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Zinc Sulphate Induced Micronucleus Test and Mitotic Index on Channa punctatus in vivo

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ABSTRACT *Zinc plays an essential role in wide range of cellular process,including defence against free redicals and mantaining genomic stability of aquatic fauna. In the present study, we analysed the induced mitotic index of dividing cells of kidney and micronuclei in peripheral erythrocytes of Channa punctatus following in vivo exposure of three different concentrations ZnSO4.7H2O. Our result revealed that Zinc induced significantly high incidences of micronuclei in peripheral erythrocytes, and inhibited mitotic index and caused considerable delay in the generation time of kidney cells in treated organisms. .Time and concentration related response of the chemical as observed in this study clearly indicated the genotoxic nature of Zinc Sulphate significantly i.e. ($p \leq 0.05$ to $p \leq 0.01$).*

KEYWORDS : ZnSO4, Genotoxic, MicronucleusTest, Mitotic index, Fish, Cytotoxic etc.

INTRODUCTION

Majority of the metals and their compounds interfere with protein/enzyme synthesis and thus may effect cell division and the nature and behaviour of the genetic apparatus(Sharma and Talukdar,1987).Compounds of some metals like cobalt, mercury, lead, Zinc and silver Chloride have induce tumours in experimental animals(Sharma and Talukdar ,1987; Nriagu, 1988). The DNA damaging property and mutagenic activity of atleast 170 metal compounds have been confirmed using various testing protocols(kanematsu et al.,1980;Hansen and stern, 1984;Leonard, 1988; Christie et. al.,1990;Garcia-Medinaet.al., 2011).

Genotoxic studies conducted in a variety of test system have failed to provide evidence for mutagenicity of zinc .However there are indications of weak clastogenic effects following Zinc exposoure (ATSDR,1990).Comperative study of single and joint action of copper and Zinc to *Synodontis clarias* and *Tilapia nilotica*(Obiokar et. al.,2010) shows significance frequency of micronuclei in all having non dose dependence.Cytogenetic and genotoxic effects of Zinc oxide nanopaticles on root cells of Allium cepa (Mamta kumara et. al., 2011) demonstrated that with the increase of pycnotic cells on the other hand MN and chromosomal aberrations index increased i.e demonstrated Zn NPS are clastogenic/genotoxic and cytotoxic agents.

Zinc supplementation is a factor,decreasing erythrocytes and hematocrit ,and increasing the platelets count in rats (Dimitrova et .al. 2010)

As to its toxicity to fish,Zinc is of middle streangth less toxic than mercury,copper and cadmium and more toxic than the nickel and lead.Its toxicity is expressed in morpho functional damage of respiratory organs(Moore Ramamurty,1997 velcheva et al ,2010) skin (Hemalata and Bannerjee 1997) and kidney (Haschek et al 2002;woodling et al.;2001)

Zinc is necessary microelement the sub lethal concentration of this metal changes biochemical parameters in fish organism which can lead to a change of the normal cell function (Gioda et al;2007,Tyagi and Srivastava,2005).Although Zinc cytotoxicity is related to the inhibition of the DNA and RNA synthesis which ,in turn jeoparadises the protein metabolism in the cell and ca++ antagonism (Walter et al;2003).

Zinc plays a central role in cellular growth and differentiation and the effects of its differentiation and the effects of its deficiency are especially pronounced in tissue and organs with a rapid turn over including immune system an during period of rapid growth,in both pre and post natality (Varin etal 2008).Zinc may be an increasing environmental problem in this sense that it is found in most vitamins and minerals supplement and various easily accessible products,including cold lozenges an increasing number of humans using dietery supplements on a regular basis(Hiaase et al;2008). dietery Zinc is generally considered to reduce the risk of cancer,but zinc supplements have been correlated with increasing risk of cancer(Grant 2008).

Genotoxicity of a compound ,usually expressed as its ability to induce DNA damage ,is often essential for mutagenesis and cancer transformation Mekinnon and Caldecott 2007). In this context zinc plays central role(Sliwinski et al ,2009) suggested that zinc may protect normal cell against DNA damaging action and increase this action in cancer cells, which indicate the dual action of this element independently of targetcell and can be useful in cancer therapy.

