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Establishment of a Hydrogen Peroxide-Induced Oxidative Damage Model in Primary Hepatocytes of Orange-Spotted Grouper (*Epinephelus coicoides*) (Postprint)

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Date: 2017-10-23T00:00:00+00:00

Abstract

This study utilized primary hepatocytes from orange-spotted grouper (Epinephelus coioides) as the experimental model, with hydrogen peroxide as the stimulus, and employed hepatocyte viability and changes in antioxidant indices as evaluation criteria to establish a stable oxidative damage model in primary hepatocytes of orange-spotted grouper. Hydrogen peroxide at concentrations of 0 (control), 100, 200, 400, 600, 800, and 1,000 mol/L was added to the primary hepatocyte culture medium for exposure durations of 2, 4, 6, 8, 12, and 24 h, comprising a total of 42 experimental groups with 10 replicates per group, to determine hepatocyte viability. Based on the determination of the appropriate hydrogen peroxide exposure time, each concentration of hydrogen peroxide (with 6 replicates per concentration) was applied to hepatocytes for the optimal duration, after which hepatocytes and culture medium were collected to measure antioxidant indices, thereby screening for the appropriate hydrogen peroxide concentration that induces oxidative damage in hepatocytes. The results demonstrated that treatment with 800 mol/L hydrogen peroxide for 8 h reduced the viability of orange-spotted grouper hepatocytes to 61.98%; compared with other groups, the 800 and 1,000 mol/L groups exhibited significantly decreased activities of superoxide dismutase, glutathione peroxidase (except for the 600 mol/L group), and catalase ($P < 0.05$), along with significantly elevated contents of malondialdehyde and lipid peroxides ($P < 0.05$), although no significant difference was observed between the 800 and 1,000 mol/L groups ($P > 0.05$). These findings indicate that a hydrogen peroxide concentration of 800 mol/L and an exposure time of 8 h can serve as suitable conditions for establishing an oxidative damage model in hepatocytes of orange-spotted grouper.

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Full Text

Establishment of an Oxidative Damage Model in Primary Hepatocytes of Grouper (Epinephelus coioides) Induced by Hydrogen Peroxide

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Abstract: This study aimed to establish a stable oxidative damage model in primary hepatocytes of grouper (Epinephelus coioides) using hydrogen peroxide (H_2O_2) as the stressor, with hepatocyte viability and antioxidant indices as evaluation criteria. Primary hepatocyte cultures were treated with H_2O_2 at concentrations of 0 (control), 100, 200, 400, 600, 800, and 1,000 mol/L for durations of 2, 4, 6, 8, 12, and 24 hours, comprising 42 experimental groups with 10 replicates each to determine hepatocyte viability. Based on the optimal exposure time determined, each H_2O_2 concentration (6 replicates per concentration) was applied to hepatocytes for the selected duration, after which cells and culture medium were collected for antioxidant index measurements to identify the appropriate H_2O_2 concentration for inducing oxidative damage. The results demonstrated that treatment with 800 mol/L H_2O_2 for 8 hours reduced hepatocyte viability to 61.98%. Compared with other groups, the 800 and 1,000 mol/L groups exhibited significantly decreased activities of superoxide dismutase, glutathione peroxidase (except for the 600 mol/L group), and catalase ($P < 0.05$), along with significantly elevated malondialdehyde and lipid peroxide contents ($P < 0.05$). However, no significant differences were observed between the 800 and 1,000 mol/L groups ($P > 0.05$). These findings indicate that treatment with 800 mol/L H_2O_2 for 8 hours provides suitable conditions for establishing an oxidative damage model in grouper hepatocytes.

Keywords: grouper (Epinephelus coioides); primary hepatocytes; hydrogen peroxide; oxidative damage model

Introduction

Grouper is one of the most important marine aquaculture species in China. However, intensive farming practices, including high-density cultivation stress, water eutrophication, and nutritional imbalances, have severely hindered industrial development in recent years [1-3]. When animals are exposed to harmful environmental factors, metabolic dysfunction occurs [4-6]. As aquatic lower vertebrates, fish are particularly susceptible to adverse environmental conditions. While appropriate stress responses can help fish adapt to their environment and improve immune function, thereby increasing aquaculture productivity [6-7], excessive oxidative stress not only impairs production performance but also induces various diseases and even mass mortality [8-10].

The liver is a vital metabolic organ in vertebrates that continuously adjusts its physiological state to adapt to environmental changes [11]. Hepatocytes can perform most liver functions [12], making them a valuable alternative to whole-animal studies for investigating toxicological effects and metabolic pathways. Cell culture models offer numerous advantages, including reduced animal usage, operational simplicity, high reproducibility, and avoidance of interference from complex in vivo factors [13]. These models have been widely applied in toxicology, pharmacology, and related fields [14-15].

