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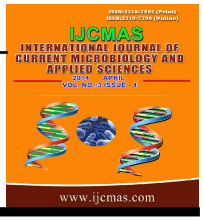

Original Research Article

Acute Effect of Mercury and Zinc Chloride on Gastrin/CCK 8 Neuropeptide in relation with behavior of freshwater snail *Bellamya bengalensis*: a microscopic study

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A B S T R A C T

Gastrin / CCK 8 nuroprtide play important role to carry out some physiological process in vertebrate and invertebrate. In the present investigation we carried out the different behavior of animal in relation with gastrin / CCK 8 immunoreactivity in the cerebral ganglia of freshwater snail *Bellamya bengalensis* against the toxicity of $HgCl_2$ and $ZnCl_2$. The immunoreactity of gastrin / CCK 8 was increased as per exposure period of $HgCl_2$ and it constant in $ZnCl_2$ exposure as compared with control. The obtained result suggests that the toxicity of $HgCl_2$ was responsible for the cellular pathology and enhancing the gastrin / CCK 8 immunoreactivity than the $ZnCl_2$. Arrested neuronal gastrin / CCK 8 was responsible for alter the behavior of *Bellamya bengalensis.* In conclusion the toxicity of $HgCl_2$ found more in relation with behavior and immunoreactivity of gastrin / CCK 8 as compared with $ZnCl_2$.

Keywords

$HgCl_2$ and $ZnCl_2$, Gastrin/ CCK8, Behavior, *B.bengalensis*

Introduction

Gastrin / CCK are first described as gastrointestinal hormone and subsequently shown to be neuropeptide as well (Rehfield, 1981). Duve and Thorpe (1984) reported gastrin and cholecystokinin (CCK) are peptides of the mammalian gastrointestinal tract, found structurally and functionally identical with COOH-terminal pentapeptid. In vertebrate, the peptides of gastrin and CCK were known for excitation and conduction of smooth muscle contraction and also involved in secretion of digestive enzymes (Janssen, 2008). Dufresne et al (2006) and Konturek et al (2003) reported Gastrin / CCK peptides are promoters for satiety centers in feeding mechanism. Thorndyke (1982), documented distribution of gastrin / CCK peptide in neurons of CG in Protochordates *Styela clava* and *Ascidiella aspersa.* He observed peripherally arranged immunoreactive fibers in CG. Pestarino (1985), investigated gastrin / CCK 8 immunoreactivity in neurons and nerve fiber (glandular lobes and in neuronal glands) of ascidian Protochordates *Styela plicata.* Most of the invertebrates are studied for observation of

gastrin / CCK peptides. Larson and Vigne (1983), initialy reported presence of gastrin / CCK peptides in the Arthropodan, A*plycia callifornica* and in the *Annelid luricus*. Similarly Bernocchi et al (1998), performed immunocytochemistry of gastrin / CCK 8 in active and hibernating snail *Helix aspersa* and found immunoreactivity in neurons of procerebrum, mesocererum and neuropil region.

According to Vigna et al (1984), Gesser and Larsson (1985), Osborne (1987) and Sonetti et al (1990) in the gastropod molluscs, gastrin / CCK 8 peptides involved in integrated sensory functions, as in the mechanism of feeding, neurosecretion, sensory and neuroendocrine communications among the cell . Gomot de et al (2000) documented that, molluscs are sentinels in ecological risk assessments for metal pollution. Neurotoxicity resulted to morphological and metabolic defects in cells (Hernadi et al., 1992; Boer et al., 1995; Wieman et al., 1996). In the metal inorganic mercury ($HgCl_2$) found neurotoxic agent (Hare et al., 1990; Umbach and Gundersen 1989).

The aim of this study was to investigate behaFvioral response of freshwater snail *B. bengalensis* in relation with immunoreactivity of neuropeptides gastrin / CCK 8 after acute induction of $HgCl_2$, and $ZnCl_2$.