Due to increasing environmental exposure to heavy metals, the need for biomonitoreeing of Zinc using sensitive genetic marker becomes parmount to forestall possible out breakof congential and genetic anomalies and disease,since the freshwater fish has recently occupied considerable position in local food menue.

The exact reason for such anomalous relationship between mutagenic and carcinogenic properties of metal compounds is difficult to suggest. In fact, there exists several possibilities. First, not all metals may really have both mutagenic as wellas carcinogenic properties. Secondly,it is possible that the carcinogenic effects of certain metal compounds may be due to the activity of their ingradients rather than metals may be due to the activity of their ingredients rathe than metals themselves(Kanematsu etal.198). Yet another possibility is that certain metals may not act directly on DNA but may cause DNA alterations because of infidelity of DNA polymerases owing to metal co-factor(Christie and Katsifis.1990).

During the last few years,fish as an vivo cytogenetic test model , has attracted the attention of quite a good number of workers for determining the mutagenic and carcinogenic potential of genetically active substances present in aquatic environment. In fact, a number of studies using fish as attest model have shown that fish can faith fully be utilised for the analysis of sister chromatid exchanges (Kligerman,1982;mohanty and Prasad ,1982) induction of micronuclei in peripheral erythrocytes (Hooftman and de Raat,1982;Manna et al;1985; sadhukhan and manna,1986;Das and Nanda,1986; Panda,1993; porichha etal,1998; Sarangi et al,2001; Choudhury et al,2006). Sperm head abnormality(Manna and Biswas,1988) and induction of lethal mutation (Manna and sadhukhan,1986) besides the usual chromosomal aberration test (Manna and Mukherjee,1989). Although the proyocol of various short term tests are yet to be standardised (carrasco et al.1990) the results obtained with some mutagen have indicated that fish may prove a very rewarding test model.

In the present study, We have ,therefore, used a commonly

occurring indigenous live fish, Channa punctatus to analyse the genotoxic potential of Zinc sulphate with the following aims(1) to compare the genotoxic effects of Zinc sulphate with those obtained in other test models and (2) to assess the further suitability of micronucleus test for biological monitoring of environmental genotoxicants. In addition, efforts have also been made in this study, to see if zinc sulphate has mito depression property as is the case with most metal compound examined so far (Sharma and Talukdar, 1987; Panda et al, 1992).

MATERIAL METHODS

Test animal:

Specimens of Channa punctatus (Bloch), measuring about 5-6 inches constitute the material source of this study. It is a very common Indian Live –fish, available all the year round in pools and water – logged marshy areas. It is very hardy and has accessory air –breathing organs and hence can survive for months in laboratory aquaria on limited ration. Moreover, it has a reasonably good mitotic index as compered to the other fresh water fish. All these features favour the use of this fish as an in vivo test model in studies relating cytogenetic effects of physical and chemical genotoxicants present in our aquatic environment.

Source of collection:

Specimens used in this study were procured from local market and maintained in laboratory aquaria at least for a week for acclimatization .Only strong and active individuals were selected and allocated at random to various groups of treatment.

Test chemical :

Analytical grade of zinc sulphate (Zn So4 .7H2O) served as test chemical. It is a compound of divalent metal which forms tetrahydral Co-ordination complexes with a Co-ordination number 4. Likes most Zinc salts it is readily soluble in water and under goes partial hdrolysation.It can replace isomorphously many and certain biological molecules.

Dose and mode of treatment:

Three concentrations of the chemical 100 ppm, 50 ppm and 20ppm solutions which correspond respectively to MC/2 and M/C5 were tested. MC is the maximum tolerable concentration of the test compound at which no death of animals beyond 10% was observed during the period of treatment. MC/2 is half and MC/5 is one fifth of the maximum tolerable concentration. The MC was determined and the test lasted for 120 hr with change of water, chemical and food every day. During 120 hr of treatment, the lowest concentration leading to maximum of 50% death or reduction food in take was noted. All specimens were given intramuscular injection of the chemical at the rate 1 ml/100g of body weight and sacrificed at intervals of 24 hr., in a120hr.experiment series. The controls received intramuscular injection of an equal amount of distilled water .Both treated and control specimens were supplied with the same amount of diet containing earthworms, chopped goat liver and rice bran. Preparations were made from at least five specimens for each concentration and sampling hours.

