Blood-brain barrier permeability to enrofloxacin in Pengze crucian carp (*Carassius auratus var. Pengze*)

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29 **Abstract:**

30 To explore the permeability of blood-brain barrier (BBB) to enrofloxacin (ENR)
 31 and the role of brain injury markers in the evaluation of brain damage, crucian carp
 32 (*Carassius auratus* var. Pengze) was employed in this work as the research object
 33 treated by a single oral administration of the antibiotic. Results showed that ENR
 34 residues could be detected in brain of crucian carp exposed to both half lethal dose
 35 (LD₅₀) and safe dose (SD) of ENR throughout the trial period, indicating that ENR
 36 had an ability of permeating the BBB of crucian carp. Evans blue (EB) dispersively
 37 distributed in brain tissue of fish exposed to SD ENR, but not in the vehicle-treated
 38 fish brain, which suggested structural failure of BBB by ENR. Expression levels of
 39 BBB-related molecules, occludin and P-glycoprotein (P-gp), were significantly
 40 down-regulated by ENR. Brain injury markers, NSE, S100B and GFAP were
 41 improved that were demonstrated by elevated contents of proteins in brain and serum.
 42 According to the data of correlation analysis, it was observed that ENR residue was
 43 closely related to S100B and GFAP, indicating that S100B and GFAP could be used as
 44 evaluation indicators for brain injury of crucian carp. All findings mentioned above
 45 suggest that ENR may cause structural change of BBB, resulting in biological brain
 46 damage in crucian carp.

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48 **KEYWORDS:** enrofloxacin, blood-brain barrier, drug residues, Pengze crucian carp

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57 1. INTRODUCTION

58 Blood-brain barrier (BBB) is considered to have a highly protective effect on CNS
59 of organisms (Pardridge 1999). In animal brain, BBB is mainly composed of brain
60 microvascular endothelial cells (BMECs), astrocytes (ACs), pericytes (PCs) and tight
61 junctions (TJs) of cells or protein skeletons (Kadry et al., 2020). BBB has almost 100%
62 barrier effect on macromolecular drugs, trans-BBB transport of macromolecules is
63 generally through receptor-mediated endocytosis (Zhang et al., 2001). However,
64 under normal circumstances, a small number of lipophile small molecules can directly
65 diffuse across BBB and enter cerebrospinal fluid (CSF) without special conditions
66 (Pardridge, 2015).

67 The intercellular insertion of TJs into BMECs also provides a protective effect,
68 separating the lumen portion from the basolateral region. Claudins and Occludins are
69 extracellular components of TJs, and key molecules of BBB. TJs is a kind of protein
70 complex mainly formed by the interaction of a variety of transmembrane and
71 cytoskeletal proteins, including claudin-1/5, occludins, zonula occludens-1 (ZO-1),
72 E-cadherin and actin, and plays an important role in biological barriers (Ward et al.,
73 2019; Wolburg et al., 2002).

74 Under physiological conditions, during effective screening of CSF and choroid
75 plexus in BBB, CSF produces small amounts of proteins that are usually retained in
76 BBB without diffusion. BBB damage can be predicted by measuring the levels of
77 these proteins in CSF. These brain-derived proteins can be used as markers of BBB
78 integrity (Neuwelt et al., 1999). The calcium-binding protein S100 Beta (S100B) is
79 mainly distributed in ACs and oligodendrocytes, and had high nerve specificity.
80 Neuron specific enolase (NSE) is expressed in neuron cytoplasm, peripheral nerves,
81 endocrine system, platelets and red blood cells, and is mainly concentrated in neurons
82 (Li et al., 2017). Glial fibrillary acidic protein (GFAP) highly expressed in ACs is a
83 kind of skeletal protein with supporting function, supports adjacent neurons and BBB
84 (Zhang, 2021). The three markers can be detected in serum if BBB is damaged

(Helmrich et al., 2020). Detection of brain-derived proteins in serum is considered to be an effective method to evaluate the degree of BBB damage. Different from TJs, brain-derived proteins can not only reflect the permeability of BBB, but also reflect the damage degree of brain exposed to drug (Helmrich et al., 2020).