Materials and Methods

Experimental animal

Adult snails *Bellamya bengalensis* (4-5 cm L, 2 - 3 cm W, 3-4 g Wt) were collected from 'Rajaram tank' near Shivaji University, Kolhapur, Maharashtra, India. They were brought to the laboratory using plastic bottles and kept for acclimatization for a weak.

Immunohistochemical study

Experimental setup

Healthy, adult acclimatized snails (4-5 cm L, 2 - 3 cm W, 3-4 g Wt) were selected for experiment. Experimental snails were divided into 5 sets (10 animals per set) as control group, set-I (intoxicated up to 24 hr), set-II (intoxicated up to 48 hr), set-III (intoxicated up to 72 hr) and set- IV (intoxicated up to 96 hr). Mean LC_{50} concentration of $HgCl_2$ (1.56 ppm) were used for animal's intoxication and the LC_{50} concentration of $ZnCl_2$ (12.70 ppm) used as a negative control. After completion of each exposure period animals were sacrifised for nervous tissue and fixed in Bouins solution for 6 - 7 hr. Fixed tissues were washed with 70 % ethanol for three days, dehydrated through ethanol-graded series, cleared in xylene and embedded in paraplast.

Serial sections (8 μm thick) of targeted tissues were simultaneously processed for immunohistochemical assay as per (Pisu et al.,,,, 2000). Sections were incubated in (3%) H_2O_2 with 10 % methanol in phosphate buffer for 7 min and 5 % of Normal Goat Serum (NGS) (GeNei Bangalore, India) for 45 min. Sections were incubated for 18 h at 4˚C in 1:500 rabbit polyclonal antibody against gastrin / CCK 8 (GeNei Bangalore, India) in 1% NGS (GeNei, Bangalore, India) and 1:50 goat antirabbit IgG (Catlog # ASIM, GeNei, Bangalore, India) for 90 min. Perioxidase – antiperiodase method by Sternberger (1970) was used for to detect the antigen antibody reactions in the neurons, it includes 1:100 rabbit PAP for

60 min and then 3,3 – diaminobenzidine tetrahydrochloride (C_{12} H_{14} N_4, 4HCl Himedia) in 0.05 M Tris / HCl buffer, pH 7.6 containing 0.01% H_2O_2. After completion sections were dehydrated in alcoholic series, cleared in xylene and mounted in fluromount. For control staining, sections were incubated in NGS instead of primary antibody with same dilution. Immunostaining was not seen in these sections.

Microscopic observation

The score of immunostained neurons form CG were assessed as Strong intensity - +++, Moderate intensity - ++, Pale / light intensity - +. The counting was done in 5 serial sections per 5 animals in each of the experimental setup (24 h, 48 h, 72 h and 96 h) under the light microscopes. Photography was carried out by Inverted and phase contrast microscope, Lawrence and Mayo. Percentage of immunostained neurons was assessed (mean ± SD) from pro-c, meso-c and meta-c in both sides of CG.

Statistical analysis

Statistical analysis was carried out by using Probit analysis method (Finney, 1971) and Graph pad InStat program.

Results and Discussion

$HgCl_2$ treated group Gastrin / CCK 8 immuno reactivity in CG of control group

The distribution of gastrin / CCK 8 in control sections were diagrammatically represented in PLATE – Fig - 2 A. Control group of animals in $HgCl_2$ set showed positive immunostaining against gastrin / CCK 8 antiserum. The obtained as, 11.56 % immunostained neurons from pro-c, 11.74 form meso-c and 10.3 from meta-c (Fig. 1 E- G). The large, small and medium sized neurons were immunoreactive for gastrin / CCK 8. In control staining, immunoreactivity was not found in the sections of pro-c, meso-c and meta-c in *B. bengalensis* (Fig. 1 B - D) (Fig. 2).

