Effect of Methanol as Cryoprotective Agent on Common Carp Fish *(Cyprinus carpio* L.) Larvae

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Abstract. Because of the lack of information about injury or malformation when the cryoprotectants agent (CPAs) used as cryoprotective agents in common carp *(Cyprinus carpio* L.) Larvae that resulting from treated embryos with different concentration of methanol (5, 10 and 15%) in different stages of embryonic development (Gastrula, Heart beat and eyed stage) were used. The results showed that there was negative correlation among the concentrations and hatching rate also malformation status was noticed. Higher defects were observed with 15% followed by 10% and finally 5% concentration for all stages. These experiments and control group were cured out under the same conditions.

Introduction

A cryoprotectant is a substance that is used to protect biological tissue from freezing damage (i.e. that due to ice formation). Methanol, also known as wood alcohol, is a commonly used organic solvent, the ingestion of which has severe potential ramifications. It is a constituent in many commercially available industrial solvents and in poorly adulterated alcoholic beverages. Toxicity usually occurs from intentional overdose or accidental ingestion and results in metabolic acidosis, neurologic squeal, and even death. Methanol toxicity remains a common problem in many parts of the developing world, especially among members of lower socioeconomic classes. Body deformations commonly occur in fish larvae, their frequency may be affected by environmental conditions, and they become visible already during embryonic development [1].

Several factors may cause an increase in frequency of body deformations in fish, such as low and high temperature [2, 3], pH changes [4, 5], low dissolved oxygen content [6] and radiation [7] .but no data about body malformation affected by cryoprotectants agent (CPA)s.

Embryonic and larval malformations are recognized as a recurring problem in fish aquaculture and represent both ethical and economical challenges for the industry [8]. A variety of natural environmental factors, such as temperature, salinity, pH, etc., have been identified as responsible for inducing deformities in fish embryos and/or larvae [9]. In addition to these, human activities and breeding in hatcheries added a series of new parameters that may exert considerable impact on developing fish [10].

Aquatic toxicology tests are designed for readily water soluble substances. However, due to the occurrence of several water-insoluble substances, the use of various solvents is needed [10]. A major concern in toxicology studies is the choice of appropriate solvents. Carrier solvents may also have toxic effects on organisms [11; 12; 13; 14]. Hence, it was necessary to set guidelines that will describe the types and the maximum allowable concentrations of the solvent to be used in experimental systems that will cause no significant impact on the observed results [15].

This study was one of series of experiments addressed the malformations and hatching rate in the common carp larvae that treated with (CPA)s in embryonic stages.

The aim of this study is to determine the toxicity (hatching rate and malformation) of different concentration of methanol on common carp *(Cyprinuscarpio* L.) larvae in order to contribute to the establishment of fish embryo cryopreservation protocols.

Material and Methods

The experiments were done using common carp *(Cyprinuscarpio* L.) larvae exposed to the methanol during embryonic development. Eggs and sperm were obtained from artificially stimulated spawning. The common carp (*Cyprinus carpio* L.) embryos were divided according to embryonic stage (Gastrula, Heart beat and Eyed stage) and exposure to three concentrations (5,10 and15%) of methanol (the concentrations of methanol were prepared as the known method) as cryoprotective agent for each stage. Followed by a washing process to remove the preservative substance. After hatching, the embryos were transferred into fiber glass tank (30 liters).

Two-three days old, the larvae were initially feed on egg custard and artemia nauplii for 7 days and the larvae weaned to dry diet until the end of experiments. Gradually, malformations were observed by microscope and manually within the period of study. Newly hatched larvae were counted and inspected.

The percentage of hatchability was calculated as a number of hatched larvae per initial number of incubated eggs. Among freshly hatched larvae the share of deformed ones was evaluated. Each deformed larva was examined and classified according to the types of malformations.

Results and Discussion

Exposure to different concentration of methanol in various embryonic stages caused different hatching rate and numerous body malformations as showed in the table (1,2,3). The hatching percentage was obtained in the control for the three stages (Gastrula, Heart beat and eyed stage this is before hatching) were (100% ,100% and 97.25%) respectively. whereas , the highest percentage of integral larvae found in gastrula stage with 5% methanol exposure was 93.11% and 89.65%was in the heart beat stage, finally, in the eyed stage found 89.19 %.

