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## Effect of *N*-ethyl-*N*-nitrosourea (ENU) toxicity on early development of Asian catfish *Clarias batrachus* (Linnaeus, 1758)

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### Abstract

The chemical mutagenesis is an efficient way to produce new mutants for future genetic improvement in aquaculture species. Although these chemicals also induce toxicity. *N*-ethyl-*N*-nitrosourea (ENU) is an alkylating agent (SN1 type) and used as a chemical mutagen to study development and physiology of fishes. Although the concentration of ENU used in common protocols to mutagenize fish is limited by its toxicity in literature. In this article, an attempt has been made to identify the ENU associated toxicity to understand the fertilization, hatchability, survivability, and early developmental deformity to provide a baseline data. The fertilization and hatching rate decreased with increased concentration of ENU in *Clarias batrachus*. The LC<sub>50</sub> value was observed as 5.7 (6.1, 5.3). The incubation periods were extended in the embryonic development with increasing concentrations of ENU. The observed deformities included unfertilized eggs, disintegrated yolk and blastodisc, retention of chorion, lordosis, retarded larvae and bent tail larvae, larva with no eye formation and larva with cardiac abnormality pooled blood in brain with tube heart. These embryos with deformities usually survived until hatching; however, embryos with more severe malformations died without hatching.

**Keywords:** *N*-ethyl-*N*-nitrosourea, Asian catfish *Clarias batrachus*

### Introduction

The recent genetic breeding technique of aquaculture fish species is motivated to discover natural mutants for improved strains (such as high performance, growth, productivity, disease resistance) produced by genetic selection, hybridization, or marker-assisted breeding approaches (Moen *et al.* 2009) [12]. To develop and identify mutant lines with desirable phenotypes are in strong need also for the understanding on developmental or physiological processes, or to discover novel signalling pathways and regulators or modulators through mutagenesis (Peng *et al.* 2014) [14]. The chemical mutagenesis is an efficient way to produce new mutants for future genetic improvement in aquaculture species. Although these chemicals also induce toxicity. *N*-ethyl-*N*-nitrosourea (ENU) is an alkylating agent (SN1 type) and a candidate compound of the Environmental Protection Agency consensus workshop (1 of 47 chemicals) chosen for *in vitro* teratogenicity test validation due to its consistent teratogenicity in *in vivo* test systems (Solomon and Faustman 1987) [19]. The chemical mutagenesis through ENU is a standard method to generate mutants, which can be used to study development and physiology of fishes. Previous studies showed that ENU associated mutagenesis protocol with different concentration requires the treatment of fish with different doses that often lead to high lethality during the treatment. Although the concentration of ENU used in common protocols to mutagenize fish is limited by this toxicity in literature (Rohner *et al.* 2011) [16]. The Asian catfish *Clarias batrachus* (Linnaeus, 1758), popularly known as Magur is a potential aquaculture candidate in Asian countries like Thailand, Philippines, Cambodia, Myanmar, China and India for its high growth rate and excellent nutritional value with high consumer preference. Previously various aspects of the foods and feeding habits, digestive physiology, artificial breeding, early developmental ontogeny and larval rearing of *Clarias* sp. have been studied extensively. Although, the present natural stock is depleting due to over-exploitation, habitat degradation and fragmentation.

Thus, large scale captive breeding is essential for the conservation of this species and fundamental pre-requisite for availability of quality fish seeds. To improve breeding performance several works have been done on the spawning behavior, spawning performance along with the effective and economical inducing agent of spawning, artificial spermiation, ovulation and maturation of gametes for better understanding and successful breeding of this species (Thakur 1976; Hossain *et al.* 2006; Goswami 2007; Sahoo *et al.* 2010). However, till now no attempt has been made to identify toxic effect of chemical mutagen on *Clarias batrachus* while using it to produce new mutants for future genetic improvement. In this article an attempt has been made to identify the ENU associated toxicity to understand the fertilization, hatchability, survivability and early developmental deformity to provide a baseline data.

