



Full length article

Met-enkephalin inhibits ROS production through Wnt/ β -catenin signaling in the ZF4 cells of zebrafish



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ABSTRACT

Opioid neuropeptides are developed early in the course of a long evolutionary process. As the endogenous messengers of immune system, opioid neuropeptides participate in regulating immune response. In this study, the mechanism that Met-enkephalin (M-ENK) inhibits ROS production through Wnt/ β -catenin signaling was investigated in the ZF4 cells of zebrafish. ZF4 cells were exposed to 0, 10, 20, 40, 80, and 160 μ M Met-enkephalin (M-ENK) for 24 h, and the cell viability was detected with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The cell viability was significantly increased by 10, 20, 40, 80, and 160 μ M M-ENK. After ZF4 cells were exposed to 0, 20, 40, and 80 μ M M-ENK for 24 h, the mRNA expression of Wnt10b, β -catenin, and CCAAT/enhancer binding protein α (C/EBP α) was significantly increased by 40 and 80 μ M M-ENK. However, the mRNA and protein expression of GSK-3 β was significantly decreased by 40 and 80 μ M M-ENK. The protein expression of β -catenin was significantly induced by 40 and 80 μ M M-ENK, while the protein expression of p- β -catenin was significantly decreased by 20, 40, and 80 μ M M-ENK. In addition, the mRNA expression of CAT, SOD, and GSH-PX was significantly increased by 40 and 80 μ M M-ENK. The levels of H₂O₂, \cdot OH, and O₂⁻ were significantly decreased, but the activity of CAT, SOD, and GSH-PX was significantly increased by 40 and 80 μ M M-ENK. The fluorescence intensity of reactive oxygen species (ROS) was decreased, and that of mitochondrial membrane potential (MMP) was increased with the increase of M-ENK concentration in ZF4 cells. The results showed that M-ENK could induce Wnt/ β -catenin signaling, which further inhibited ROS production through the induction of C/EBP α , MMP, and the activities of antioxidant enzymes.

1. Introduction

There are various pathogens and stress factors in water environment, which easily stimulate the inflammatory responses in fish species [1,2]. Reactive oxygen species (ROS), mainly including hydroxy radical (\cdot OH), superoxide anion (O₂⁻), and hydrogen peroxide (H₂O₂), are tightly associated with the innate immunity of fish [3–7]. The antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), catalase (CAT), play a key role in reducing the damage from oxidative stress [8,9].

Opioid neuropeptides are developed early in the course of a long evolutionary process. It is known that opioid neuropeptides participate in the cross talk between the nervous and immune systems. As the endogenous messengers of immune system, opioid neuropeptides are involved in regulating the immune response [10–12]. Moreover, opioid neuropeptides are not restricted in the immunocytes of mammalian organisms. It has been shown that opioid neuropeptides are also present in the immunocytes of invertebrates [13]. Under the stressful stimuli, the immune response can be altered by endogenous opioid neuropeptides through binding to the opioid receptors on inflammatory cells

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[14,15]. As one of the main opioid neuropeptides, Met-enkephalin (M-ENK) participates in regulating immune response through binding to a special subtype of delta receptor [16,17].

In the Wnt/ β -catenin signaling pathway, GSK-3 β plays a key role in regulating β -catenin accumulation. The action of GSK-3 β participates in the processes of β -catenin phosphorylation and lead to the degradation of β -catenin [18,19]. However, the inactivation of GSK-3 β causes the cytosolic accumulation of β -catenin, which regulates diverse transcription factors related to immune response [20,21]. However, there is little information on the molecular mechanism of M-ENK regulating oxidative stress in fish species. For the function of M-ENK on the production of ROS in fish remains unknown, it is essential to investigate whether M-ENK regulates ROS production through Wnt/ β -catenin signaling pathway. In this study, the mechanism that M-ENK regulates ROS production via Wnt/ β -catenin signaling was to be investigated in the zebrafish ZF4 cells.

2. Materials and methods

2.1. Cell culture

The ZF4 cells of Zebrafish were obtained from China Zebrafish Resource Center (CZRC) (Wuhan, China), and maintained at 28 °C with 5% CO₂ in DMEM:F12 (Gibco) supplemented with 10% fetal bovine serum.

2.2. MTT assay

M-ENK was purchased from Sigma (St. Louis, Mo.). The ZF4 cells were seeded onto 96-well plates (6×10^3 cell/well). ZF4 cells were treated by different concentration of M-ENK (0, 10, 20, 40, 80, and 160 μ M), and cultured at 28 °C with 5% CO₂ in DMEM:F12 (Gibco) supplemented with 10% fetal bovine serum for 24 h. Cellular viability was detected with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. 20 μ L MTT (5 mg/mL) and 100 μ L dimethyl sulfoxide was added to the 96-well plate. Finally, a Spectra MAX microplate reader was used to detect the absorbance at 595 nm. Six replicate wells were used for each concentration and the MTT assay experiment was repeated six times. Cell growth rate was expressed as absorbance relative to that of untreated controls.

2.3. M-ENK treatment

Based on the result of MTT, ZF4 cells were cultured in the basal culture medium containing 0, 20, 40, and 80 μ M M-ENK for 24 h. Then cells were collected for gene expression and ROS analysis, respectively.