Enrofloxacin (ENR) is widely used in animal disease control. Its common products include raw powder, sodium salt, hydrochloride and lactate. However, the antibiotic residue and toxicity to organisms have attracted more attention, such as hepatorenal toxicity (Zhai and Li, 2017; Chen et al., 2019). In aquatic organisms, especially in fish, ENR can accumulate in fish, which impacts growth and development (Chen et al., 2019). Presently, as a member of fluoroquinolones (FQLs), ENR toxicity research mainly focuses on liver, kidney and edible animal tissues, effect on brain tissue or central nervous system (CNS) is still unknown.

Our previous work investigated the toxicity of difloxacin, another member of FQLs, to *Carassius auratus gibelio*, and found that both difloxacin doses, 2480 and 20 mg/kg, could lead to abnormal expression of inhibitory neurotransmitter γ -aminobutyric acid related anabolic genes, indicating that difloxacin influenced central nervous system (Ruan et al., 2014). ENR has most of the physical and chemical properties of FQLs, such as high lipophilicity, long biological retention, and low degradation rate (Wei et al., 2007). So it can be assumed that ENR maybe mediate impact on brain or CNS.

Pengze crucian carp (*Carassius auratus* var. Pengze), an economic species in China, was selected as the experimental object to explore the effects of ENR on the BBB permeability in fish.

2. MATERIALS AND METHODS

2.1. Experimental animals

The fish (83.60 ± 3.72 g), healthy and free from parasites, were purchased from a farm in Pengze county, Jiangxi Province, and temporarily raised for two weeks with

112 plenty of oxygen which was pumped into the water. The feeding frequency was twice
113 per day. During the experiment, the aquaculture water was aerated by artificial aerator,
114 and the dissolved oxygen was stabilized at 6.50 ± 0.50 mg/L, and the water
115 temperature and pH were controlled at $25 \pm 1^\circ\text{C}$ and 7.50 ± 0.30 . Feeding was stopped
116 24 h before the experiment. Fish were divided randomly into three groups, control
117 group (fish were orally administrated by normal saline), LD50 group (fish were orally
118 administrated by 1949.84 mg/kg ENR) and SD group (fish were orally administrated
119 by 194.98 mg/kg ENR), respectively (Zhao et al., 2013).

120 **2.2. Determination of ENR residue by HPLC**

121 5.0 g sample and 30 g anhydrous sodium sulfate were homogenized in 30 mL
122 acidified acetonitrile with a high-speed tissue masher. The homogenate sample was
123 placed in a triangular flask with glass beads (2~3 particles/bottle), shook on a shaker
124 for 15 min (120 rpm), centrifuged at 4500 rpm for 15 min. The supernatant was mixed
125 with 30 mL acidified acetonitrile, centrifuged at 4500 rpm for 15 min to harvest
126 supernatant.

127 25 mL n-hexane was added into the supernatant and shaken for 5 min. The lower
128 layer of acetonitrile was transferred to the flask and evaporated to dry at 55°C . The
129 residue was fully dissolved with 1.0 mL mobile phase and transferred to a 1.5 mL
130 centrifuge tube, centrifuged at 4500 rpm for 5 min. The supernatant was filtered by
131 $0.45\ \mu\text{m}$ microporous membrane, and the filtrate was subjected to HPLC assay
132 according to the Announcement of Ministry of Agriculture and Rural Affairs of China
133 [No. 783] (2006).

134 **2.3. Evans blue staining**

135 After the OCT embedding agent infiltrates the tissue, the tissue was frozen and
136 embedded on the quick-freezing table. Tissue was sectioned after the OCT embedding
137 agent turned white and hardens. The sample was fixed on the slicer, the tissue surface
138 of the sample was trimmed and then finely cut, and the thickness of the section was
139 adjusted to 8-10 μm . After the sections were transferred to a clean slide for labeling,

140 the red evans blue (EB) distribution (EB appears red under green light) was observed
141 under the confocal laser scanning microscope at 405 nm excitation wavelength.