While oxidative stress responses have been studied in several economically important fish species, including common carp [16] (*Cyprinus carpio*), tilapia [17] (*Oreochromis mossambicus*), spotted snakehead [18] (*Channa punctatus*), and catfish [19] (*Silurus asotus*), research on liver oxidative damage in grouper remains scarce. Based on current domestic and international literature, this study utilized primary cultured hepatocytes from grouper as experimental material and measured various antioxidant indices to determine optimal conditions for establishing an H_2O_2-induced oxidative damage model.

Materials and Methods

Experimental Materials

Grouper (*Epinephelus coioides*) with a body weight of (50 ± 2) g and length of (13.0 ± 0.8) cm were obtained from the Marine Aquaculture Experimental Station of Jimei University.

Main Reagents

H_2O_2 stock solution, 0.25% trypsin, L-15 medium, phosphate-buffered saline (PBS), penicillin (10,000 IU/mL), streptomycin (10,000 g/mL), amphotericin B, insulin, and fetal bovine serum were purchased from Gibco (USA). Trypan blue and dimethyl sulfoxide (DMSO) were obtained from Shanghai Jierui Biological Engineering Co., Ltd. Assay kits for all measured indices were purchased from Nanjing Jiancheng Bioengineering Institute. All other chemical reagents were of analytical grade.

Primary Hepatocyte Culture

Primary hepatocytes were isolated from grouper using the method described by Luo et al. [20]. Cells in logarithmic growth phase were adjusted to a density of 2×10^5 cells/mL in complete medium (L-15 medium) and seeded into 25 cm^2 culture flasks containing L-15 medium supplemented with 20% fetal bovine serum (FBS). Cultures were maintained at 25 °C in a 5% CO_2 incubator.

Cell viability was determined using a hemocytometer. Cells were mixed 1:1 with 0.5% trypan blue; viable cells appeared transparent and oval, while dead cells stained blue. Viability was expressed as the percentage of viable cells among

total counted cells. Only primary hepatocytes with viability > 90% were used for subsequent experiments.

Experimental Design

A 96-well cell culture plate was used, with each well receiving 100 µL of cell suspension at a concentration of 2×10^5 cells/mL. H_2O_2 was added to primary hepatocyte cultures at final concentrations of 0 (control), 100, 200, 400, 600, 800, and 1,000 mol/L for exposure durations of 2, 4, 6, 8, 12, and 24 hours. This comprised 42 groups with 10 replicates each for viability determination.

Hepatocyte viability after H_2O_2 treatment served as the primary criterion for preliminary screening of optimal exposure time. Based on the selected exposure time, each H_2O_2 concentration (6 replicates per concentration) was applied to hepatocytes, after which both cells and culture medium were collected for antioxidant index measurements to further identify the appropriate H_2O_2 concentration for inducing oxidative damage [21-22].

Measurement of Hepatocyte Viability and Antioxidant Indices

Cell viability was determined using the MTT colorimetric method [23]. After measuring optical density (OD) values, hepatocyte viability was calculated using the following formula:

Hepatocyte viability (%) = 100 × OD_{570} nm of H_2O_2-treated group / OD_{570} nm of control group

Glutathione peroxidase (GSH-Px) activity was measured using the dithiobis-nitrobenzoic acid method, superoxide dismutase (SOD) activity by xanthine oxidase method, catalase (CAT) activity by colorimetric method, malondialdehyde (MDA) content by thiobarbituric acid method, and lipid peroxide (LPO) content by fluorometric assay. Detailed procedures for all indices followed the instructions provided with the assay kits from Nanjing Jiancheng Bioengineering Institute.

Data Processing

All data were organized and calculated using Origin 2015 and expressed as mean ± standard error (mean ± SE). Statistical analysis was performed using SPSS. Data were analyzed by one-way ANOVA when conditions were met. If significant differences were detected among groups, Duncan' s multiple range test was used for post-hoc comparisons. Differences were considered significant at $P < 0.05$ and non-significant at $P > 0.05$.

Results

Effects of H_2O_2 Concentration and Exposure Time on Hepatocyte Viability

As shown in Table 1 , hepatocyte viability decreased with increasing H_2O_2 concentration at each exposure time. When exposure time $\geq$ 8 hours, viability in the 600–1,000 mol/L groups was significantly lower than in the 0–400 mol/L groups at the same time point ($P < 0.05$).

At H_2O_2 concentrations of 800 and 1,000 mol/L, viability decreased dramatically when exposure time increased from 2–4 hours to 6–24 hours, dropping from 89.83% and 88.98% at 2 hours to 44.53% and 40.88% at 24 hours, respectively. This corresponded to cell death rates of 54–60%.