$HgCl_2$ induced gastrin / CCK 8 immunoreactivity in CG

Cerebral neurons of *B. bengalensis* found immunostained and increased their reactivity as per exposure period. The distribution of these increased immunoreactive neurons as per exposure periods were diagrammatically shown in Fig. 3 A - D). After 24 hr, 16. 94% neurons were intensive and moderately immunostained form pro-c, whereas neurons of meso-c and meta-c showed slightly increased immunoreactivity as compared with control (12.9 % and 11.42 % of neurons) (Fig. 4 A - C). After 48 hr, gastrin / CCK 8 immunoreactivity in pro-c and meta-c found significantly enhanced as compared with meso-c (Fig. 4 D - F). Immunoreactivity was prominent after 72 hr, it includes 34.96% from pro-c, 26.14% in meso-c and 21.62% meta-c (Fig. 4 G-I). Almost 60 % CG neurons were positively immunostained after 96 h. Pro-c has 60% immunostained neurons, whereas meso-c were 42.7% and meta-c 21.22 (Fig. 4 J- L). The data of percent immunoreactivity in control and $HgCl_2$ induced CG was expressed in Fig. 2.

$ZnCl_2$ treated group
Gastrin / CCK 8 immunoreactivity in CG of control group

In $ZnCl_2$ control group, scattered immunoreactive neurons were observed in

CG of *B. bengalensis* (Fig. 5 A), 11.84% of neurons from pro-c, 11.28% neurons of meso-c and 9.54% from meta-c were immunostained (Fig. 5 E - G). Figure - 5 B – D Immunoreactivity was not found in pro-c, meso-c and meta-c incubated in NGS instead of primary antibody (Fig. 6).

$ZnCl_2$ induced gastrin / CCK 8 immunoreactivity in CG

The distribution of immunostained neurons after 24, 48, 72 and 96 hr from cerebral ganglia were diagrammatically shown in Fig. 7 A-D. Immunoreactivity of gastrin / CCK 8 was found slightly increased after 24 hr of exposure in CG (Fig. 8 A- C). After 48 and 72 hr, moderately increased immunoreactivity found, it includes 13.44% and 14.54% form pro-c, 13% and 13.88% from meso-c respectively (Fig. 8 D - I). After 96 hr, moderately increased immunostaining were observed in pro-c, meso-c and meta-c, it includes 13.8 %, 15.26% and 10.58% respectively (Fig. 8 J – L). The alterations in gastrin / CCK 8 immunoreactivity of cerebral neurons in control and ZnCl2 induced were graphically represented in Figure – 6.

Comparative account of gastrin / CCK 8 immunoreactivity in CG of *B.bengalensis* after exposure of $HgCl_2$ and $ZnCl_2$

At comparative level, gastrin / CCK 8 immunoreactivity of neurons were enhanced in intoxication of $HgCl_2$ as compared to $ZnCl_2$. After exposure of $HgCl_2$, almost 60 % cells were immunostained for gastrin / CCK 8 in different regions of CG in freshwater snail *B. bengalensis*. In $ZnCl_2$ induced CG gastrin / CCK 8 immunoreactivity of neurons were less as compared to $HgCl_2$ treated CG. The immunoreactivity of neurons from CG for gastrin / CCK 8 found increased as per exposure period. The score of staining intensity of immunoreactive neurons from different exposure period were documented in Table – 1.

The present findings confirm our previous demonstration of Gastrin / CCK8 immunoreactivity in CG of molluscs and further demonstrate the neuronal reactivity of gastrin / CCK 8 was increased after exposure of $HgCl_2$ than the $ZnCl_2$ (Londhe and Kamble 2013).