142 **2.4. Concentration detection of ENR in brain tissue**

143 The weight of crucian carp brain tissue was accurately weighed. After
144 homogenizing the crucian carp brain tissue, 3 mL PBS and 3 mL 60% trichloroacetic
145 acid were added. After fully mixing with a vortex oscillator, the tissue was left
146 standing at room temperature for 30 min. After centrifugation at 3000 r/min for 10
147 min in a low temperature high speed centrifuge, the supernatant was harvested. A
148 series of standard working solution of 200 μ L and sample supernatant were added into
149 the 96-well plate. The absorbance at 632 nm was measured by setting the blank well
150 to zero in the plate.

151 **2.5. Quantitative polymerase chain reaction (qPCR)**

152 The primers (β -actin, S100B and GFAP) for qPCR were listed in table 1, and
153 β -actin was used as the reference gene. The reactions were performed in a
154 thermocycler (Bio-Rad, USA) with the following profile: one cycle for 30 s at 95 $^{\circ}$ C,
155 followed by 39 cycles for 5 s at 95 $^{\circ}$ C and 30 s at corresponding temperature (Table 1),
156 and 10 s at 95 $^{\circ}$ C, and then 5s at 65 $^{\circ}$ C, then 5s at 95 $^{\circ}$ C. The qPCR was performed in
157 the CFX-96 real-time PCR system (Bio-Rad, USA). The qPCR reactions with 20 μ L
158 total volume per reaction (10 μ L TB Green Premix, 6.4 μ L double distilled water, 0.8
159 μ L of each primer, and 2 μ L cDNA templates) were set up according to the
160 manufacturer's protocol. Relative gene expression was analyzed using the $2^{-\Delta\Delta C_t}$
161 method.

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Table 1 Information of qPCR primers

Gene	Primer sequences	Gene sequence number	Temperature (°C)
<i>β-actin</i>	F: TACGTTGCCATCCAGGCTGTG R: CATGGGGCAGGGCGTAACC	M_24113.1	55-60
S100B	F: GGGAACCATCATTGAGGTGT R: TGCTCGGTAAGAGTCGGA	XM_026235739.1	58.0
GFAP	F: ACCTGCTCAATGTCAAAGTGGC R: GTTCGCACAACTATGCTCCTCTC	XM_026208575.1	56.5

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165 2.6. Western blot

166 Brain tissue homogenate containing RIPA and PMSF was centrifuged at 4°C, 12000
167 rpm for 10 min. Supernatant was determined total protein concentration by BCA
168 method, mixed with loading buffer, and then denatured for 10 min by boiling water
169 bath. Samples were subjected to SDS-PAGE, and transferred to PVDF membrane.
170 Following treatment with methanol and skimmed milk powder solution, membrane
171 was probed with primary antibody (1:1000). After washing with PBST, membrane
172 was incubated in labeled secondary antibody solution (1:500), and then exposed to
173 chemiluminescence fluid in a dark room followed by analysis with Bio-Rad gel
174 imaging system.

175 2.7. Detection of serum markers of brain injury

176 According to ELISA manual for experimental operation (MEIMIAN Biological
177 Reagent Co., LTD, Wuhan), serum markers of brain injury were determined.

178 2.8. Data analysis

179 Data were expressed as mean ± SD, and SPSS 17.0 was used for statistical analysis.
180 One-way ANOVA and LSD were used to make multiple comparisons for evaluating
181 the statistical significance. $P < 0.05$ was taken as statistical significance.

182 3. RESULTS

183 3.1. ENR can pass BBB in brain crucian carp.

184 According to the Figure 1, ENR residues in brain tissues from the two treated
185 groups of fish were significantly different, the peak values of ENR were $712.46 \pm$
186 $14.18 \mu\text{g/g}$ in LD₅₀ group and $31.04 \pm 0.36 \mu\text{g/g}$ in SD group, respectively. In brain
187 tissue of LD₅₀ treated fish, ENR underwent rapid biotransformation, while in brain of
188 SD dose exposed animals, the elimination rate of ENR was slow. The data suggest
189 that ENR can diffuse across BBB into brain tissue.