2.4. Real-time quantitative polymerase chain reaction

Total RNA was extracted from ZF4 cells using Trizol reagent (Invitrogen, USA). A PrimeScript™ RT Reagent Kit (Takara, Japan) was used to transcribe RNA to cDNA, and the SYBR® Premix Ex Taq™ II (Takara, Japan) was used to quantify the expression level of genes. The

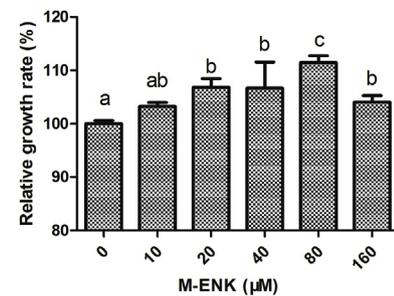


Fig. 1. Effect of M-ENK on the cellular viability of ZF4 cells. Values are expressed as means \pm SEM. (n = 6). Statistically significant differences are denoted by different letters ($P < 0.05$).

primer sequences for Wnt10b, β -catenin, GSK-3 β , C/EBP α , CAT, SOD, GSH-PX, and β -actin were designed and listed in Table 1. Real-time PCR was carried out in a quantitative thermal cycle (ROCHE, Light-Cycler®480, Switzerland). The $2^{-\Delta\Delta CT}$ method was used to analyze the differences of relative gene expression according to the protocol of Livak and Schmittgen [22].

2.5. Western blot analysis

ZF4 cells were cultured in basal culture medium containing 0, 20, 40, and 80 μ M M-ENK for 24 h. The ZF4 cell samples were prepared and the membranes were probed with the primary antibody and the goat anti-rabbit IgG-HRP conjugated secondary antibody as the method of Tian et al. [23]. Rabbit polyclonal antibodies *anti*- β -catenin (Cat. No. 8480, 1:1000), *anti*-phospho- β -Catenin (Ser675) (Cat. No. 4176, 1:1000), and *anti*-GSK-3 β (Cat. No. 12456, 1:1000) were purchased from Cell Signaling Technology Inc. Rabbit polyclonal antibody *anti*- β -actin (sc-1615, 1:10000) was purchased from Santa-Cruz Inc. Densitometry analyses were performed with the Image J software (National Institutes of Health).

2.6. Immunofluorescent microscopy of ZF4 cells

The cells (1×10^5 cell/ml) were seeded into each petri dish (35 mm, specially used for confocal microscopy), and cultured in basal culture medium containing 0, 20, 40, and 80 μ M M-ENK for 24 h. ZF4 cells were fixed with paraformaldehyde and incubated with *anti*- β -catenin antibody (Cat. No. 8480, Cell Signaling Technology Inc., 1:200) and the FITC dye secondary antibody (Santa-Cruz, 1:400), respectively. Finally, 5 μ M DRAQ5™ (Cell Signaling Technology Inc.) was used to stain nucleus, and the immunofluorescence of β -catenin was analyzed with a laser scanning confocal microscope (Leica TCS SP2, Germany).

2.7. Assay the level of ROS and the activities of antioxidant enzymes

Cells from each culture dish were homogenized and the supernatants were collected for ROS analysis. Protein concentration was assayed at 595 nm according to the method of Bradford [24]. The

Table 1

Real-time quantitative PCR primers for genes of zebrafish.

Target gene	Forward (5'-3')	Reverse (5'-3')	GenBank
Wnt10b	TCCTGAAACAGGCTCGAAGT	GCTGCTCACTTGCACACATT	AY182171.1
GSK-3 β	TCTGCTCACCCTTTCCTTTC	CTCCGACCCACTTAACCTCCA	NM_131381.1
β -catenin	GGAGCTCACCAGCTCTCTGT	TAGCTTGGGTCGTCTGTCT	NM_001001889.1
CEBP/ α	CACAACAGCTCCAAGCAAGA	AATCCATGTAGCCGTTGAGG	BC063934.1
CAT	CAAGGTCTGGTCCCATAAA	TGACTGGTAGTTGGAGGTA	BC051626
SOD	GTCGCACTTCAACCTCA	TCCTCATTGCCACCCTTCC	BC055516
GSH-PX	AGATGTCATTCTGCACAGG	AAGGAGAAGCTTCTCAGCC	AW232474
β -actin	CCGTGACATCAAGGAGAAGC	TACCGAAGATTCCATACCC	AF057040.1