In the present study, distribution of gastrin / CCK 8 peptide within neurons of different parts of Cg i.e. pro-c, meso-c and meta-c in freshwater snail *B. bengalensis* were observed. Similarly Osborne (1982), reported immunoreactivity of CCK in the meta-c of CG in snail *Helix aspersa*. Pisu et al (2000) documented, gastrin / CCK 8 immunoreactivity in the CG and buccal ganglia (BG) of snail *Helix aspersa.* Duve and Thorpe (1984), reported gastrin / CCK 8 immunolabeled neurons and axonal processes of neuropil in brain and thoracic ganglia of blowfly in *Calliphera vomitera*. We found, different types of neurons were involved in the immunostaining against vertebrate antiserum of reactively gastrin / CCK 8 from pro-c, meao-c and meta-c in *B. bengalensis.* Schalze et al., (1975) and Altrup (1987), noticed immunoreactivity in large and giant neurons of CNS in *Helix pomatia* reacted with antiserum of gastrin cholecystokinin. Pisu et al (2000), reported less gastrin / CCK 8 immunoreactivity in medium and small sized neurons of meso-c and meta-c of CG in fully active snail *Helix aspersa.* Among control group, meso-c neurons in CG of all experimental animals involved in gastrin / CCK 8 immunoreactivity. Comparatively, pro-c

Fig.1 Distribution of gastrin / CCK 8 immunoreactive neurons in the CG of *B. bengalensis* form $HgCl_2$ control group. **A**- Diagrammatic presentation of immunoreactive neurons for gastrin / CCK 8 in different regions in CG. LCG – left cerebral ganglia, RCG- Right cerebral ganglia, Pro-c- procerebrum, Meso-c- mesocerebrum, Meta-c – metacerebrum, ○- negative neurons, ● –moderately immunoreactive neurons, ● - strongly immunoreactive neurons, **B – D** Sectional view showed no immunoreactivity in pro-c, meso-c and meta-c sections, 400X. **E - G** Gastrin / CCK 8 immunoreactivity in pro-c, meso-c and meta-c of CG in control group, 400X.

