鱼类氟中毒研究

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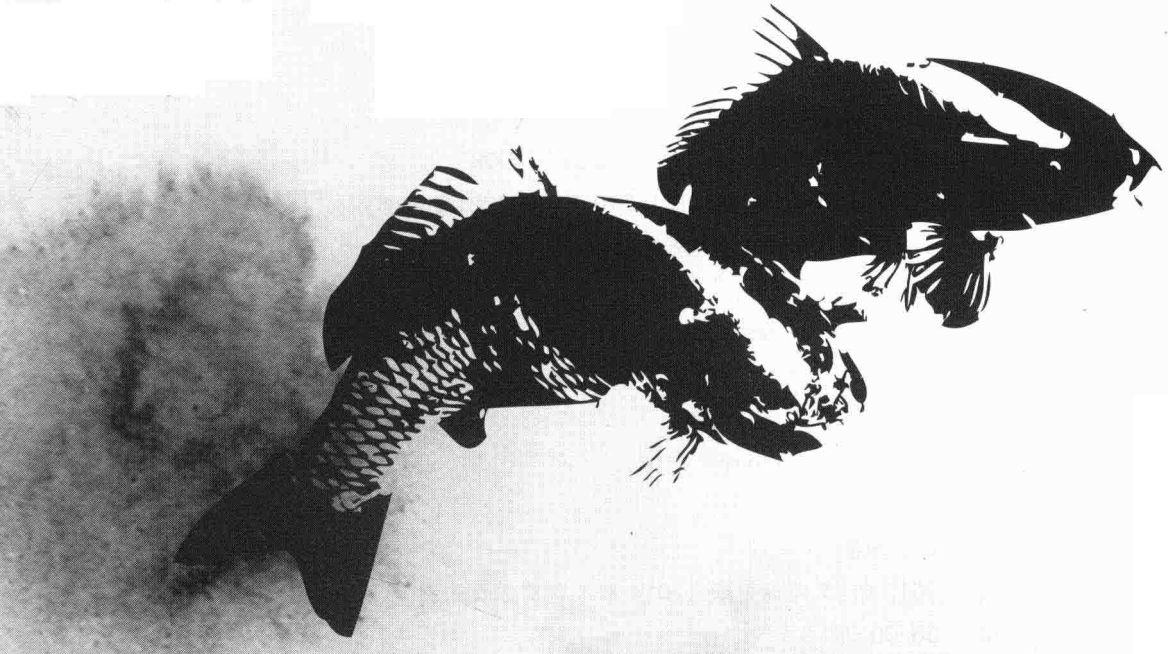

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前　言

地方性氟中毒（其后简称地氟病）是一种危害我国居民身体健康最为严重的地方性疾病。在一些重病区，居民氟斑牙患病率几乎达100%，氟骨病患病率也高达80%。因此，我国已被定为世界上受氟危害最严重的国家之一。随着工业的发展，人为活动排放的氟也已大大增加，在相当多的地区，大气降水中的氟含量已明显增加。人类的活动已经明显地改变了全球氟的自然循环状况。目前，引起世界关注的温室效应与全球气候变暖、南极上空臭氧空洞、酸雨等问题，都与人为排放的氟化物有密切的关系。

当水体中氟含量大于1.0mg/L时，称为氟超标，也称高氟水。饮用高氟水很容易引起氟中毒。在我国除了上海市、海南省、台湾省到目前还没有发现氟中毒外，其他各省、市、自治区都有不同程度的流行。

水生生态系统中的氟污染主要是由于工业活动（例如，磷矿的采集和加工，铝的加工制造）和农业活动（氟污染的化肥和杀虫剂的使用）所造成的。江河水含氟量为0.1～0.5mg/kg，地下水由于受地理环境的影响含氟量变化较大，在我国含氟低的地区为0.007～0.2mg/kg，含氟高的地区则达32～40mg/kg。据报道，我国氟中毒病区地下水中氟含量一般为2.3～8.0mg/L，我国温泉水中氟含量通常超过5mg/L，甚至可高达45mg/L；含氟工业排放废水中氟含量高达96.8mg/L，有的甚至高达3 000～5 000mg/L。

