioxidant properties (Kojima et al., 1981; Martínez-Beamonte et al., 2018; Tatewaki et al., 2016). Due to the properties of high cell membrane absorption, high bioavailability, and relatively low cost, plant-derived SQ has the potential to be applied as a feed additive for pigs (Martínez-Beamonte et al., 2018; Tatewaki et al., 2016). A previous study found that oral administration of SQ improved weight gain and final BW in a rodent model of oxidative stress induced by cyclophosphamide (Senthilkumar et al., 2006). Of note, a recent study has shown that dietary SQ supplementation alleviates the growth arrest of broiler chickens caused by diquat-induced oxidative stress (Chen et al., 2021). As expected, in this study, we obtained the SQ from the plant and verified that the plant-derived SQ supplementation at 2000 mg/kg efficiently alleviated diquat-induced growth retardation, which prompts us to further explore the potential protective roles of SQ against acute oxidative damages.

Oxidative stress is usually defined as the disturbance of redox homeostasis. MDA is an important end product of lipid peroxidation, the level of which indirectly reflects the degree of oxidative damage (Tsikas, 2017). The activity of antioxidant enzymes such as SOD, GSH-Px, and CAT, can reflect the antioxidant capacity (Sies, 2015). In this study, the diquat challenge decreased the activities of SOD and GSH-Px and increased the levels of MDA in the serum of piglets. Of note, SQ supplementation effectively alleviates the poor antioxidant status of the serum caused by diquat. Similarly, oral administration of SQ was reported to improve the antioxidant status of rats challenged with cyclophosphamide (Senthilkumar et al., 2006). In this study, we found that plant-derived SQ supplementation improved the overall antioxidant status of the piglets, which may contribute to improving the growth performance of piglets under normal feeding conditions. These findings indicate that plant-derived SQ may be used as an effective and low-cost antioxidant for feed formulas in the future. The liver is a major organ susceptible to oxidative stress (Ferro et al., 2020). In this study, we found that SQ supplementation alleviated the increase in MDA levels and the decrease in the SOD and GSH-Px activities in the liver caused by diquat, which suggests that SQ supplementation improves the antioxidant status of the liver against oxidative stress. For the muscle tissue, we also found that SQ supplementation alleviated the increase of MDA levels and the decrease of SOD activity caused by diquat. Overall, SQ supplementation improved the antioxidant capacity and alleviated oxidative damage in piglets during the diquat challenge.

Oxidative stress usually leads to intestinal damage and dysfunction. Of note, a previous study has shown that dietary SQ supplementation markedly increased SQ accumulation in the intestinal mucosa, which implies that SQ has great potential for protecting the intestine from oxidative damage (Tilvis and Miettinen, 1983). Evidence suggests that diquat-induced oxidative stress leads to abnormal morphology and dysfunction of the small intestine (Cao et al., 2019). DAO is an intracellular enzyme of intestinal epithelial cells. When small epithelial cells are damaged, DAO will be released into the blood. Serum DAO levels reflect the damage degree of the intestinal epithelium (Luk et al., 1981). Serum D-lactate levels are positively correlated with the degree of intestinal epithelial injury, which directly reflects the permeability of intestinal epithelium (Günel et al., 1998). Similarly, we found that diquat challenge induced the atrophy of jejunal epithelium, shortening of villi, deepening of crypts, intestinal hyperpermeability (reflected by higher serum DAO and D-lactate levels), and deficiency of tight junction proteins (ZO-1, occludin, and claudin-3) in the piglets. Importantly, SQ supplementation effectively alleviated these adverse effects caused by diquat. These results suggest that SQ supplementation alleviates diquat-induced intestinal oxidative damage, which helps alleviate the growth retardation induced by acute oxidative stress. To date, studies have shown that SQ has a beneficial role in some diseases such as atherosclerosis, cancer, and diabetes (Lou-Bonafonte et al., 2018). A previous study found that dietary SQ supplementation alleviated DSS-induced colitis by downregulating p38/MAPK and nuclear factor kappa-B signaling pathways (Sánchez-Fidalgo et al., 2015). Another study reports that SQ alleviated LPS-induced dysfunction of the intestinal epithelial barrier in vivo and in vitro (Felices et al., 2019). To the best of our knowledge, we found that SQ supplementation effectively alleviated oxidative stress-induced intestinal damage in piglets for the first time.

The small intestinal epithelium is one of the fastest renewing tissues, renewing every 3 to 5 d, which acts as a dynamic barrier against challenges from the luminal environment (Sato et al., 2009). The balance between the apoptosis of small intestinal epithelial

cells shedding from the villi and the replenishment of cells from the crypt is the basis for maintaining the barrier function of the intestine (van der Flier and Clevers, 2009). The present results showed that diquat injection significantly increased the apoptosis of jejunal epithelial cells, which could be one of the mechanisms involved in diquat-induced jejunal epithelial atrophy and the decrease of jejunal villi height. This finding is similar to the previous one, which showed that diquat induced apoptosis of intestinal porcine epithelial cells (IPEC-1) in vitro (Jin et al., 2021). Notably, in our study, SQ supplementation in feed effectively alleviated diquat-induced apoptosis of the jejunal epithelial cells and intestinal hyperpermeability in piglets. In line with our in vivo results, our in vitro results demonstrated that SQ alleviated oxidative stress-induced apoptosis in IPEC-J2 cells. Moreover, we further demonstrated that SQ could help scavenge the intracellular ROS in IPEC-J2 cells under oxidative stress, which agrees with a previous study that showed that SQ decreased intracellular ROS levels of MCF10A human mammary epithelial cells in a dose-dependent manner (Warleta et al., 2010). Of note, the SQ supplementation decreased the content of MDA and increased the activities of SOD and GSH-Px both in the oxidative-stressed jejunal mucosa and IPEC-J2 cells. These results consistently suggest that SQ improves the antioxidant capacity of the intestinal mucosa and alleviates acute oxidative stress-induced excessive apoptosis of intestinal epithelial cells.

In conclusion, we found that plant-derived SQ supplementation improved growth performance under normal feeding conditions and effectively alleviated diquat-induced acute oxidative stress, growth retardation, and small intestinal injury in piglets. These findings may provide an efficient strategy for alleviating oxidative stress-induced inferior growth performance and intestinal damage in piglets.

Author contributions

Junquan Tian: Conceptualization, Methodology, Investigation, Writing-original draft. **Qian Jiang**: Writing-original draft, Writing-review & editing, Funding acquisition. **Xuetai Bao**: Validation, Writing-review & editing, Writing-original draft. **Fan Yang**: Validation, Writing-review & editing. **Yuying Li**: Validation, Writing-review & editing, Writing-original draft. **Haihun Sun**: Conceptualization, Writing-review & editing. **Kang Yao**: Conceptualization, Supervision, Writing-review & editing, Funding acquisition. **Yulong Yin**: Conceptualization, Supervision, Writing-review & editing, Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.09.001.

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