## Materials and Method

**Artificial breeding procedure:** Healthy and gravid brood fishes (Avg. weight; female 150±10.5 gm, male 120±8.5 gm) were collected during the breeding season (July-August) from a local fish farm of West Bengal, India. Fishes were stocked in a pond with a surface area of 0.10 to 0.13 ha and a water depth of 165 to 180 cm. The males were selected based on pointed and reddish genital papilla, while females by a round and reddish papilla, softness of abdomen and uniform size of intra-ovarian oocytes. Artificial breeding was carried out following the method of Roy *et al.* (2019)<sup>[17]</sup>. Fertilized eggs were obtained by manual stripping and then washed with freshwater for activation. After that diluted sperm solution (normal saline) were added to the eggs and mixed. The eggs were spread on nylon mesh putting inside the flow through aquaria (130 cm X 66 cm X 12 cm) of 6 mm thickness and incubation was carried out. The water parameters were studied during the experiment with the values of Dissolved Oxygen 7.8 mg L<sup>-1</sup>, Total Ammonical Nitrogen 4 mg L<sup>-1</sup>, pH 6.5 ± 0.5 and temperature 26 ± 0.5 °C.

**ENU preparation and application:** Ethylnitrosourea (Sigma-Aldrich) was stored in the dark at -20 °C as stock solution. It was dissolved in Hank's balanced salt solution (pH 7.4) to get working solution ranging from 0.5 mM to 10 mM immediately before use. The test solutions were added directly to embryo at final concentrations ranging from 0.5 mM to 10 mM in dark for 2 hrs of exposure. Control embryos were only exposed to Hank's solution. From each breeding set around two hundred fertilized oocytes were randomly sampled to study fertilization and hatching rate, survival rate and larval deformities. Three replicates were taken for each set of experiment.

**Determination of Fertilization Rate:** This was calculated by counting the total number of fertilized eggs in each replicate and expressing it as a percentage of the total number of eggs. This can be mathematically expressed as: % fertilization rate = Number of fertilized eggs / Total number of eggs x 100.

**Determination of Hatching Rate:** This was successfully carried out by counting the total number of hatched eggs in each replicate and expressing it as a percentage of the total number of eggs. This can be mathematically expressed as: % hatching rate = Number of hatched eggs / Total number of eggs x 100.

**Embryonic and Larval malformation:** The embryonic and larval developmental deformities were studied each hour under the light microscope (Olympus Trinocular Microscope XSZ156T) mounted with digital camera (Olympus SZ-10 Digital Camera, 18x Optical Zoom). The parameters were studied qualitatively including yolk and blastoderm disintegration, notochord flexion, retarded larvae, bent tail, lordosis, cardiac malformation.

**Statistical analysis:** The fertilization and hatching ratio of embryos and larvae were presented as percentage value. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to calculate the statistical significance of differences among treatments. Statistical significance was set at the  $p < 0.01$  level. Statistical analyses were performed using SPSS ver 17.0. The LC<sub>50</sub> values were calculated using probit analysis.

## Results and Discussion

The responses of *in vitro* systems to chemical agents has got recent public and scientific interest to identify and characterize *in vitro* alternatives to whole-animal testing. The fertilization and hatching rate decreased with increased concentration of ENU in *Clarias batrachus*. The study showed that lowest fertilization rate (18.22±4.56%) was observed in 10mM ENU treated fish with lowest mean hatching rate of 10.33±3.11% ( $p < 0.01$ ) (Table 1). The LC<sub>50</sub> value was observed as 5.7 (6.1, 5.3). The incubation periods were extended in the embryonic development with increasing concentrations of ENU. Similar observation i.e. decreased fertilization and hatching success was also reported with increasing concentrations of Formalin in *Clarias gariepinus* (Akhpoilih and Adebayo 2010) and sumithion in *Danio rerio* (Rahman *et al.* 2020)<sup>[15]</sup>. Michael *et al.* (2011)<sup>[11]</sup> reported that reduced hatchability might be related with the hindered development of embryos as an important effect of the toxicant. It may be due to inhibition of the tetraspanin cd63 gene that caused deficiency in secretion of proteolytic enzymes essential for controlling of the chorion. In African catfish embryos, lowered hatching rate was observed after exposure to various concentrations of buprofezin and endosulfan (Agbohessi *et al.* 2013; Marimuthu 2013)<sup>[1, 9]</sup>. There was a significant increase in the frequency of embryo and larval deformities exposed to ENU at 26±0.5°C for 2 hrs with an increased concentration of ENU (Table 2). The observed deformities included unfertilized eggs (Fig 1B) disintegrated yolk and blastodisc (Fig1C&D), retention of chorion (Fig1E), lordosis (Fig1F), retarded larvae and bent tail larvae (Fig 1G&H), larva with no eye formation and larva with cardiac abnormality pooled blood in brain with tube heart (Fig I & J). These embryos with deformities usually survived until hatching; however, embryos with more severe malformations died without hatching. ENU reacts initially to form a reactive intermediate through a rate-limiting reaction to develop different spectrum of developmental toxicity undergoing first-order nucleophilic substitution reactions being more potent developmental toxins (Solomon and Faustman 1987)<sup>[19]</sup>. Early Life Stage (ELS) tests have been proposed as faster and more cost-efficient bioassays for testing the potential toxicity of chemicals in fish. However, the various embryonic and larval stages of fish differ in their susceptibility due to physiological and biochemical differences (McKim 1995)<sup>[10]</sup>. Previous embryotoxicity study also reported concentration-dependent decrease in viability