Methods of preparation and analysis of micronucleusTest:

The fish were cut in the caudal region and smears of peripheral blood were made on grease free clean slides after mixing with a drop of 0.1 %tri sodium citrate solution. The thus prepared smear was dried rapidly by waving in the air and kept for at least 1 hour in dust and moisture free conditions; I ml of 0.15% Leishman`s stain solution (prepared by dissolving 150 mg of Leishman`s powder in 100 ml of methyl alcohol) was poured on to the slides which were kept for 90 minutes in a closed chamber; A double amount of distilled water (neutral pH) was then added to each slide and mixed thoroughly with the aid of a Pasteur pipette; The slides were kept in this condition for 5 minutes in a covered container followed by washing with distilled water of neutral pH; The slides were then blotted dry with filter paper, kept in xylene for 5 minutes and finally mounted in DPX for observations. In addition to the method described above, some representative slides were also stained in feulgen instead of leishmn`s stain The procedure adopted is as: Air-dried smears were rensed in cold 1N Hcl; They were then hydrolysed in 1N HCl for 10 minutes at $50^0$c, rinsed in cold 1N HCl followed by another rinse in distilled water of neutral pH;The slides were then transferred to Schiff`s reagent (Feulgen solution) and kept for 1 hour in dark;The slides were then rinsed in distilled water (neutral pH) and stained in 1% aqueous light green solution for one minute, washed, dehydrated and mounted in DPX

Method of cytological preparations for mitotic index:

Kidney in fish per se serves as haemopoetic organ and is known to possess fairly large number of dividing cells. For the analysis of mitostatic effect of the test chemicals therefore the kidney of the control as well as treated specimens were individually collected in 0.56%potassium dichloride solution, minced and flushed repeatedly to obtain a thin suspension. After about 30 minutes, the cell suspension in KCl solution was centrifused at 1500 RPM for 15 minutes, the supernatant was discarded and the residual material was fixed in aceto alcohol mixture (1:3) for at least 45 minutes with two changes in the fixative..The thin suspended cells in aceto-alcohol mixture were dropped on to Chilled slides (stored in 50% alcohol in freezer) and flame dried. The dried slides were stained in 2% Giemsa (stock solution diluted with Sorensen`s buffer at pH 6.8), the superfluous stain was washed in tap water and dried in air.

Methods of observations and statistical Analysis:

All slides were coded and randomized before observations. From each specimen 4000 erythrocytes (1000 per slide) were scored under (100 x) oil immersion objective. Following the protocol Fenech et al (2003).The diameter of the micronucleus (MN) showed be less than one third of the main nucleus; MN showed be separated from or marginally overlap with main nucleus as long as there is clear identification of the boundary; the non-refractile particles which resembled with main nucleus in all respects were considered to be micronuclei.

The mean frequency of micronuclei and standard deviation for each group were calculated. The data obtained were subjected to student`t`test to calculate the significance of treated values over control. In addition a one way analysis of variance (ANOVA) test was also applied for time-responsive studies; as per the formula.

RESULTS

MICRONUCLEUS TEST

The erythrocytes of *channa* punctatus, like those of other fish, are fairly large and have centrally placed round nuclei and sizeable cytoplasm. The nucleus to cytoplasm ratio is 1:6.These features facilitated easy scoring of the MN. The size as well as the location of micronucleus with in the cytoplasm varied from cell to cell but the shape was round in almost all the cells. Each affected erythrocyte contained one very small dot–like micronucleus (MN) placed very close to the main nucleus (Figs.1-3).The frequency of cell with more than one, micronucleus was very rare. In addition to the incidence of MN, a few erythrocytes exhibited different kinds of nuclear anomalies. For example while in some cells micronucleus was connected to the main nucleus by a chromatin bridge thus giving the appearance of a conically projected nucleus, others had a bilobed nucleus or a normal nucleus with a notch in its body (Fig.4).such anomalies were not included in preparations from control specimens and hence were not included in quantitative analysis. Fig.5 shows the incidence of MN in the peripheral erythrocytes of *Channa* punctatus following intramuscular injection with different concentrations of Zinc Sulphate and in controls. Evidently, the peripheral erythrocytes of the treated specimens from all sampling hours have relatively higher frequency increases with the increase in the dose and duration of treatment. While the frequency ranges from 0.04 to 0.07 in controls, it varies from 0.07 to 0.15, from 0.09 to 0.20 and from 0.14 to 0.34 respectively in specimens injected with 20, 50 and 100 ppm solution of Zinc Sulphate at the rate of 1ml per 100g body weight (table-1) statistical