我国淡水渔业养殖用水主要来自于地下水、井水、温泉水等天然水体，而这些水体在部分地区的含氟量较高。高浓度的氟对水生生物包括鱼类存在毒害效应。鱼类终生生活在水中，可以直接从水中吸收氟，是较易受到氟毒害的靶生物。研究表明，过量的氟能对其血液指标、形态和行为指标以及细胞结构产生影响，会导致鱼类发生各种急性与慢性效应，如生长发育减缓、死亡率升高、鳃、肝、脑等发生病理学变化、不同组织氟的积累与畸形、鱼体代谢紊乱等。目前，世界上大多数国家均已经知道了人类饮水的氟含量标准，而旨在保护水生生物的标准很少，仅见美国（4.0mg/L）、德国（0.7mg/L）、加拿大（0.12mg/L）和中国（1.0mg/L）的建议值，可见相关研究尚不足以在全球范围内达成共识。可

见，开展氟对鱼类毒性的研究迫在眉睫。

鲤鱼，*Cyprinus carpio*（Cypriniformes，Cyprinidae），为鲤形目、鲤科、鲤属的一种经济鱼类，在全国各地均有分布，是我国最重要的养殖鱼种之一。而且，鲤鱼在我国水氟含量较高的地区，如山西省、内蒙古自治区、云南省和贵州省等均是特别重要的水产养殖对象。然而，氟对鲤鱼的毒性还未引起广泛关注。所以，我们以鲤鱼为模式动物，来研究氟中毒对鱼类的毒性效应。

2010 年，作者进入山西农业大学兽医学博士后流动站进行博士后研究，第一次接触了鱼类的氟中毒。我选择在全国分布范围广、适应性强的鲤鱼作为研究对象，首先进行了氟对鲤鱼的急性毒性试验，确定了鲤鱼 96h 的 LC_{50} 为 321.6mg/L，这表明，鲤鱼对氟化物具有相对较高的耐受性。在此基础上，我们以保证受试鱼体不死亡为前提，并结合环境中氟浓度变化，确定了最终的实验浓度。实验浓度确定之后，首先研究了水氟长期暴露下在鲤鱼不同组织中的积累，发现氟在鲤鱼组织中的含量随着暴露浓度的升高和暴露时间的延长而增加，氟在鲤鱼检测组织中的分布情况为鳃 > 肝 > 脑 > 肾 > 肌肉 > 肠，其中，在鳃中的含量最高。随后研究了氟对鲤鱼生长性能、体组成和生化指标的影响，发现氟能对鲤鱼的生长、血清渗透压、体组成及血液生化指标产生显著的影响，导致生长和食物利用效率的降低，表明高浓度的氟能对鲤鱼构成威胁。

为了验证氟对鲤鱼不同组织的毒性效应，我们首先以氟蓄积量最高的鳃为研究对象，检测了氟长期暴露于鲤鱼之后对鳃的毒性效应，结果表明，90d 的暴露之后，Na^+-K^+-ATPase 和 Ca^{2+}-ATPase 活性随着暴露剂量的增加而降低。此外，随着氟暴露剂量的增加，SOD 活性被显著抑制，而 MDA 含量显著升高，暗示氟促进了鲤鱼的氧化应激。组织病理学观察显示氟对鲤鱼鳃组织和氯细胞造成了损伤，且随着暴露剂量和时间的增加，损伤加重。为了进一步揭示氟对鲤鱼鳃毒害效应的机制，观察了氟化物长期暴露后鲤鱼鳃组织中 MAPKs 信号通路激酶表达变化情况，氟化物长期暴露导致鲤鱼鳃组织中磷酸化 ERK 及磷酸化 JNK 表达改变，这可能与慢性氟中毒导致的鳃损伤有关系，进一步揭示了慢性氟中毒鳃组织损伤的分子机制。