and increasing flexure, cardiac, optic, and cephalic malformations in Medaka by ENU (Solomon and Faustman 1987) [19]. A significant higher proportion of notochord deformity and pericardial edema was detected at 2.5 mg/l malathion and higher on *C. gariepinus* larvae (Lien *et al.* 1997) [8]. Nguyen and Janssen (2002) described embryo larval toxicity tests in *Clarias gariepinus* by using various toxicants where growth was highly disrupted followed by developmental abnormality, larval survival, embryo survival and hatching. Exposures of stinging catfish larvae to sumithion produced deformities including irregular head shape, lordosis, yolk sac edema, body arcuation, tissue ulceration, etc. The mortality rates of larvae were significantly

increased in response to increase in sumithion concentrations (Shahjahan *et al.* 2017) [18]. Similar finding was observed in case of African catfish with increased concentration of formalin due to its potential toxicity, persistence and bioaccumulation (Svobodova, 1993; Akhpoilih and Adebayo 2010). It has been reported that spinal curvature (lordosis, kyphosis and scoliosis) might result from differential accumulation of toxicants and lack of neuromuscular coordination due to decreased collagens in the spinal column, changing amino acid composition or due to down regulations of *pkt7* gene, a critical regulator of wnt signaling (Ekrem *et al.* 2012; Hayes *et al.* 2014) [4, 5].

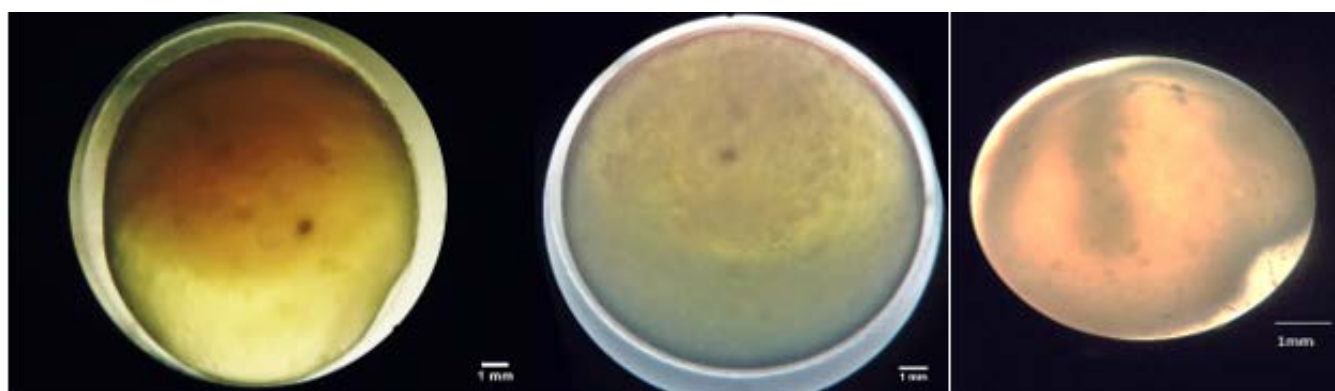
**Table 1:** Fertilization and hatching percentage in *C. batrachus* exposed to different concentrations of ENU