The number of malformed embryos were increase with increasing concentration of methanol, whereas, the number of hatched embryo were decrease. The obtained results referred into multiple malformations ,such spinal deformity, semi-operculum, head defected ,blind and others defects were observed.

Many studies referred to the effects of the exposure to the methanol on body organs such the causes of lordosis in *C. carpio* [16] That found could not be established but in a number of marine fish lordotic deformation is noted to occur only in fish that had no gas in their swim bladder. In several fish species the spinal malformation was found to be associated with the absence of a functional swim-bladder [17]. the head appeared larger or smaller in comparison to normal fish that appeared to be due to ossification and compression of bones. There were various deformations of the head, including beak like appearance. Stump body is caused by an abnormal ossification of the trunk vertebrae. Mortality of larval fish can be high if the teratogenic defects are severe enough to impair critical body functions [18].

Methanol is toxic by two mechanisms. First, methanol (whether it enters the body by ingestion, inhalation, or absorption through the skin) can be fatal due to its depressant properties poisoning. Second, in a process of toxication, it is metabolized to formic acid (which is present as the formate ion) via formaldehyde in a process initiated by the enzyme alcohol dehydrogenase in the liver[19] Methanol is converted to formaldehyde via alcohol dehydrogenase (ADH) and formaldehyde is converted to formic acid (formate) via aldehyde dehydrogenase (ALDH). The conversion to formate via ALDH proceeds completely, with no detectable formaldehyde remaining [20]. Formate is toxic because it inhibits mitochondrial cytochrome c oxidase, causing the symptoms of hypoxia at the cellular level, and also causing metabolic acidosis, among a variety of other metabolic disturbances [21]. Fetal tissue will not tolerate methanol. However, in some cases the abnormalities may not be life threatening and the malformations can persist into juvenile and adult life stages [21].

Table (1): Number of malformed larvae resulting from the exposed embryos to methanol at gatrula stage

Concentration %	No. of the used embryos	Number of hatched Larva	Defected Larvae	Percentage of malformed Larvae %	No. and % of Integral Larvae
Control (0)	37	28	0	0 %	28a (100%)
5%	37	29	2	6.89 %	27a (93.11%)
10%	37	28	4	14.28 %	24b (85.74%)
15%	37	25	7	24 %	18c (67.57%)

$X^2 = 9.04$ $X^2 = 9.43$
$P< 0.01$ $P< 0.01$
df = 3 df = 3

Table (2): Number of malformed larvae resulting from exposed embryos to methanol at heart beat stage.

Concentration %	No. of the used embryos	Number of hatched Larva	Defected Larvae	Percentage of malformed Larvae %	No. and % of Integral Larvae
Control (0)	37	34	0	0	34a (100%)
5%	37	31	4	12.46	27a (87%)
10%	37	29	3	10.3	26b (89.65%)
15%	37	19	3	21	15c (51.35%)

$X^2 = 15.96$ $X^2 = 2.53$
$P < 0.01$ $P < 0.01$
df = 3 df = 3

Table (3): Number of malformed larvae resulting from exposed embryos to methanol at eyed stage.

Concentration %	No. of the used embryos	Nummber of hatched Larva	Defected Larvae	Percentage of malformed Larvae %	No. and % of Integral Larvae
Control (0)	37	36	0	0	36a (97.25%)
5	37	33	6	18.18	27a (89.19%)
10	37	25	9	36.0	16b (67.57%)
15	37	18	7	38.8	36c (48.65%)

$X^2 = 19.3$ $X^2 = 12.76$
$P < 0.01$ $P < 0.01$
df = 3 df = 3

Table (4): Correlation coefficients of hatched embryo compare with control group.

Concentration%	5%	10%	15%
Control (0%)	0.97	0.72	0.65

Table (5): The type and number of malformed larvae resulting from exposed embryo to methanol

N	Type of malformation	Number of defected larvae
45	Semi – operculum	5
	Spinal deformity	16
	Head deformity	4
	Blind	6
	Yolk sac swelling	6
	Stum body	4
	Multiple defects	4

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