In this study, a viability range of 50–65% was selected as the criterion for model establishment [21]. The H_2O_2 concentrations and exposure times falling within this range were 800 mol/L for 8 hours (61.98% viability), 800 mol/L for 12 hours (50.19% viability), and 1,000 mol/L for 8 hours (60.08% viability). These results indicate that treatment with 800–1,000 mol/L H_2O_2 for 8 hours concentration-dependently reduced hepatocyte viability to a moderate degree. Therefore, 8 hours was selected as the appropriate exposure time.

Validation of Oxidative Damage Model Using Primary Hepatocyte Antioxidant Indices

To validate the reliability of the grouper oxidative damage model, antioxidant indices were measured in primary hepatocytes after 8 hours of exposure to various H_2O_2 concentrations. As shown in Table 2 , GSH-Px, CAT, and SOD activities in primary hepatocytes gradually decreased with increasing H_2O_2 concentration. Except for GSH-Px activity in the 600 mol/L group, the 800 and 1,000 mol/L groups exhibited significantly lower GSH-Px, CAT, and SOD activities compared with other groups ($P < 0.05$), though no significant difference was observed between the 800 and 1,000 mol/L groups ($P > 0.05$). The control group showed the highest GSH-Px, CAT, and SOD activities, significantly higher than all other groups ($P < 0.05$).

H_2O_2 also affected lipid peroxidation in grouper hepatocytes, as reflected by changes in LPO and MDA contents. As shown in Table 2, LPO and MDA levels exhibited opposite trends to antioxidant indices, gradually increasing with H_2O_2 concentration. Except for the 100 mol/L group, which showed no significant difference from the control ($P > 0.05$), all other H_2O_2 treatment groups were significantly higher than the control ($P < 0.05$). No significant differences were observed among the 200, 400, and 600 mol/L groups ($P > 0.05$) or between the 800 and 1,000 mol/L groups ($P > 0.05$).

Various methods have been reported for establishing H_2O_2-induced oxidative damage models in cells such as cardiomyocytes and hepatocytes [21], yet studies on oxidative damage models in grouper primary hepatocytes are extremely

limited. Moreover, different studies employ different evaluation indices and criteria for assessing oxidative damage. The MTT assay offers advantages including good reproducibility, high specificity, accuracy, and rapidity, with OD values reflecting both cell number and proliferation rate, making it suitable for model evaluation [24-25]. However, criteria vary across studies. For establishing oxidative damage models, cell viability should be neither too low nor too high. Excessively low viability indicates massive cell death and irreversible damage, which is unsuitable for studying antioxidant mechanisms, while excessively high viability suggests cells are in exponential growth with robust activity, resulting in insufficient oxidative damage for subsequent experiments [26]. Therefore, an appropriate degree of cellular injury is essential for model establishment.

H_2O_2 induces oxidative damage through generation of various oxygen radicals [such as superoxide anion radicals ($O_2^{-}\cdot$) and hydroxyl radicals ($\cdot OH$)] and promotion of lipid peroxidation. As H_2O_2-induced oxidative damage and intracellular antioxidant processes progress, the organism' s antioxidant capacity becomes imbalanced [27]. H_2O_2 causes oxidative damage that disrupts biological membranes, disturbs intracellular homeostasis, inactivates enzymes, accelerates protein denaturation, and subsequently impairs cellular antioxidant enzyme activity and capacity [28].

Using a hepatocyte viability range of 50–65% as the criterion [21], we preliminarily screened H_2O_2 concentrations and exposure times. In this study, treatment with 800 mol/L H_2O_2 for 8 hours yielded a viability of 61.98%, meeting the criterion. Based on this, antioxidant indices (GSH-Px, SOD, CAT activities and MDA, LPO contents) were used as validation criteria at the 8-hour time point. Treatment with 800 mol/L H_2O_2 significantly reduced antioxidant enzyme activities (GSH-Px, SOD, and CAT) and increased lipid peroxidation products (MDA and LPO) compared with the control group. These results demonstrate that 800 mol/L H_2O_2 induces significant changes in lipids and enzyme systems without causing complete cell death, making it the optimal concentration. Our findings are consistent with those of Jin [21], who established an H_2O_2-induced oxidative damage model in dairy cow mammary epithelial cells. Additionally, we observed no significant changes when H_2O_2 concentration was increased from 800 to 1,000 mol/L, indicating that 800 mol/L H_2O_2 is sufficient to induce obvious oxidative stress in grouper hepatocytes and can be used as the standard concentration for model establishment. Based on both cell viability and antioxidant index results, we conclude that the optimal conditions for establishing an H_2O_2-induced oxidative damage model in grouper primary hepatocytes are 800 mol/L H_2O_2 for 8 hours.

In the H_2O_2-induced oxidative damage model of grouper hepatocytes, both cell viability and antioxidant indices (GSH-Px, SOD, CAT activities and MDA, LPO contents) can serve as markers for oxidative stress. The suitable conditions for establishing this model are treatment with 800 mol/L H_2O_2 for 8 hours.

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