此外，我们研究了氟长期暴露对鲤鱼肝组织氧化应激、组织病理学变化、细胞凋亡及 Bcl-2 和 Bax 蛋白表达的影响。结果表明，90d 的暴露之后，SOD 和 GSH 活性受到抑制，而 MDA 含量显著升高，表明氟引起鲤鱼发生了氧化应激。显微观察结果表明，随着氟暴露浓度的增加及暴露时间的延长，肝组织及细胞器的损伤加重。细胞凋亡指数与氟浓度呈正相关（$r = 0.995$）。水中氟离子浓度与

Bcl-2 的表达及 Bcl-2/Bax 呈负相关（r = −0.98，r = −0.96）。氟浓度与 Bax 表达呈正相关（r = 0.96）。以上结果表明，鲤鱼能耐受相对高水平的氟浓度，但氟的长期暴露对鲤鱼肝组织造成了损伤。肝细胞凋亡是氟诱导肝组织发生组织病理学变化过程的一个重要的诱因。

本项目研究了不同浓度氟化物暴露 90d 后对鲤鱼肾脏氧化应激、细胞凋亡及 Caspase-3、Caspase-8 和 Caspase-9 蛋白表达的影响。结果显示，SOD 和 GSH 活性呈现剂量-时间依赖性下降，而 MDA 含量呈剂量-时间依赖性增加，这说明随着鲤鱼肾脏发生形态学变化及明显的细胞凋亡，氟诱导鲤鱼发生氧化损伤，特别是在高剂量组。氟暴露也显著提高了 Caspase-3、Caspase-8 和 Caspase-9 蛋白的表达。以上结果表明，氟的长期暴露引起氧化应激，损伤鲤鱼肾脏结构，通过线粒体途径或死亡受体途径导致肾脏细胞凋亡。

最后，研究了氟化钠对鲤鱼脑组织的抗氧化系统、组织结构和细胞凋亡的影响。结果发现，暴露 90d 后，SOD 和 GSH 活性主要表现为抑制，而 MDA 水平在整个暴露实验期间均呈增高趋势。脑组织切片的显微观察结果显示，氟化钠能导致鲤鱼脑组织血栓形成和各细胞层结构发生病理学改变，并具有剂效相关性。生物检测结果显示：随着氟化钠暴露剂量增大，鱼脑细胞凋亡率的增加与 MDA 水平的升高呈正相关关系（r = 0.996745），而 Bcl-2 的表达减弱，与 SOD 和 GSH 活性呈正相关关系（r = 0.919766 和 r = 0.98896）。

本书与已出版的其他关于氟的专著不同，收入的论文全部是作者对鲤鱼氟中毒问题进行第一手研究的结果。几年来，我们针对中国部分养殖水体中氟含量过高的实际问题，运用多种分子生物学知识，对高氟对鲤鱼的毒性进行了研究，为阐明氟对水生生物的影响提供基础资料，并为水体氟标准的制定提供理论依据。

目前，整个系列研究在中外文杂志上发表论文 5 篇，包括《Aquatic toxicology》《Chemosphere》《Environmental toxicology and Chemistry》《环境科学学报》《山西农业大学学报》。此外，还有几篇文章准备投稿。这些研究之所以能够持续进行，要感谢方方面面的支持。首先要感谢博士后导师王俊东教授对我的支持与帮助，正是他对氟研究的坚定信念促使我在鲤鱼氟中毒研究方面不断深入。同时还要感谢支持上述研究的中国博士后科学基金（2 项：2012M520601，2013T60267），山西农业大学科研启动基金（XB2009003），山西农业大学博士后基金（92462），山西农业大学科技创新基金（2009005）。

最后，还要感谢我的爱人陈剑杰、中国科学院沈阳应用生态研究所的谢凌天研究员以及我的两位硕士研究生贾如辉和薛文娟在本实验及论文发表过程中的

帮助。

全书共分为十一章：第一章介绍氟中毒的历史和危害；第二章介绍氟对鲤鱼的急性毒性及安全浓度研究；第三章介绍水暴露下氟在鲤鱼不同组织中的积累；第四章介绍氟对鲤鱼生长性能、体组成及生理生化指标的影响；第五章介绍氟对鲤鱼鳃组织的毒性影响；第六章介绍氟对鲤鱼鳃组织 MAPKs 信号通路相关基因及蛋白表达的影响；第七章介绍氟对鲤鱼肝细胞凋亡及 Bcl-2 和 Bax 蛋白表达的影响；第八章介绍氟对鲤鱼肾脏细胞凋亡及 Caspase 蛋白表达的影响；第九章介绍氟对鲤鱼脑抗氧化系统及细胞凋亡的影响；第十章介绍氟对牙齿的影响及饮水加氟的认识过程；第十一章介绍氟化物的检测。