analysis of the data, however, reveals that the increase in the frequency is significant only in the specimens injected with 100ppm solution. Multiple variance analysis reveals that Zinc Sulphate induces micronuclei in dose dependent manner. The calculated F value for dose (F=17.966; df=3.12; p 0.05) is greater than the tabulated value.

MITOTIC INDEX

The effect on Zinc Sulphate on cell division is slightly more pronounced. As can be judged from the histogram of mitotic index (Fig.6), there is a distinct decrease in the frequency of dividing cells in kidney of all specimens from treated series as compared to the controls. The frequency of dividing cells ranges from 1.99 to 1.89, from 1.94 to1.74 and from 1.84 to1.62 in specimens injected with 20, 50 and 100 ppm solution at the rate of 1ml per 100g body weight while it is varies between 2.04 and 2.01 in controls (Table-2).Statically, the decrease in the frequency is not only significant in the specimens injected with 100 ppm solution but also in specimens sacrificed at 96 and 120 hour following injection with 50 ppm solution. Multiple variance analysis reveals that the effect of zinc sulphate on the mitotic index is dose but not period dependent. The calculated F value for dose (F=17.025; df3.12; p>0.05) is greater than the tabulated

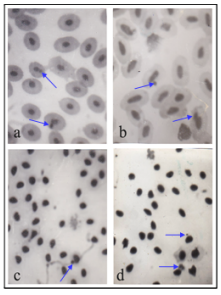


Fig 1 : a, b and c show single peripheral erythrocyte micronuleus in *Channa punctatus*, treated with $ZnSO_4$. Fig d shows peripheral erythrocytes of *Chana punctatus* with single bilobed or notched micronuclei

TABLE -1 Frequency of micronuclei in peripheral erythrocytes of Channa punctatus following their exposure to different concentrations of ZnSO4 for varying period of time

Period of exposure	Controls	Treated micronucleus series		
		MC/5	MC/2	MC
24h	0.04 ± 0.02	0.07 ± 0.04	0.09 ± 0.05	0.14 ± 0.06a
48h	0.05 ± 0.03	0.09 ± 0.05	0.12 ± 0.07	0.18 ± 0.07a
72h	0.05 ± 0.04	0.10 ± 0.05	0.13 ± 0.06	0.24 ± 0.09a
96h	0.06 ± 0.04	0.12 ± 0.06	0.15 ± 0.07	0.29 ± 0.11b
120h	0.07 ± 0.05	0.15 ± 0.07	0.20 ± 0.10	0.34 ± 0.10b

TABLE-2 Frequency of mitotically dividing cells in kidney of Channa punctatus following their exposure to different concentrations of ZnSO4 for varying period of time

Period of exposure	Controls	Treated micronucleus series		
		MC/5	MC/2	MC
24h	2.04 ± 0.07	1.99 ± 0.08	1.94 ± 0.08	1.84 ± 0.11a
48h	2.01 ± 0.08	1.96 ± 0.07	1.89 ± 0.09	1.78 ± 0.10a
72h	2.02 ± 0.08	1.94 ± 0.09	1.86 ± 0.11	1.74 ± 0.12a
96h	2.00 ± 0.09	1.91 ± 0.10	1.80 ± 0.11a	1.69 ± 0.12b
120h	2.01 ± 0.07	1.89 ± 0.08	1.73 ± 0.12a	1.62 ± 0.15b

Fig.2- Incidence of micronuclei of peripheral erythrocytes of Channa punctatus exposed to different concentrations of Zinc Sulphate for varying periods of time and in control