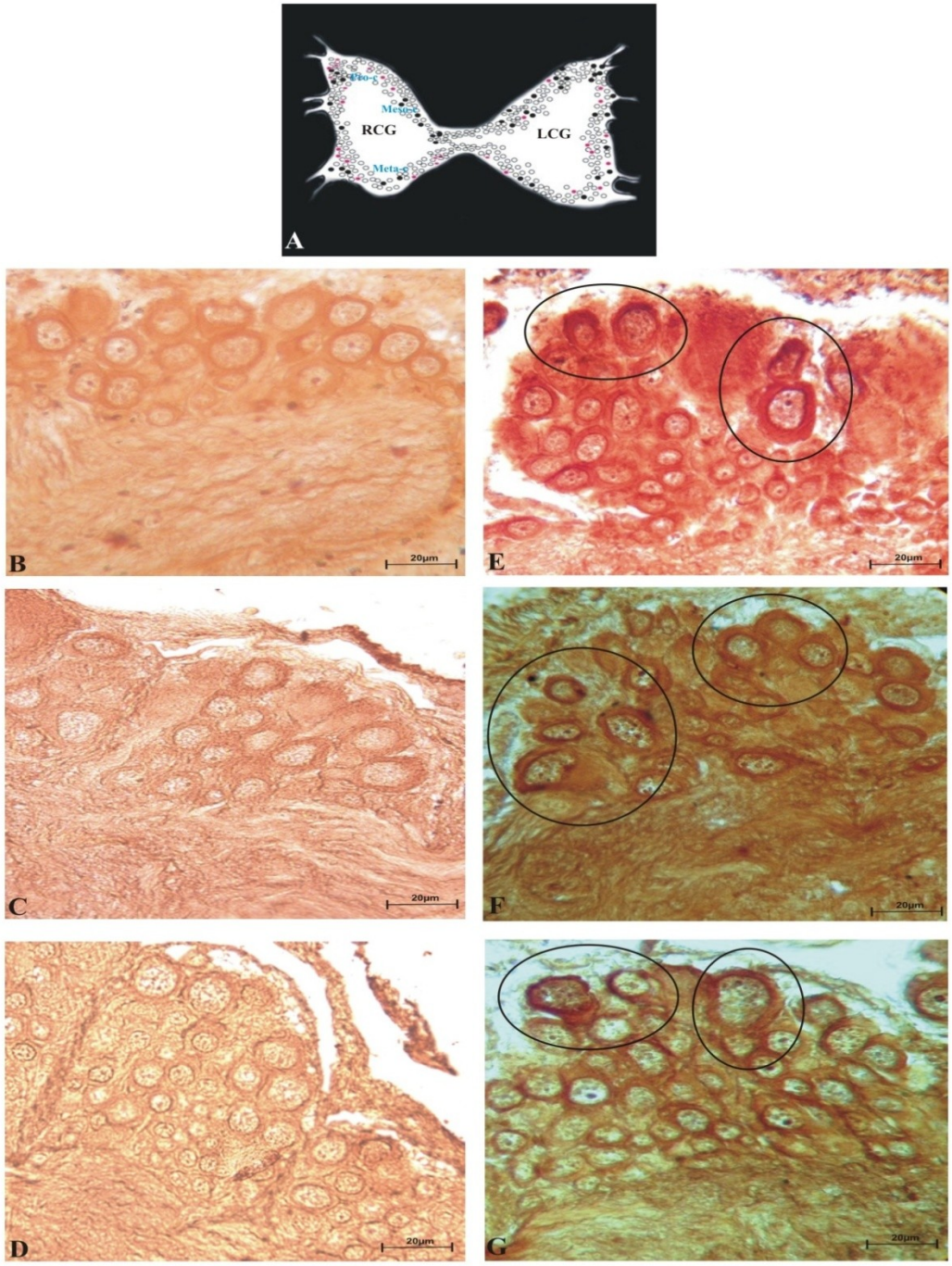

Fig.2 $HgCl_2$ induced gastrin / CCK8 immunoreactivity in cerebral regions of *B. bengalensis*. *** - P<0.001(Significant), **- P< 0.01 (Less significant) and NS - P<0.05 (Non significant).

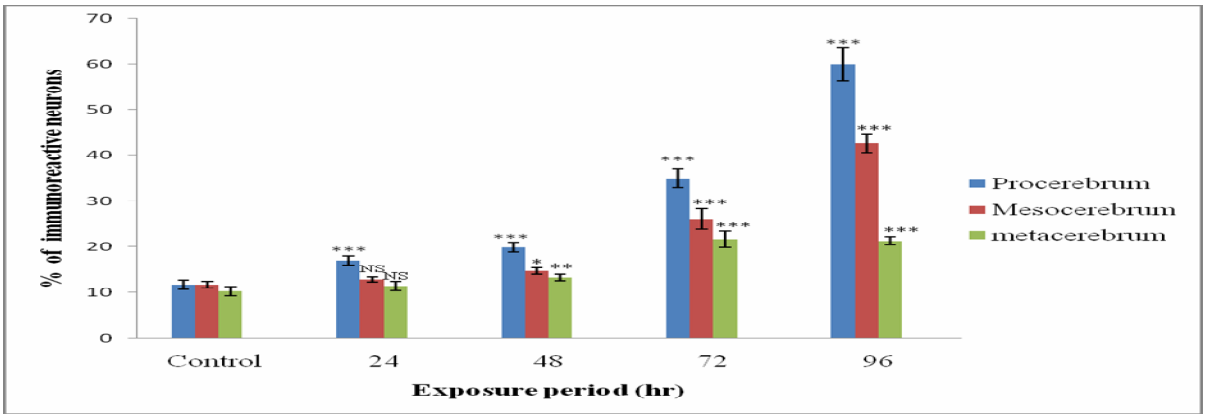

Fig.3 Distribution of gastrin / CCK 8 immunoreactive neurons in CG at different exposure period of $HgCl_2$ in snail *B. bengalensis.* A- D– Immunoreactive neurons in CG *Semperula maculata* after 24, 48, 72 and 96 hr of exposure respectively. ○ - negative neurons, ● - moderately immunoreactive neurons, ● - strongly immunoreactive neurons.