曹谨玲

2013 年 8 月

Abstract

Fluorosis is a kind of endemic zoonosis, existing all over the world. There is a long history of the records about fluorosis in China, especially in Shanxi province. About 100 thousand years ago, the dental fossil of ancient people living in Xujiayao village of Yanggao county in Shanxi province, who were in the epoch evolving from ape man into homo sapiens, has the vestige of fluorosis. In addition, contemporaneously, fluososis also appeared in Ding village of Linfen city in Shanxi province. Ji kang (A. D. 223 ~ 262), a luminary in Jin dynasty, said "Teech become brown, when live in Shanxi" in his works.

Internationally, serious damage to livestock colcano erupting in Iceland has been lasting for several hundreds years. Early in 1694, the pozzuolana owing to eruption resulted in animal fluorosis in Iceland. The local farmers Oddur Iiriksson and Eenedikt Peturss described the toxicity symptom of animal disease called "volcano teeth" for the first time: tooth staining and incisive tooth defection as a result of excessive wear. These descriptions became the first report of tooth damage. In 1845, after Hekla volcano erupted, exostosis was found in bones of sheep which die of ingesting pozzuolana containing fluoride.

As for human fluorosis, there had a recordation of mottled teeth in medical literature since early 1771. It was not known that such dental problems were resulted from drinking water containing fluoride until 1931. So far as we know, fluorosis widely exists in more than 50 countires over Asia, Africa, Europe, North America, South America, and Oceania. Endemic fluorosis can be found every city and province in China, except Hainan and Shanghai. Therefore, on the 20th Conference of the International Society for Fluoride Research (ISFR) held at Beijing, Secretary General then, said "the environmental fluoride problem in China is the most serious in the world." According to the data jointly issued by health department, development and innovation committee and ministry of finance, up to the end of 2004, the amount of fluorosis patients was the most a-

mong all kinds of endemic deseases. The population of dental fluorosis was 39.50 million, and fluorosis of bone was 2.90 million. It's meant that based on the total population of 1.4 billions, one in thirty people got fluorosis on average.

The history of industrial fluoride is not very long. Early in 1855, there was a case of compensation for a loss of polluted plant because of the exhaust gas from one German smelter industries. However, it is until 1907 that the real toxic source was the fluoride in the gas. Early in 1930s, a Denmark scholar, Professor P. Flemming Moller first reported that changes of bone X ray of cryolite workers, and suspected that it is the results of fluoride. In 1932, he named this disease as fluorosis firstly. Soon, Doctor Kaj E Roholm fully confirmed the harm effects of fluoride on the cryolite workers. Later, he published his famous monograph *Fluorine Intoxication*.

After he had consulted lots of related literature, secret and official documents of World War Two and word of Generals survived from that war, in 2004, Chrstopher Bryson wrote his book Fluoride Deception, which revealed the scared bad story of American fluoride that concealed for 60 years. Chrstopher believed that Manhattan project in 1944 led to serious fluoride pollution. In order to get fissionable uranium-238 from stable uranium -235, huge amount of UF6 were used in Manhattan project. During that very period, those factories discharged high concentration of fluoride and polluted the around areas. In this book, he discovered how industrial groups contrived the "safe" fluoride to protect the pollutant fluoride and to win the industrial fluoride hazard suit.

In China, the industrial pollution happened in 1960s. Fluorosis was firstly found in grazing lands around the industrially polluted region of Baotou of Inner Mongolia. Symptoms of severe fluorosis in herbivore included excessive tooth wear and bone lesion. Commonly, the molar were woren down to the gum line. Exostosis developed on cervical rib. Dental lesion influenced their foraging, and lead to malnutrition, low productivity such as infertility and miscarriage, and even death. According to the investigation in 1974, the area of the fluorosis hazard was up to 100 housand square kilometers. The amount of animal fluorosis was more than 6 000 thousands. Because the economic value of animal like cattle, horse, and sheep became less, livestock farming in this area lost its balance. Finally, there was only sheep industry left. All these seriously restricted the development of their livestock farming and improvement of herdsmen's living standard.