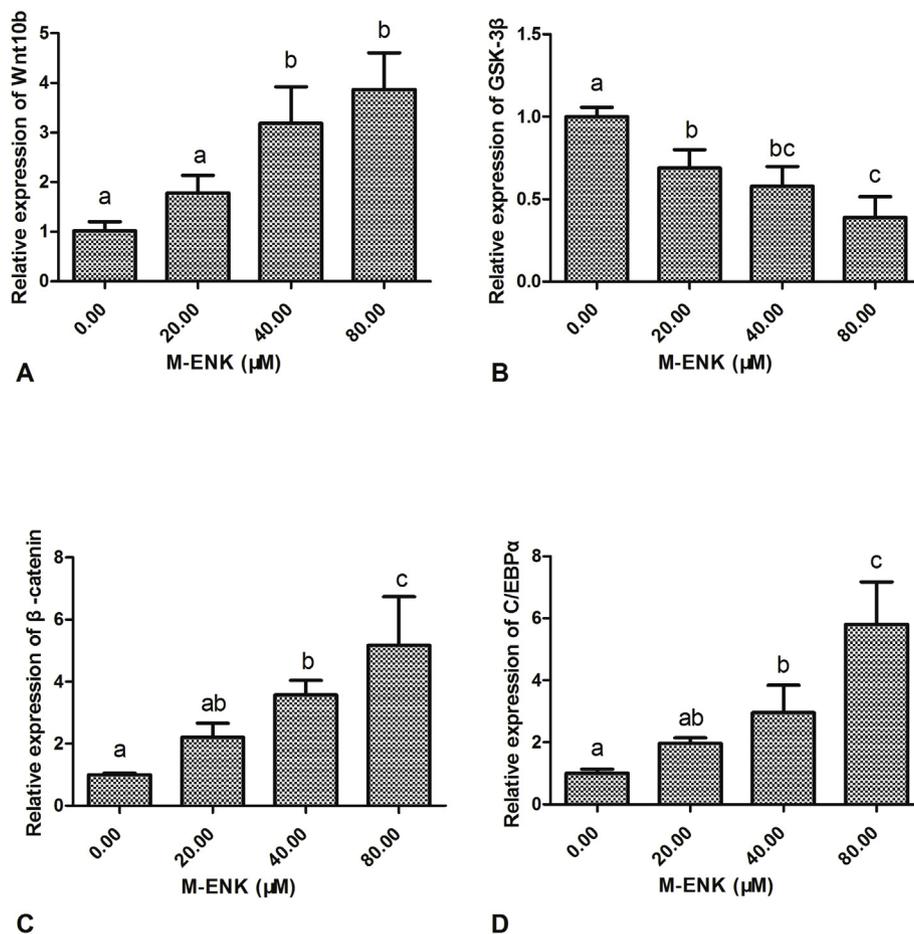


Fig. 2. Effect of M-ENK on the mRNA expression of Wnt10b, GSK-3β, β-catenin and C/EBPα in ZF4 cells of zebrafish. (A) Wnt10b; (B) GSK-3β; (C) β-catenin; (D) C/EBPα. Values are expressed as means ± SEM. (n = 6). β-actin is the reference gene. Statistically significant differences are denoted by different letters (P < 0.05).

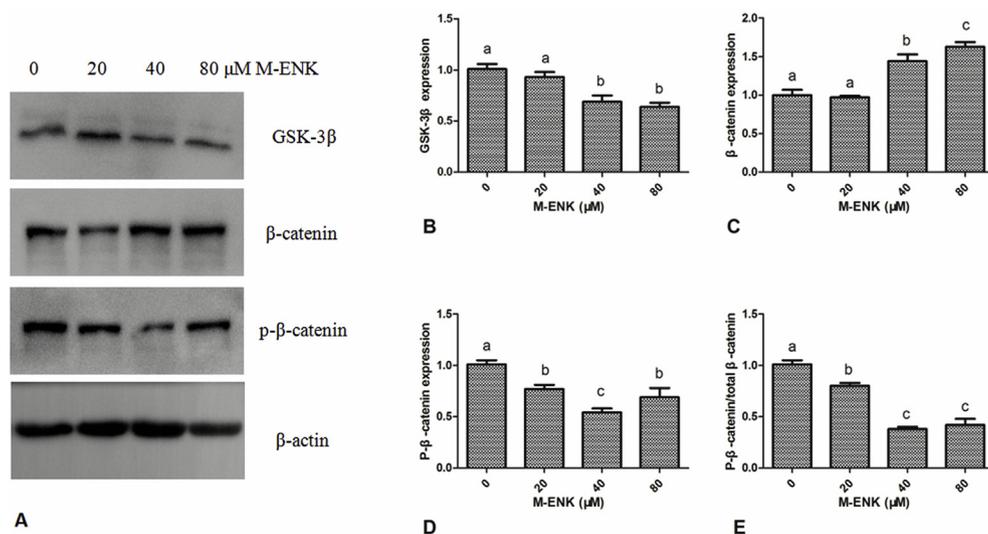


Fig. 3. Effect of M-ENK on the protein expression in the ZF4 cells of zebrafish. (A) Protein bands of western blot; (B) GSK-3β; (C) β-catenin; (D) p-β-catenin; (E) p-β-catenin/total β-catenin. Antibodies anti-β-catenin, anti-phospho-β-Catenin (Ser675), and anti-GSK-3β were purchased from Cell Signaling Technology Inc. Antibody anti-β-actin was purchased from Santa-Cruz Inc. Values are expressed as means ± SEM. (n = 3). Statistically significant differences are denoted by different letters (P < 0.05).

content of H₂O₂ and the generation of O₂^{•-} and ·OH were examined with the reagent kits (NanJing JianCheng Bio Inst, Nanjing, China). The content of H₂O₂ was determined at 405 nm for it formed the stable complex with ammonium molybdate. Based on the oxidation of hydroxylamine by the xanthine-xanthine oxidase system, the generation of O₂^{•-} (U/g protein) was assayed at 550 nm using the xanthine oxidase method. The generation of ·OH was detected at 550 nm, which was expressed as U/mg protein. One U means that 1 mg protein generates

1 mmol/L of hydrogen peroxide per min.

The activity of SOD was detected at 550 nm according to McCord and Fridovich [25], with one U of SOD corresponding to the quantity of enzyme that caused 50% inhibition of cytochrome c reduction (U/mg protein). The activity of CAT was detected at 405 nm, and one U of CAT is the enzyme that decomposes 1 mmol/L of hydrogen peroxide per min [26]. According to the method of Flohe and Gunzler [27], GSH-PX activity was assayed at 412 nm based on the rate of NADPH oxidation.