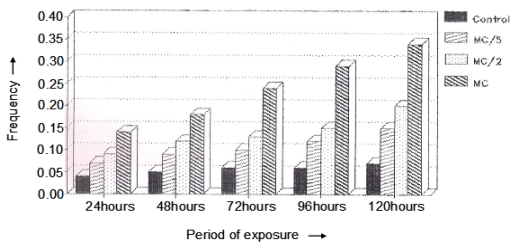
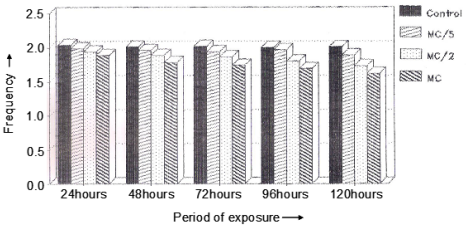


Fig.3- Frequency of Mitotic index in kidney cells of Channa punctatus exposed to different concentrations of Zinc Sulphate for varying periods of time and in control



DISCUSSION

Calesnick and Diman 1988). Zinc reduced the extent of DNA damage induced by H^2O^2 normal human Lymphocyte (Sliwinski et al 2009) It can play an important role into the inducing and progression of prostate cancer and malignant cells to apoptosis induced by cytotoxic agents (Costello and Franklin 1988; 2000; yanet al 2008)The genotoxic (Mutegencicity, conrcinicity and teratogencity) properties of zinc compounds have been reviewed by Hansen and Stern (1984) and Leonard et al.. (1986).Many earlier authors in different parts of the world (vosyliene and svecevicius,1995,vosy line et al 2003 Kazlauskine et al,2003, Kazlauskiene and Burba 1997,otitoloju2001,oyewo,1998) have similarly on served and recoded differential toxicity of heavy metals compounds against different test systems

In vitro genotoxicity tests have yielded contradictory results. Kalinina et al. (1977) reported zinc chloride (Zncl2) to be a frame shift mutagen in Salmonella assay whereas in the rac assay with Bacillus subtills it has been found to be inactive (Nisioka, 1975, Kanematsu et al.1980). In mammalian cells in culture Zncl2 has been found to be a weak clastogen in human lymphocytes (Deknudt and Deminati 1978) but did not induce mutation at the thymidne kinase locus in L 5178 Y mouse lymphoma cells (Amcher and Paillet, 1980). In vivo genotoxicity studies with zinc hav generally yielded negative results. Duknudt and Gerber (1979). However, have shown that Zncl2 and induce chromosome aberration in bone marrow cells of calcium deficient mice but not in mice fed with standard diet. Moutchen-Dahmen (1963) observed that treatment with Zncl2 enhanced the yield of chromosome aberrations induced by Ethyle-methane sulphonate. Similarly, zinc sulphate has been found to produce no genotoxic effect when fed to whole animal. It has also been found to give negative result in salmonella test, Drosophila sex linked recessive lethal test and bone marrow micronucleus assay in mice (Gocke et al., 1981).

Cytogenetic and genotoxic effect of zinc oxide nanoparticles on root cells of Allium cepa (mamta kumari etal 2011) observed that with the increasing concentrations of ZnO Nano particles Mitotic Index deceased with the increase of pycnotic cells. On the othenhand MN and chromosomal aberration index in creased. Sadhukhan (1990), on the other hand, observed elevated rate of micronucleus in both peripheral blood and kidney cells of fish treated with 1 ml of 150 ppm of ZnSo4 solution. Genotoxicity of ZnSo4 has also been demonstrated using Drosophila wing Indian cat fish (Heteropneustes fossilis)exposed to mitomycin c and paper mill effluent (Das and Nanda,1986) andSpot test (Anand Kumar, 1993). Zinc at higher concentration can be (welkell et al 1986 uobet et.al.1988. cales nick and diman 1988).In the present study, the inciduence of the particles notes as MN in the cytoplasm of the blood cells in zinc sulphate treated fish looked like chromatin bodies so far as the staining behaviour is concerned and were Feulgen positive. Moreover, the incidence of such particles were very low in control specimens. These facts clearly indicate that the particules were of chromosomal origin and had certainly been induced by Zinc Sulphate.