Treatments	Control	0.5 mM	1mM	5 mM	10 mM
% of fertilized egg	94.62±2.05 <sup>a</sup>	88.83±0.08 <sup>b</sup>	76.38±3.16 <sup>c</sup>	59.26±1.54 <sup>d</sup>	18.22±4.56 <sup>e</sup>
% of hatchability	85.56±1.33 <sup>a</sup>	75.89±3.14 <sup>b</sup>	64.23±2.7 <sup>c</sup>	45.35±0.54 <sup>d</sup>	10.33±3.11 <sup>e</sup>

Data are means ± S.E (n=3 replicate per treatment) expressed as % of total number of eggs for fertilized egg and total hatched larvae for survival. Letters with the different superscript in the same row differed significantly ( $p < 0.01$ ).

**Table 2:** Details the frequency of specific types of deformities in *C. batrachus* exposed to ENU

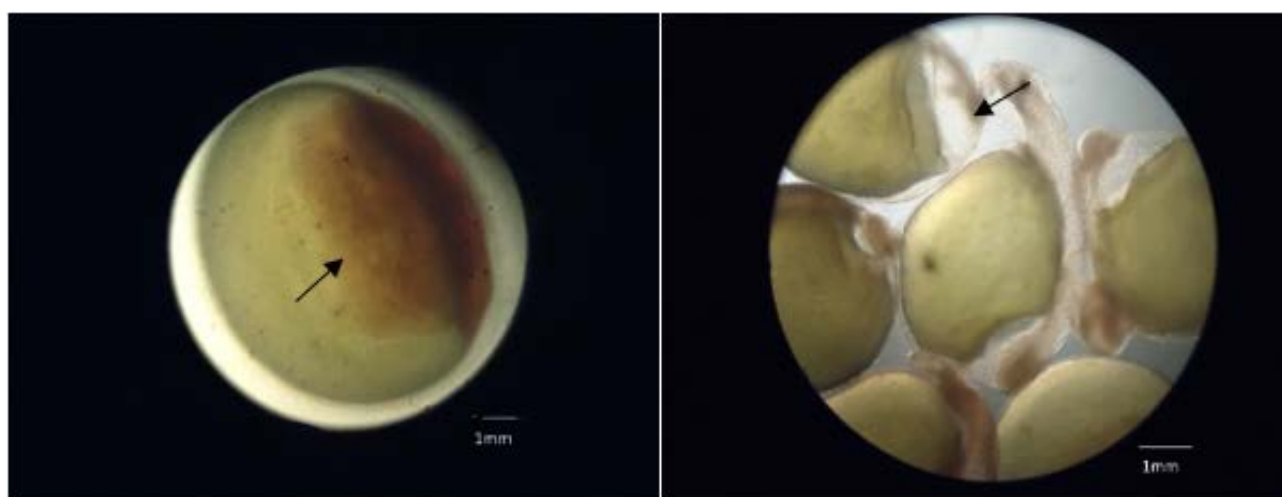
Concentration	Yolk disintegration	Blastodisc degeneration	Retention of chorion	Lordosis	Retarded larvae	Bent tail	No eye formation	Abnormal heart
Control	0	0	0	0	0	0	0	0
0.5 mM	5	9	10	8	10	4	7	4
1mM	14	17	15	17	13	16	18	17
5 mM	45	49	51	45	42	48	43	48
10 mM	80	92	89	90	82	86	92	94