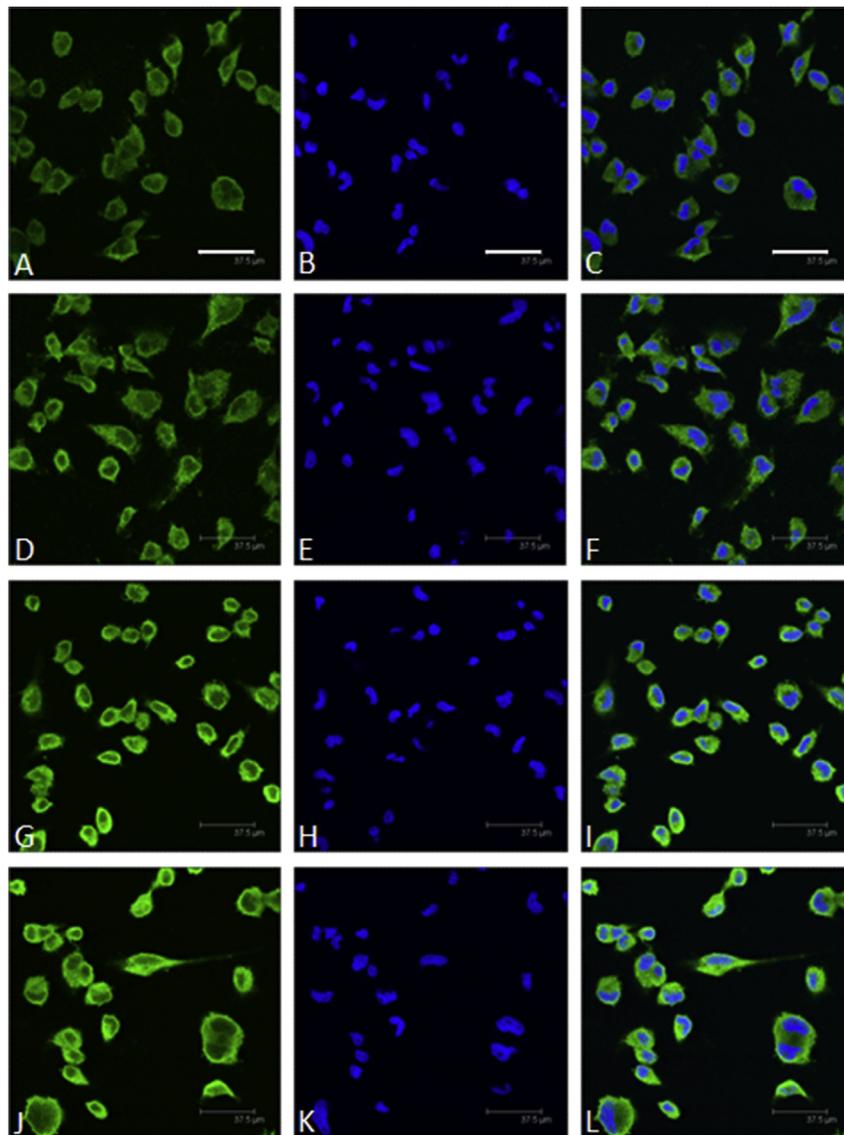


Fig. 4. Effect of M-ENK on the immunofluorescence of β -catenin in the ZF4 cells of zebrafish. (A, D, G, J): β -catenin, green; (B, E, H, K): nucleus, blue; (C, F, I, L): green and blue merger. Bar: 37.5 μ m.

One U of GSH-PX was the amount of enzyme that depleted 1 μ mol GSH in 1 min.

2.8. Loading the fluorescent indicator of ROS

The cells (1×10^5 cell/ml) were seeded into each petri dish (35 mm, specially used for confocal microscopy), treated with 0, 20, 40, and 80 μ M M-ENK for 24 h, and washed with PBS. The distribution of ROS was detected with 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes). Cells were incubated with 10 μ M DCFH-DA for 30 min, and washed with PBS.

2.9. Loading the fluorescent indicator of mitochondrial membrane potential (MMP)

The cells (1×10^5 cell/ml) were seeded into each 35 mm petri dish and treated with 0, 20, 40, and 80 μ M M-ENK for 24 h. To detect MMP, cells were loaded with 10 μ M Rhodamine-123 (Molecular Probes) at 37 $^{\circ}$ C for 30 min, and washed with PBS.

2.10. Confocal imaging

The fluorescence of ROS and MMP was measured with a laser-scanning confocal microscope (Leica TCS SP2, Germany) at an excitation wavelength of 488 nm, respectively. Images were obtained with a 40 \times objective lens. ROS and MMP were investigated in the green and yellow channel, and the fluorescence intensity of ROS and mitochondria in ZF4 cells was quantified with Leica Confocal Software, respectively.

2.11. Statistics

Data were expressed as mean \pm standard error of means (SEM). The significance of the differences was evaluated by one-way ANOVA, followed by Tukey's multiple range test with SPSS16.0 Software. $P < 0.05$ were considered statistically significant.