Adding calcium fluorophosphates to forage also lead to economic loss of husbandry

in given time and areas. Early in 1930s to 1970s, there were huge amount of literature which reported that calcium fluorophosphates additives can cause sever cow fluorosis. In 1930 Reed O. E. and C. F. Huffman firstly reported that the content of fluoride in crude phosphorite was near 3.5%, and described the bone and dental lesion in cow which had been fed with such additives for more than 5 years.

Fluoride ions have caused widespread endemic fluorosis in humans and animals, however little attention was paid to the effect of fluoride ions on aquatic organisms. Fish take up fluoride directly from water and are susceptible to fluoride contamination of their environment. Acute toxicity experiment on common carp was carried out to explore the safe criteria of fluoride to fish. The fish were exposed to concentrations of 200mg/L, 263.4mg/L, 346.9mg/L, 456.9mg/L, 600mg/L of fluoride (in the form of NaF). Toxic symptoms of fluoride in common carp included abnormal motility, loss of equilibrium, changes in gill ventilation. The mortality rate of juvenile carp was raised with the increase of exposure concentration and exposure time. The LC_{50} of fluoride ions to carp at 24 h, 48 h, 72 h and 96 h, 120 h were 381.614mg/L, 313.344mg/L, 333.388mg/L, 321.583mg/L, 351.775mg/L, respectively. The safe concentration of fluoride for carp was 3.22mg/L.

The tissue distributions of fluoride of the common carp (*Cyprinus carpio*) chronically exposed to fluoride was examined. Carp were exposed to a range of aqueous fluoride (35 to 124mg/L) and sampled at 30, 60 and 90 days. The accumulation of fluoride in the tissues increased with the level and duration of exposure. Steady state was not achieved under the experimental conditions. The gills accumulated the highest levels of fluoride followed by the liver > brain > kidney > muscle > intestine.

Effects of fluoride on growth performance, body composition and biochemical measurements of *C. carpio* were evaluated. Our results showed that growth, serum osmolality, body composition, and biochemical measures in the blood were significantly affected by fluoride. Weight gain rate and specific growth rate in the exposed fish decreased significantly compared with those of the control fish. Fluoride significantly reduced the levels of crude protein and crude lipids. The major ion levels in the serums of fluoride-exposed fish were severely disturbed, resulting in a lower osmolality. All the biochemical parameters measured in the blood were affected by the exposure to fluoride. Total protein, albumin, globulin and glucose in fish exposed to 63.6mg/L, 77.7mg/L and

124.4mg/L were significantly lower than those in the control fish. AST and ALT were markedly increased in exposed fish compared to the control ones.

The toxicity of fluoride in the gills of the common carp (*Cyprinus carpio*) chronically exposed to fluoride was examined. Carp were exposed to a range of aqueous fluoride (35 to 124mg/L) and sampled at 30, 60 and 90 days. A dose-dependent inhibition was observed for the enzyme activities of Na^+-K^+-ATPase and Ca^{2+}-ATPase in the gills after the fish were exposed for 90 days. Also, accumulation of fluoride was associated with the inhibition of superoxide dismutase (SOD) activities and a dose-dependent stimulation of malondialdehyde (MDA) levels in the gill tissues, suggesting that fluoride promoted oxidative stress in the fish. Microscopic examinations revealed injuries to gill tissues and chloride cells, with the severity of injury increasing with exposure concentration.

