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Effects of metronidazole on proopiomelanocortin a gene expression in zebrafish

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ABSTRACT

The Metronidazole (MTZ), a widely used antibiotic for treating variations of infections, recently is applied in a powerful tool for specifically ablating cells or tissues when combined with *E. coli* nitroreductase (NTR). Although some undesired biological effects on eukaryote cells have been reported previously, the toxicological mechanism of MTZ has not been uncovered yet. In current study, we found that MTZ can induce *proopiomelanocortin a* (*pomca*) expression in zebrafish larvae. The effect of MTZ is in stage-sensitive and dose-dependent manner. A pro-proliferation activity of MTZ on *pomca*-expressing cells in the pituitary at larval stage was also observed. Furthermore, up-regulated levels of *prolactin* (*prl*) and *glycoprotein hormone subunit α* (*gsuα*) were also observed after the MTZ treatment. Therefore, utilizing our zebrafish as *in vivo* model, we concluded that MTZ can interfere the endocrine signals in the pituitary hormone genes expression. Our current results raised the cautions to the intensively application of MTZ in clinical practices and biomedical researches.

1. Introduction

Metronidazole (MTZ), is a widely used nitroimidazole antibiotic not only for various simple infections induced by anaerobic bacteria and protozoans ((Chacko and Bhide, 1986; Freeman et al., 1997), but clinically combined with other chemotherapeutic agents to reduce risk of perioperative infections post-surgery (Willis et al., 1976, 1977a,b). The undesired biological effects of MTZ are mild to human, but many concerns are still present about its possible mutagenic and carcinogenic effects based on the fact that it is a proven mutagen to bacteria, genotoxic to human cell lines, and carcinogenic to animal models as well (Andersen et al., 1981; Bendesky et al., 2002; Bostofte et al., 1981; Roe, 1983; Sadowska et al., 2013a). Lack of power epidemiological studies until now excludes MTZ out of the big human carcinogen family (IARC, 1987). Also, the large use of the medicinal substance will definitely introduce into environments and their effects on living organisms are unresolved (Lanzky and Halling-Sorensen, 1997).

Zebrafish (*Danio rerio*), an outstanding model contributes not only to molecular mechanisms of vertebrate development, but to evaluation of putative endocrine disruption chemicals (Ji et al., 2012; Sun et al., 2010a). Recently, utilizing MTZ and an *Escherichia coli* derived nitroreductase (NTR), one substrate-enzyme pair, has been developed as a powerful tool to specifically ablate target cells or tissues for regenerational studies in zebrafish model especially (Curado et al., 2007). The applicability of the MTZ/NTR system is based on two points, one is that MTZ is nontoxic and tolerant to target cells and the other is that NTR can convert MTZ into a cytotoxic product (Pisharath, 2007). Until now, this technology has been successfully applied to ablate doze kinds of cells and tissues including bone, retina, heart, insulin-expressing cells, kidney, skin and gonad in embryonic and adult zebrafish, medaka, frog, and even ringed worms (Choi et al., 2011; Curado et al., 2008; Dranow et al., 2013; Fraser et al., 2013; Hsu et al., 2010; Montgomery et al., 2010; Pisharath and Parsons, 2009; Pisharath et al., 2007; Veedin-Rajan et al., 2013; Willems et al., 2012; Zhao et al., 2009).

The anterior pituitary gland, a key organ of the vertebrate endocrine system, regulates body homeostasis, stress and other physiological processes, mainly via secreting circulating hormones (Perez-Castro et al., 2012). These hormone secreting pituitary cells and the biosynthesis and secretion of hormones are prone to chemical disruption (Diamanti-Kandarakis et al., 2009). Previous study showed that Nitroimidazole derivatives such as MTZ, affected cAMP mediated signaling and thus the release and biosynthesis of several anterior pituitary hormones in primary pituitary cell culture (Stalla et al., 1989). Furthermore, MTZ had been suggested

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to disrupt some functionality of the endocrine system such as spermatogenesis, and reproduction related hormones, FSH and LH in rat (Grover et al., 2001; McClain et al., 1989; Stalla et al., 1989).

Our current study here mainly probed the effect of MTZ on the transcriptional expression of key pituitary hormone genes in the zebrafish pituitary during embryonic stages. We found that MTZ was a pro-proliferation agent for *pomca*-expressing cells in the zebrafish anterior pituitary. Our results strongly suggest a caution on the use of the MTZ/NTR dual system in pituitary cell ablation for regeneration and developmental biology research. The endocrine-disrupting effects of the MTZ found in our *in vivo* zebrafish models also urge more efforts should be made on such potential side effects when applying overdose MTZ in clinical practice and also effect of MTZ in waters on living organisms.

2. Methods and materials

2.1. Zebrafish maintenance

The zebrafish wild-type adults were in-crossed and maintained in a temperature controllable environment. And healthy pairs of adults were crossed to produce off-springs. Embryos and larvae were incubated in egg water, and staging of zebrafish was according to our previous study (Sun et al., 2010a). The generation of the transgenic lines *Tg(tshβ:EGFP) and Tg(pomca:EGFP)* were described previously (Ji et al., 2012; Sun et al., 2010a). All of the experimental procedures were approved by the Animal Care and Use Committee at Institute of Hydrobiology of Chinese Academy of Sciences (Approval Protocol No. IHB20100042) and were in accordance with the National Guiding Principles for the Care and Use of Laboratory Animals.