190
191 **Figure 1.** Residual amount of ENR in the brain of crucian carp (n=10). ** expresses $P < 0.01$
192 between LD₅₀ group and SD group.
193

194 3.2. ENR promotes BBB permeability to EB in crucian carp.

195 Cerebral slices of crucian carp in the control group showed that EB was clearly
196 visible in brain microvessels (Figure 2), and the contour boundary of vascular was
197 clear, which verified that EB did not penetrate brain microvessels into glial cells. In
198 Figure 2, the permeability of brain microvessels was increased after ENR treatment,
199 and EB was diffused in the brain microvessels of crucian carp, indicating that EB
200 diffused into the glial cells. As shown in Figure 2, after treatment with ENR of SD,
201 EB content in crucian carp brain was significantly increased ($P < 0.01$).

Figure 2 Distribution and content of EB in brain of crucian carp. EB content in the brain of crucian carp after treatment with SD of ENR for 96 h. ** expresses $P < 0.01$.

3.3. ENR represses expressions of occludin and P-gp, but promotes expression of GFAP, NSE and S100B in crucian carp brain.

Data in Figure 3 present that contents of crucian carp BBB related proteins, occludin and P-gp, were significantly down-regulated by single oral administration of ENR at LD₅₀ and SD ($P < 0.01$), moreover LD₅₀ ENR was observed more obvious inhibition than SD ENR ($P < 0.01$). Meanwhile, ENR exposure elevated protein contents of GFAP and NSE in animal brain ($P < 0.01$ or $P < 0.05$), LD50 ENR was more effective in improving expression of NSE than SD ENR, significant difference between LD50 treated and SD treated fish was observed in NSE ($P < 0.01$), not in GFAP ($P > 0.05$). Apart from augment of protein content, ENR induced expression of GFAP was supported by increased transcript product ($P < 0.01$, Figure 4).

Detection of transcript products also revealed that S100B was promoted by ENR ($P < 0.01$, Figure 4), the translation level could be reflected by serum content of S100B, markedly high S100B protein content was observed after treatment with ENR ($P < 0.01$, Figure 5). Data in Figure 5 also showed that ENR increased NSE and GFAP protein contents in serum. LD₅₀ ENF caused extremely significant increase of GFAP protein content in serum of crucian carp ($P < 0.01$), but the change of GFAP protein content in SD group was unstable, and the difference of GFAP protein content between the two groups was extremely significant ($P < 0.01$).

Figure 3. Expressions of BBB-related protein in the brain of crucian carp. Protein contents were determined by western blot analysis. * or ** expresses $P < 0.05$ or $P < 0.01$, respectively.

Figure 4. Expression of S100B and GFAP genes in crucian carp. Transcriptional levels were assessed by qPCR method. ** expresses $P < 0.01$.

Figure 5 Serum contents of brain injury markers in crucian carp. Protein contents were determined by ELISA detection kits. ** expresses $P < 0.01$.

3.4. Correlation analysis between ENR residues and serum S100B, NSE and GFAP

Data in Table 2 present that, according to the correlation between ENR and the contents of three serum brain injury markers analyzed by Spearman rho in SPSS, the

correlation between ENR and S100B, NSE and GFAP were 0.853 ($P < 0.01$), 0.140 and 0.657 ($P < 0.05$), but no close correlation was observed between ENR residue and NSE ($P > 0.05$).

Table 2 Correlations between ENR residues and serum S100B, NSE and GFAP

item	correlation coefficient	significant level
ENR residues / S100B	0.85	$P < 0.01$
ENR residues / NSE	0.14	$P > 0.05$
ENR residues / GFAP	0.66	$P < 0.05$

4. DISCUSSION

The barrier permeability of FQLs has been reported for a long time. Escudero and co-workers detected the presence of difloxacin in goat milk, demonstrating the ability of difloxacin to penetrate the hemothorax barrier into the udder (Escudero et al., 2011). It is reported that corresponding drugs can be detected in the brain tissue of rats after FQLs perfusion (Jaehde et al., 1993). In this work, ENR residue was detected in brain tissue of crucian carp exposed to ENR, two doses of LD₅₀ and SD, peak values of ENR residue were 712.46 µg/g and 31.04 µg/g in LD₅₀ group and SD group, respectively. As a member of FQLs, ENR could stay in the brain tissue of crucian carp in large quantities, indicating that ENR possesses high BBB permeability.