Further Zn can act as aneugen. Hartwel et al. 2000, obiakar etay 2010) resulting micronucleus formatron and / or can result in chromosomal reanangement (Hartwall et al 2000) Thus, results obtained in this study are identical to those obtains in peripheral

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blood of Eastern mudminnow, (Umbra pygmea) exposed to ethylmethane sulphonate (a well known mutagen clastogen) contaminated water (Hooftman and deraat 1982) certain pesticides (Manna and Biswas, 1986). Thus our results support the view that the microneucleated erythrocytes in fish, as in mammals Hayashi et al., 1988, Pati and Bhunya, 1989: Dhingra et al., 1990), do, in fact, enter the peripheral circulation and persist there atleast for certain period of time.With in vivo test systems most workers in the past have used a double or multiple injections or treatment with the agent in studies relating induction of micronulei. In the present study, however, the chemical was injected only once instead of twice or thrice. Nevertheless, the micronucleated cells were found to persist in animals sacrificed every after 120h of exposure. This indicates that the rate of elimination of the agent or its metabolites in very slow. In other words, zinc sulplate has relatively longer half-life as compared to other heavy metals. Another reason which could be attributed to the persistence of MN for longer duration may be that the disintegration of the chromatin fragment in affected cells in fish is not as fast as is the case with other in vivo systems, especially mammals. Equally important reason may be a very low of metabolic activity of fish per se. the fact that Mohanty as Prasad (1982) observed quite a good number of sister chromatid differentially stained metaphases in Channa punctuatus ever after 3 days of exposure to single injection of BrdU at the rate of 50mg of body weight strongly suggests that the later possibility is the most likely an explanation. Whatever may be the reason, the present study clearly indicate the suitability of assessing the peripheral blood of fish for the induction of MN as a short term test for bio-monitoring of environmental genotoxicants (*clastogens and/ or spindle poisons*) including waterborne ones, as also suggested by some previous workers (Hooftman and Raat, 1982; Das and Nanda, 1986, Manna and Biswas, 1986)

Production of MN by an agent has been attributed to its clastogenic or spindle poisoning effect or both (Heddle, 1973; Schmid, 1976), Higher incidence of MN in erythrocytes of the specimens exposed to zinc sulphate at all the sampling hours coupled with a clear –cut dose related response clearly demonstrates its genotoxic nature. Further support in favour of genotoxic potential of zinc sulphate comes from our data on mitotic index. In fact, we observed relatively low rate of mitotically dividing cells in kidney of all specimens exposed to different concentration of zinc slphate. Furthermore, mitotic index in the treated series decreased with increase in the dose of duration. The present investigation, however, suffers from the limitation of not having data on chromosome aberrations and hence we are not in a position to suggest whether the induction MN in treated series was due to its clastogenic or spindle poisoning effect. However, dose related response as well as progressive increasing in the frequency of micronucleated cells (MNC) with concurrent increase in the exposure time with no sign of recovery upto 120 hours of the treatment as observed in this study rules out the possibility of the impairment of spindle apparatus. They also suggest that micronucleus producing mode action of Zinc Sulphate could probably be an expression of its clastogenic action.

As pointed out earlier, the results of short terms mutagencity assays for zinc are equivocal. The exact reason for the conflicting results is not well understood. It is possible

That difference in the results obtained by different workers may be due to the different form of zinc tested and the possible interaction between medium compounds and the zinc form used. For instance, when inorganic zinc i.e. Zncl2 or ZnsO4 is added to growth medium. The zinc is completely dissociated and numerous constitutents of the medium, especially serum proteins and capable of binding free Zinc (Parisi and Vallee, 1970). Thus inorganic Zinc may be bound to the medium component and the unavailable for transport into the cell. On the other hand, when Zinc is tested as an organic complex, there exists a competition between that complex and medium constituents for the Zinc. If the organic compound bind Zinc with a higher affinity than the medium components. The Zinc may transported as organic complex. Subsequent metabolism within the cell would than release Zinc. Thus the presence or absence of a mutagenic response in a short term assay would depend on the type of medium, the type of cell and the binding constant of the metal complex.

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