A

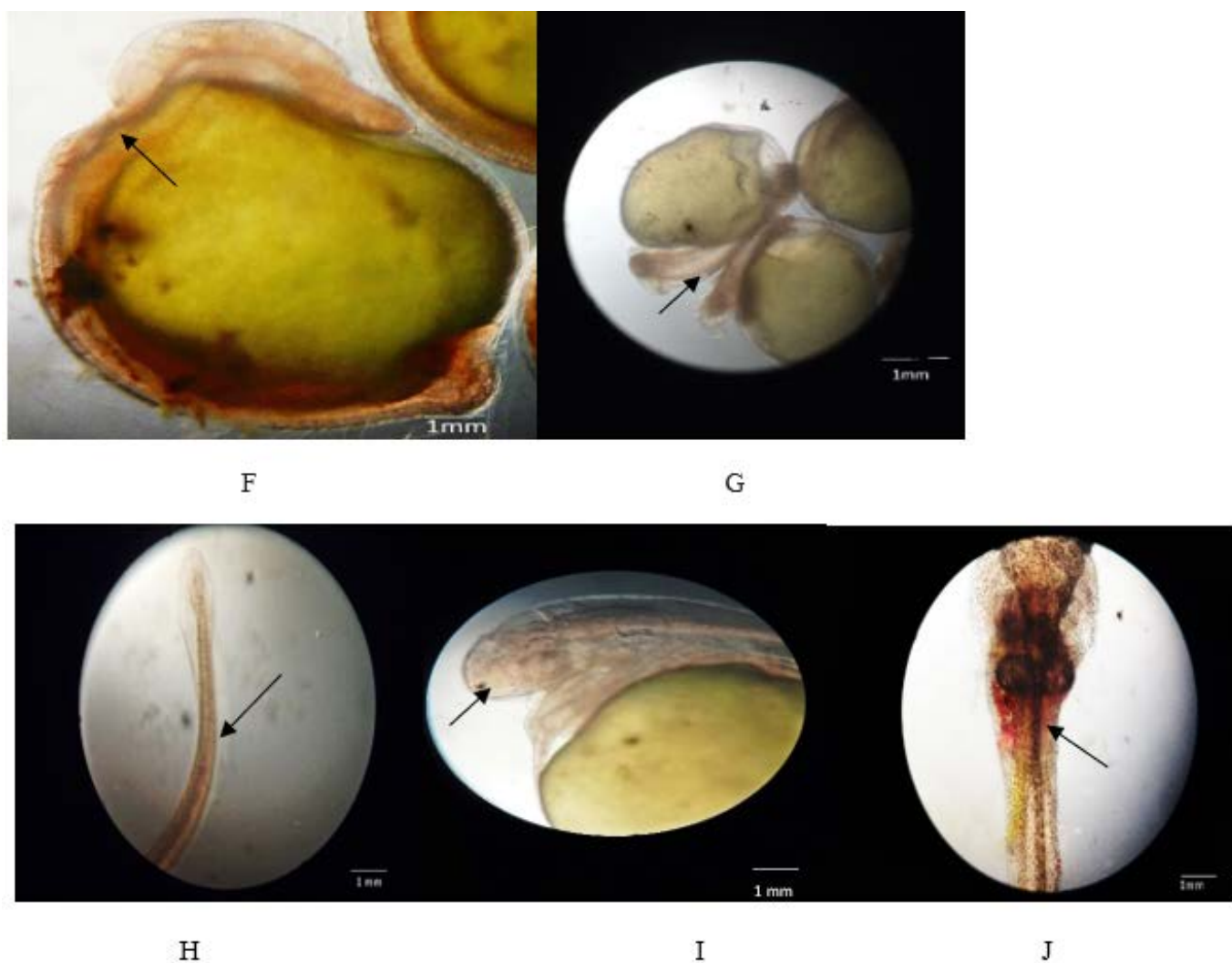
B

C



D

E



**Fig 1:** Developmental deformities observed in *C. batrachus* upon exposure to ENU (A) fertilized egg (B) unfertilized egg (C) disintegrated yolk (D) disintegrated blastodisc (E) retention of chorion (F) lordosis (G) retarded larvae (H) bent tail larva (I) larva with no eye formation (J) larva with cardiac abnormality pooled blood in brain

### Conclusion

In recent years there is an increasing demand of exotic hybrid magur (*Clarias gariepinus* x *Clarias macrocephalus*). About 90% of fish farmers adopted this fish in early nineties without any knowledge on the production technology due to its higher muscle mass and faster growth rate. Although these exotic species and GMOs are considered to have possible ecological risks in new environment and can be allergenic to consumers (Baruah *et al.* 1999) [3]. In this aspect *N*-ethyl-*N*-nitrosourea (ENU) can act as a mutagenic agent to apply TILLING technology (Kennedy & O'Bryan 2006; Kuroyanagi *et al.* 2013)<sup>[6, 7]</sup> for breeding practices to produce useful phenotypes such as rapid growth, increased muscle mass and disease resistance in *Clarias batrachus*. Hence to apply this mutagen we must first determine the proper concentration of application and this study is the first report which showed increasing concentration decrease the fertilization and hatching ratio along with deformities of egg and larvae.

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