2.2. Chemicals and treatment procedures

The MTZ powder (Cat. No. M-1547) purchased from Sigma, USA. The preparation of the MTZ solution (10 mM) and procedures for the MTZ treatments were described previously (Curado et al., 2008).

2.3. DIG-RNA probe synthesis and whole mount in situ hybridization assay

pGEMT-easy vector (Promega, USA) carrying the partial mRNA sequences of genes *gh, gsuα, pomca, tsh* and *prl* were constructed and linearized for probe template preparation. Then Digoxigenin (DIG) labeled RNA probes were synthesized using DIG-11-UTP (Product No. 03359247910, Roche, Mannheim, Germany) and T7 RNA polymerase (Cat. No. EP0111, Thermo Scientific, Rockford, USA). Well synthesized probes were stored at −70 °C for whole mount *in situ* hybridization assay. Briefly, the fixed embryos and larvae were permeated by Protease K treatment and then were incubated with DIG probes overnight at 65 °C. Anti-DIG AP antibody was added and at last NBT/BCIP staining was used for colorization. The concrete protocol was based on Ref. Sun et al. (2010a).

2.4. Cyro-sectioning

The wild-type and transgenic zebrafish larvae were fixed in 4% PFA and mounted in hot 1–2% agarose for a precise orientation. The well trimmed fish agarose block was immersed into Tissue-Tek OTC (Sakura, Japan) and sectioned (10 μm thickness) by the cryotome (Leica CM3050S, Germany). The sections were collected by ice-cold polylysine coated slides.

2.5. Cell proliferation detection assay

The recovered frozen sections were blocked with 10% normal goat serum in phosphate buffered saline containing 0.1% Tween (PBST), and then incubated 4 °C overnight with the primary rabbit antibody against phospho-histone 3 (pH3) (at 1:200 dilution, Cat. No. SC-8656-R, Santa Cruz Biotechnology, USA). Followed by several PBST washes, the CY3 tagged goat antibody against rabbit IgG (at 1:150 dilution, Cat. No. BA-1032, Boster, Wuhan, China) was added and incubated in the dark for one hour at room temperature. Then the sections were coverliped with 50% glycerol/PBST and stored at 4 °C for microscopy examination.

2.6. Flow cytometric assessment of pomca- and prl-positive cells in pomca:EGFP/prl:RFP transgenic zebrafish

The *prl*:RFP and *pomca*:EGFP were generated as previous descriptions (Liu et al., 2006; Sun et al., 2010). The prl:RFP transgenic fish were kindly provided from Dr. Shuo Lin laboratory. The *prl*:RFP/*pomca*:EGFP transgenic zebrafish were generated via crossing the *prl*:RFP and *pomca*:EGFP transgenic lines in the laboratory. Twenty-five *prl*:RFP/*pomca*:EGFP larvae in each group after the treatments at 7 dpf are euthanatized by lethal administration of benzocaine. Heads are dissected in sterile PBS and dissociated for 30 min at 37 °C with trypsin (1 mg/mL) and DNase I (10 U/mL) in D-Hanks buffer containing 0.22 mg/mL EDTA. After 200 μL of trypsin inhibitor was added (10 mg/mL), the samples are vortexed for 5 s followed by centrifugation (1 min at 800 rpm), washed 3 times with PBS and re-suspensed in PBS. The cell suspension was filtered through a 50-μm filter (Filcon GmbH, Taufkirckin/Munich, Germany) and sorted with a flow cytometer (FACSAria III; BD Biosciences).

2.7. Fluorescence and confocal microscopy observation

All above images involving the application of microscopy were collected either by a basic fluorescence microscope (Leica, Germany) or a LM710 confocal microscope (Zeiss, Germany) and photographed by built-in CCDs. All images were processed by the Adobe Photoshop software.

2.8. Intensity quantification of pomca expression

The whole mount *in situ* hybridization images were processed for intensity quantification analysis using ImageJ software (NIH, USA). The relative intensity of *pomca* expression was calculated by the ratio of respective signal spot areas vs. the largest one. And the ratios were further plotted using Microsoft Excel.

2.9. RT-PCR and quantitative real-time PCR

Two groups of 30 lavae at 3.5 dpf were randomly selected for MTZ treatments. The pooled total RNA from the whole larvae body of the 30 lavae was extracted as one sample using the TRIzol reagent, treated with RNase-free DNase I for 30 min at 37 °C, and extracted with phenol/chloroform. A RevertAid First Strand Kit (Thermo Scientific) and oligo(dT) primers were used to synthesize cDNA in a reaction volume of 20 μl. The procedure of the Real-time PCR was performed following the methods described previously (McClain et al., 1989). The mRNA level of each gene in each sample was first calculated in reference to the *β-actin* transcript, which was amplified as an internal control, and then represented as the fold increase relative to the control samples. The sequence information of the primers were provided in Table 1.