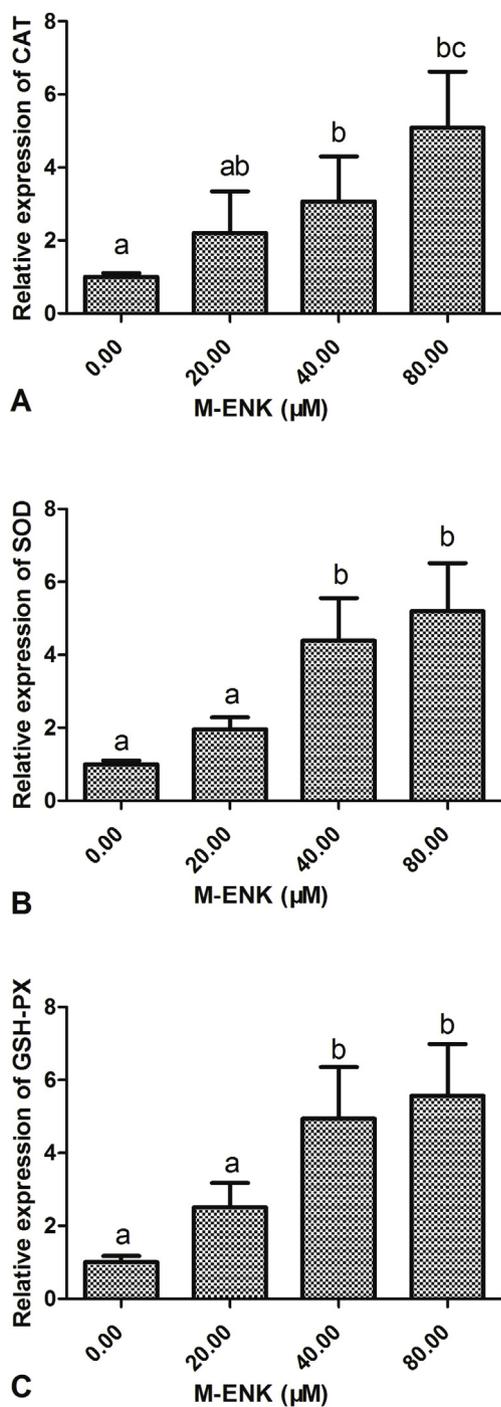


Fig. 5. Effect of M-ENK on the mRNA expression of CAT, SOD, and GSH-PX in ZF4 cells of zebrafish. (A) CAT; (B) SOD; (C) GSH-PX. Values are expressed as means \pm SEM. ($n = 6$). β -actin is the reference gene. Statistically significant differences are denoted by different letters ($P < 0.05$).

3. Results

3.1. Effect of M-ENK on the cell viability

In this study, the ZF4 cells were exposed to 0, 10, 20, 40, 80, and 160 μM M-ENK for 24 h. The results indicated that the cell viability was significantly increased by 20 and 40 μM M-ENK (Fig. 1). In addition, the cell viability was significantly increased by 80 and 160 μM M-ENK (Fig. 1). The highest cell viability was observed at 80 μM M-ENK (Fig. 1).

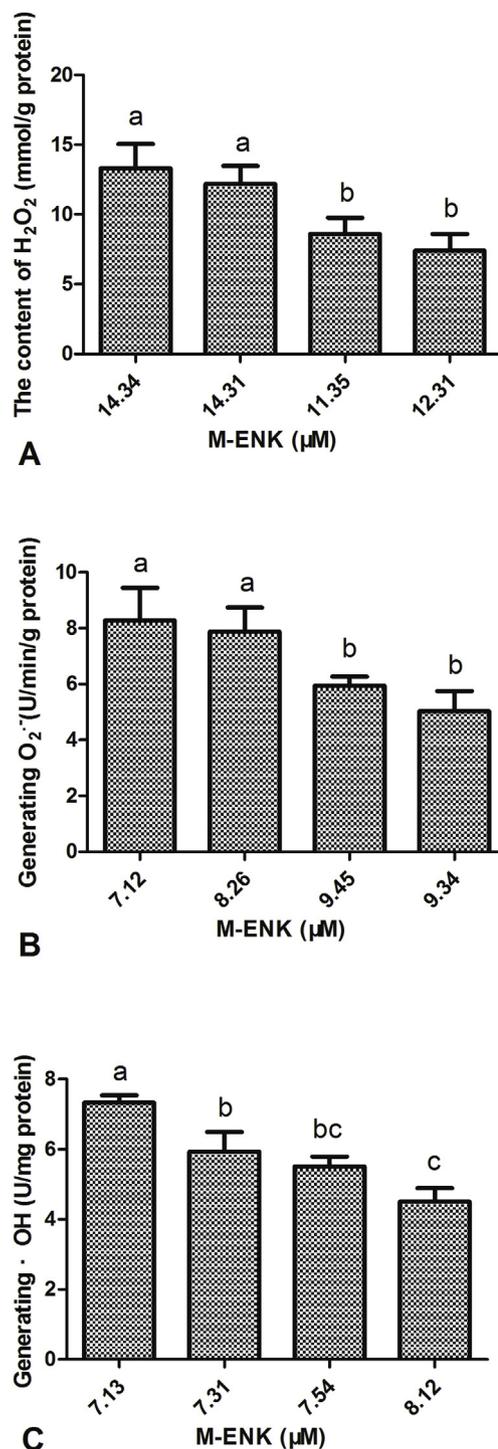


Fig. 6. Effect of M-ENK on the level of H_2O_2 , $\text{O}_2^{\cdot-}$, and $\cdot\text{OH}$ in ZF4 cells of zebrafish. (A) H_2O_2 ; (B) $\text{O}_2^{\cdot-}$; (C) $\cdot\text{OH}$; Values are expressed as means \pm SEM. ($n = 6$). Statistically significant differences are denoted by different letters ($P < 0.05$).