EB can locate in the injured site of BBB and has advantages of quantification (Saunders et al., 2015). Structurally, permeability of BBB is mainly controlled by BMECs, TJs and matrix membranes, it is difficult for substances to penetrate through the intact BBB into brain tissue. BBB permeability is easily affected by a variety of factors. For instance, unconventional means, such as heat stress, noise and ultrasound can increase BBB permeability of mice (Ohta et al., 2020; Sun et al., 2021). Chen et al. found that intraperitoneally injected muskone could change the ultrastructure of BBB in zebrafish (Chen et al., 2020). FQLs have high lipid solubility, so they can

263 penetrate BBB and enter the brain easily (Ruan et al., 2014). According to the
264 findings of this work, ENR exposure promoted EB diffusion, indicating that ENR
265 improved permeability of BBB, then resulted in EB crossing BBB. This result further
266 confirms that ENR can cross BBB via changing the permeability of BBB.
267 Consequently, ENR penetrates BBB of the fish via both lipid diffusion and changing
268 BBB permeability.

269 BBB permeability is regulated by a variety of factors, such as protein modification
270 site changes (Goncalves et al., 2013), temperature (Uchida et al., 2019), heavy metals
271 (Wang et al., 2017) and drugs (Chen et al., 2020), which can affect the structure and
272 function of BBB. Occludin is an important molecules of tight junction widely
273 distributed in body barrier. As an important gene in TJs, occludin is highly expressed
274 in BBB and plays key role in the regulation of BBB function (van Leeuwen et al.,
275 2018; Zhang et al., 2010). P-gp, a transporter of BBB, plays an important role in
276 maintaining stability of internal environment of brain (Ding et al., 2021). In this study,
277 it was found that expression of occludin in TJs of crucian carp BBB was extremely
278 significantly down-regulated by ENR. This result suggested that the change of BBB
279 permeability to ENR might be mediated by inhibiting expression of occludin in
280 crucian carp brain.

281 P-gp is an important transporter of BBB. Clinically, P-gp inhibitors are used to
282 extend retention time of drugs in brain (Nobili et al., 2012). This work revealed that
283 ENR not only reduced the expression of TJs-related gene occludin, but also
284 significantly inhibited P-glycoprotein, resulting in more ENR existed in brain tissue of
285 Pengze crucian carp and consequent brain injury. S100B, NSE and GFAP have been
286 recognized as marker molecules for astrocytes and neurons in brain, so their
287 expression levels reflected the degree of brain injury. Our findings showed that ENR
288 improved expression of S100B, NSE and GFAP in crucian carp brain, and higher
289 contents of them were observed in serum. This result suggests that ACs are in a state
290 of activation. ACs can be regulated by some drugs. Resveratrol is beneficial for brain

injury caused by oxygen and sugar deprivation via inhibiting expression of S100B and GFAP, and consequent activation inhibition of ACs (Liu et al., 2020). Sevoflurane can induce expression of GFAP and activation of ACs and, resulting in neurological disorders in mice (Zhang, 2020).

5. CONCLUSION

In the present study, we firstly revealed that ENR improved BBB permeability of crucian carp by mediating structure disruption, consequently more ENR entered brain tissue through BBB. Elevated ENR residue induced brain injury was related to activation of ACs and neuron injury.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The entire experimental procedure was approved by the Animal Care Commission of the College of Animal Science and Technology, Jiangxi Agricultural University, China.

AUTHOR CONTRIBUTIONS

Conceptualization, F.Z., J.R. and H.L.; formal analysis, F.Z., R.W., J.H. and H.Z.; methodology, F.Z. and R.W.; resources, L.W.; writing—original draft, F.Z.; writing—review and editing, J.R. and H.L.

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