The effects of fluoride on the expression of MAPKs transcription pathways in carp gills were evaluated. The expressions of MAPKs in gills were examined using QRT-PCR, Western blotting and immunohistochemistry methods. The Caspase-3 activity was increased in a dose-dependent pattern. QRT-PCR showed that the mRNA levels of ERK1, ERK2, and p38 in the fish from the treatment groups decreased significantly compared with the control fish for 90 days of exposure ($p < 0.05$). Western blotting indicated that p-ERK1/ERK1 and p-ERK2/ERK2 in treatment groups decreased markedly in fish exposed to 63.3mg/L, 77.7mg/L, 124.4mg/L fluoride relative to those of the control group ($p < 0.05$). p-JNK/JNK in groups exposed to 63.3mg/L, 77.7mg/L, 124.4mg/L fluoride increased significantly ($p < 0.05$). Immunohistochemistry demonstrated that the expression of p-ERK and p-ERK/ERK increased with fluoride levels ($p < 0.05$) while the expression of p-JNK and p-JNK/JNK decreased with fluoride levels ($p < 0.05$). Our results demonstrated that the changes of the expression of p-ERK and p-JNK in the gills of carp after the chronic exposure to fluoride might be connected to gill impairment induced by chronic fluorosis.

The oxidative stress, histopathological changes, apoptosis, and Bcl-2 and Bax expression in the livers of the common carp (*Cyprinus carpio*) chronically exposed to fluoride were evaluated. Our results showed that after 90 days of exposure, the inhibition of SOD, GSH activities and a dose-dependent stimulation of MDA levels in the liver tissues indicated that fluoride caused oxidative stress in the fish. Microscopic examinations

showed that damages to the liver tissues and cell organelles in the liver tissues increased with exposure concentration and duration. A positive correlation was observed between the apoptosis index and fluoride levels in the livers ($r = 0.995$). There was a negative correlation between the fluoride concentration of water and the expression of Bcl-2, Bcl-2/Bax ($r = -0.98$, $r = -0.96$). A positive correlation was showed between the fluoride concentration of water and the expression of Bax ($r = 0.96$) after 90 days of exposure. Our results suggested that the common carp could tolerate relatively high levels of fluoride but adverse effects of fluoride occurred in the livers of the fish after 90 days of exposure. The apoptosis of liver cells had an important causative role in the process of fluoride-induced pathological changes of liver.

The oxidative stress, apoptosis, and protein expressions of Caspase-3, Caspase-8 and Caspase-9 in kidney of the carp juveniles exposed to 0mg/L, 40mg/L, 80mg/L, 120mg/L, 160mg/L of fluoride (in the form of NaF) for 90 days were investigated. The results showed that dose- and time-dependent decrease of SOD and GSH and dose- and time-dependent increase of MDA were observed in the carp juveniles, which suggested that fluoride induced oxidative damage accompanied with morphological changes and significant apoptosis in fish exposed to fluoride, especially in the higher doses. Fluoride exposure also significantly elevated the protein expressions of Caspase-3, Caspase-8, and Caspase-9. In conclusion, these results indicate that chronic exposure to fluoride causes oxidative stress, damages the kidney structure, and results in renal apoptosis by mitochondrial pathway or death receptor pathway.

The physiological toxicity of sodium fluoride on antioxidant system, organizational structure and apoptosis of brain in Cyprinus carpio Linnaeus were studied. Results showed that the superoxide dismutase (SOD) and glutathione (GSH) activities were induced with the concentrations of sodium fluoride increasing firstly and then inhibited after 30 days exposure, but the SOD and GSH activities were all decreased after 60 and 90 days exposure, respectively. The content of lipid peroxides (MDA) was increased during the whole test period. After 90 days of exposure, histopathological changes of brain tissue were observed. It was found that thrombosis and structural changes in the cell layers were resulted from the exposure of sodium fluoride. The biological investigation results showed there were positive correlation between the cell apoptosis rate and the MDA levels ($r = 0.9968$), but with dosage increasing, Bcl-2 protein concentration de-

creased, which was positive correlated with SOD and GSH activities (r = 0.9198, 0.9889) .

Taken together, chronic exposure to elevated concentration of fluoride caused a suite of detrimental effects in *C. carpio*, which might lead to a decrease in growth and food utilization efficiency, damages of various tissues. Our results implied that high levels of fluoride could pose a threat to carp in the field.

Key words: Fluoride; Common carp; Toxicity; Histopathological changes

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