3.2. Effect of M-ENK on the mRNA expression of Wnt10b, GSK-3 β , β -catenin, and C/EBP α

Compared to the control group, the mRNA expression of Wnt10b was significantly increased by 40 and 80 μM M-ENK in the ZF4 cells of zebrafish (Fig. 2A). No significant difference was observed on the mRNA expression of Wnt10b between the control and 20 μM M-ENK (Fig. 2A). However, the mRNA expression of GSK-3 β was significantly decreased by 20, 40, and 80 μM M-ENK (Fig. 2B). In addition, the

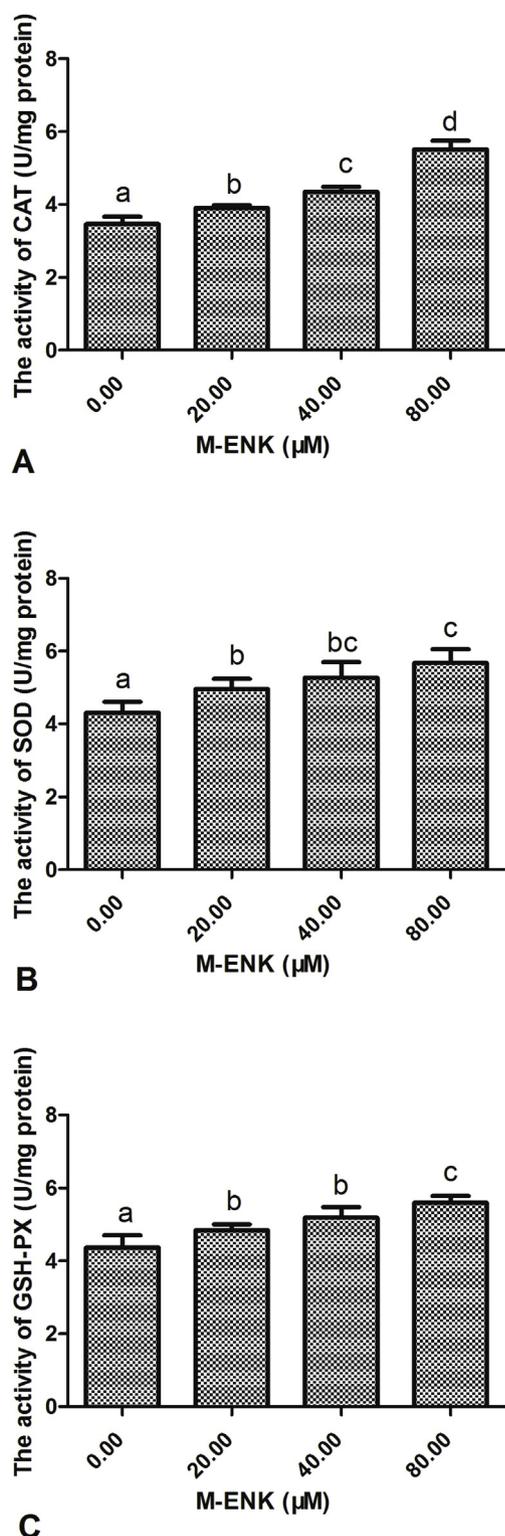


Fig. 7. Effect of M-ENK on the activity of CAT, SOD, and GSH-PX in ZF4 cells of zebrafish. (A) CAT; (B) SOD; (C) GSH-PX. Values are expressed as means \pm SEM. (n = 6). Statistically significant differences are denoted by different letters ($P < 0.05$).

mRNA expression of β -catenin and C/EBP α was significantly induced by 40 and 80 μ M M-ENK (Fig. 2C and D). The highest mRNA expression of β -catenin and C/EBP α was found at 80 μ M M-ENK (Fig. 2C and D).

3.3. Effect of M-ENK on the protein expression of GSK-3 β , β -catenin, and p- β -catenin

The protein bands of western blot were shown on Fig. 3A. Compared to the control group, the protein expression of GSK-3 β was significantly decreased by 40 and 80 μ M M-ENK (Fig. 3B). However, the protein expression of β -catenin was significantly induced by 40 and 80 μ M M-ENK (Fig. 3C). No significant difference was observed on the protein expression of GSK-3 β and β -catenin between the control and 20 μ M M-ENK (Fig. 3B and C). In addition, the protein expression of p- β -catenin and the level of p- β -catenin/total β -catenin were significantly decreased by 20, 40, and 80 μ M M-ENK (Fig. 3D and E).

3.4. Effect of M-ENK on the immunofluorescent of β -catenin

The result of immunofluorescent microscopy of ZF4 cells indicated that the protein expression of β -catenin was induced by 20, 40, and 80 μ M M-ENK (Fig. 4). The highest expression of β -catenin was found at 80 μ M M-ENK (Fig. 4).

3.5. Effect of M-ENK on the mRNA expression of CAT, SOD, and GSH-PX

Compared to the control, the mRNA expression of CAT in the ZF4 cells of zebrafish was significantly increased by 40 and 80 μ M M-ENK (Fig. 5A). No significant difference was observed on the mRNA expression of CAT between 40 and 80 μ M M-ENK in ZF4 cells of zebrafish (Fig. 5A). Moreover, the mRNA expression of SOD and GSH-PX was significantly increased by 40 and 80 μ M M-ENK (Fig. 5B and C). No significant difference was observed on the mRNA expression of SOD and GSH-PX between 40 and 80 μ M M-ENK in ZF4 cells of zebrafish (Fig. 5A).

3.6. Effect of M-ENK on the level of ROS

The level of H₂O₂ was significantly decreased by 40 and 80 μ M M-ENK (Fig. 6A). Nevertheless, the level of H₂O₂ was not significantly decreased by 20 μ M M-ENK (Fig. 6A). The level of O₂⁻ was significantly decreased by 40 and 80 μ M M-ENK (Fig. 6B), no significant difference was found in ZF4 cells treated by 20 μ M M-ENK (Fig. 6B). In addition, the level of \cdot OH was significantly decreased by 20, 40, and 80 μ M M-ENK (Fig. 6C).

3.7. Effect of M-ENK on the activity of CAT, SOD, and GSH-PX

The activity of CAT was significantly increased by 20, 40, and 80 μ M M-ENK in ZF4 cells of zebrafish (Fig. 7A). In addition, the activity of SOD and GSH-PX was significantly increased by 20, 40, and 80 μ M M-ENK in ZF4 cells of zebrafish (Fig. 7B and C). The highest activity of CAT, SOD, and GSH-PX was observed at 80 μ M M-ENK in ZF4 cells of zebrafish (Fig. 7A–C).

3.8. Effect of M-ENK on the fluorescence intensity of ROS

Labeled with DCFH-DA, ROS distribution was clearly detected in ZF4 cells (Fig. 8A–D). The fluorescence intensity of ROS was decreased with the increase of M-ENK concentration (Fig. 8E). When ZF4 cells were exposed to 40 and 80 μ M M-ENK for 24 h, the fluorescence intensity of ROS was significantly lower than that of the control (Fig. 8E).

3.9. Effect of M-ENK on the fluorescence intensity of MMP

The fluorescence intensity of MMP in ZF4 cells was increased with the increase of M-ENK concentration (Fig. 9A–D). Moreover, the fluorescence intensity of MMP was significantly higher than the control when ZF4 cells were exposed to 20, 40, and 80 μ M M-ENK for 24 h (Fig. 9E). No significant difference was observed on the fluorescence

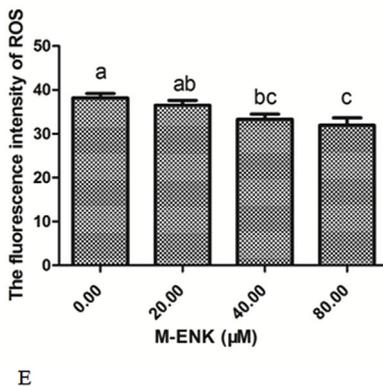
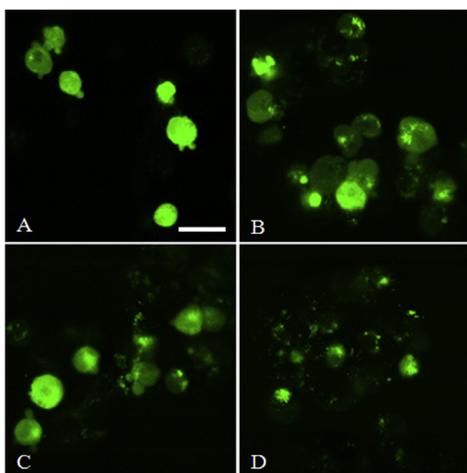


Fig. 8. Effect of M-ENK on the fluorescence intensity of ROS in ZF4 cells of zebrafish. (A) Control; (B) 20 μM M-ENK; (C) 40 μM M-ENK; (D) 80 μM M-ENK; (E) Effect of M-ENK on ROS fluorescence intensity. Values are expressed as means ± SEM. (n = 20 cells). Statistically significant differences are denoted by different letters ($P < 0.05$).

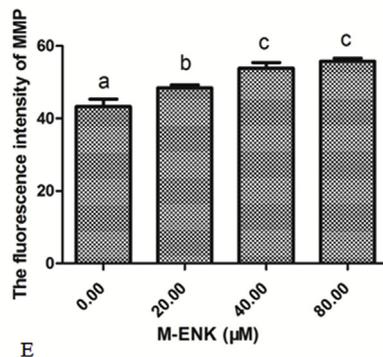
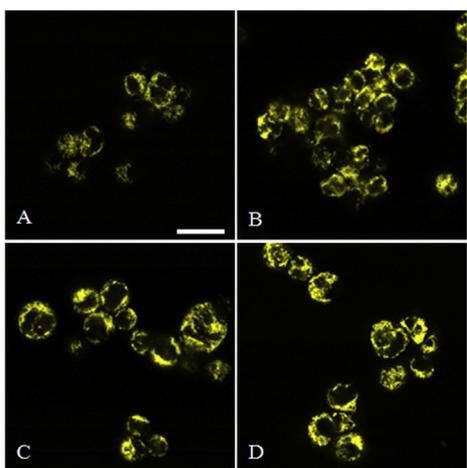


Fig. 9. Effect of M-ENK on the fluorescence intensity of MMP in ZF4 cells of zebrafish. (A) Control; (B) 20 μM M-ENK; (C) 40 μM M-ENK; (D) 80 μM M-ENK; (E) Effect of M-ENK on the fluorescence intensity of MMP. Values are expressed as means ± SEM. (n = 20 cells). Statistically significant differences are denoted by different letters ($P < 0.05$).

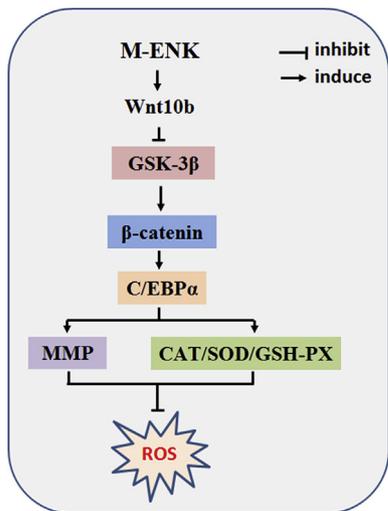


Fig. 10. The mechanism that M-ENK inhibits ROS production through Wnt/β-catenin signaling in zebrafish ZF4 cells. M-ENK induces Wnt/β-catenin signaling, which further inhibits ROS production through the induction of C/EBPα, MMP, and the activities of antioxidant enzymes.

intensity of MMP in ZF4 cells treated by 40 and 80 μM M-ENK (Fig. 9E).

4. Discussion

In this study, the ZF4 cells were exposed to 0, 10, 20, 40, 80, and

160 μM M-ENK for 24 h. The results indicated that the cell viability was significantly increased by the different concentration of M-ENK. The highest cell growth was observed at 80 μM M-ENK. The result indicated that the growth of ZF4 cells was related to the concentration of M-ENK. M-ENK could induce cell growth at the suitable concentration.

If there was no Wnt ligand in the canonical Wnt signaling pathway, β-catenin could be degraded by GSK-3β [28,29]. However, when Wnt ligand binding to membrane receptor, GSK-3β was impeded, which led to β-catenin accumulation [30,31]. The action of GSK-3β could phosphorylate β-catenin and lead to the degradation of β-catenin [18,19]. Thus GSK-3β plays a key role in regulating Wnt/β-catenin signaling [32,33]. In the present study, the mRNA expression of Wnt10b was significantly increased by 40 and 80 μM M-ENK, while that of GSK-3β was significantly decreased by 40 and 80 μM M-ENK in the ZF4 cells of zebrafish. In addition, the mRNA expression of β-catenin was significantly induced by 40 and 80 μM M-ENK. Moreover, the protein expression of GSK-3β was significantly decreased, while that of β-catenin was significantly induced by 40 and 80 μM M-ENK. The protein expression of p-β-catenin and the level of p-β-catenin/total β-catenin were significantly decreased by 20, 40, and 80 μM M-ENK. Our result indicated that 40 and 80 μM M-ENK could induce Wnt/β-catenin signaling in the ZF4 cells of zebrafish (Fig. 10). In the previous study, it was found that the expression of C/EBPα was regulated by β-catenin [34,35]. For the mRNA expression of C/EBPα was significantly increased by 40 and 80 μM M-ENK, β-catenin could induce the expression of C/EBPα in the ZF4 cells of zebrafish (Fig. 10).

As ubiquitous intracellular messengers, ROS play a significant role in inducing mitogenic response [36,37]. However, the overproduction

of ROS affects the physiological function of lipid and protein, which further results in diseases, apoptosis, as well as the ageing process [38,39]. In this study, the level of H_2O_2 and $O_2^{\cdot-}$ was significantly decreased by 40 and 80 μM M-ENK. In addition, the level of $\cdot OH$ was significantly decreased by 20, 40, and 80 μM M-ENK. Labeled with DCFH-DA, the distribution of ROS was clearly detected in ZF4 cells, and the fluorescence intensity of ROS was decreased with the increase of M-ENK concentration. The present results indicated that M-ENK could decrease the levels of ROS in the ZF4 cells of zebrafish. In addition, the mRNA expression of CAT, SOD, and GSH-PX was significantly increased by 40 and 80 μM M-ENK. The activity of CAT, SOD, and GSH-PX was significantly increased by 20, 40, and 80 μM M-ENK. In the previous study, it is found that ROS were regulated by Wnt/ β -catenin signaling [40–43], and Wnt/ β -catenin signaling regulated oxidative stress [36,37]. In this study, Wnt/ β -catenin signaling was induced and the level of ROS was decreased by M-ENK in the ZF4 cells of zebrafish. It showed that M-ENK may inhibit the level of ROS via Wnt/ β -catenin signaling, and M-ENK participated in regulating oxidative stress in the ZF4 cells of zebrafish (Fig. 10).

ROS generation has a necessarily close connection with the organelles of mitochondria [44]. Moreover, mitochondria is involved in regulating cell apoptosis, and the loss of MMP is a crucial step in apoptosis [39,45,46]. In this study, the fluorescence intensity of MMP was significantly higher than the control when ZF4 cells were exposed to 20, 40, and 80 μM M-ENK. Our results indicated that MMP may participate in regulating ROS production, and M-ENK may inhibit ROS generation through increasing MMP in ZF4 cells (Fig. 10).

5. Conclusion

M-ENK promoted the growth of ZF4 cells, and cell growth was related to the concentration of M-ENK. M-ENK could induce Wnt/ β -catenin signaling, which further inhibited ROS production through the induction of C/EBP α , MMP, and the activities of antioxidant enzymes.

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