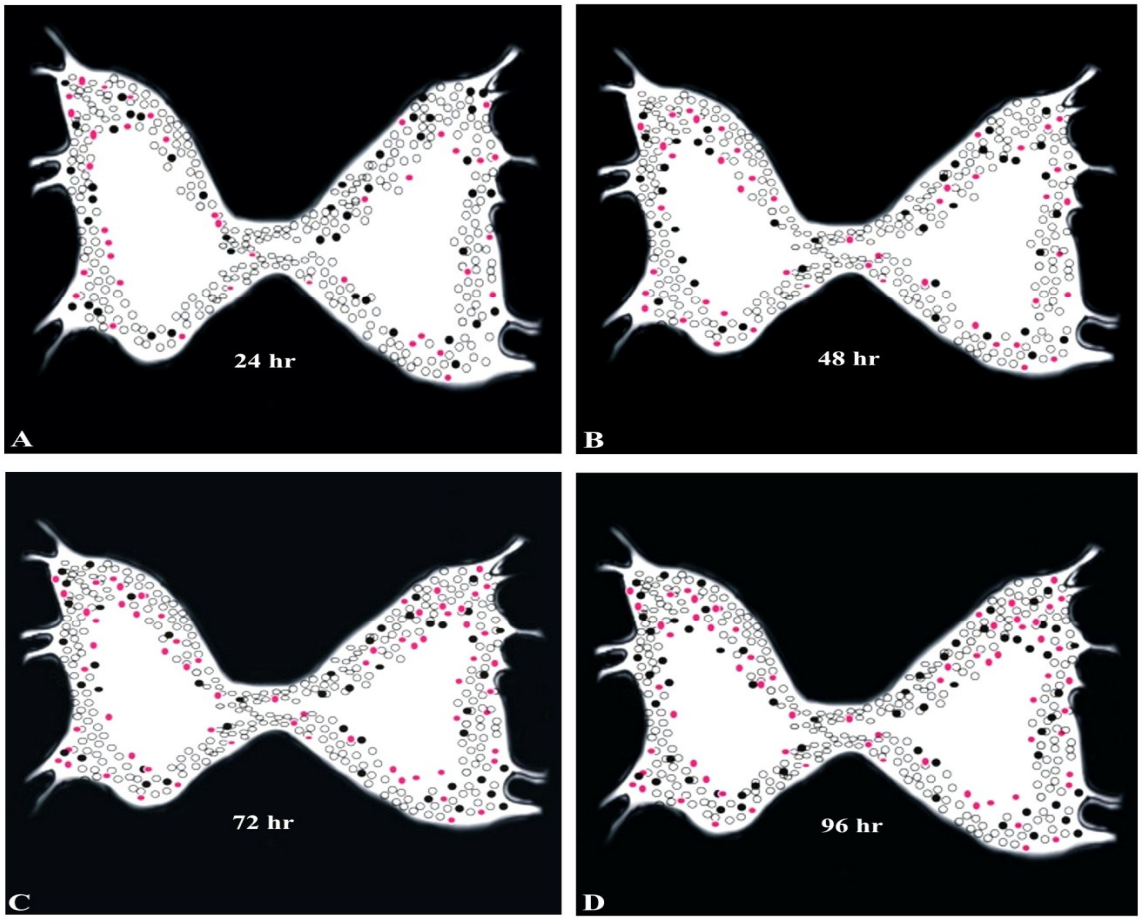

Fig.4 $HgCl_2$ induced gastrin / CCK 8 immunoreactivity in CG of snail *B. bengalensis* at different exposure period. **A - C** Showed 24 hr of gastrin / CCK 8 immunoreactivity, 400X. **D- F** 48 hr of gastrin / CCK 8 immunoreactivity, 400X. **G- I –** 72 hr of gastrin / CCK 8 mmunoreactivity, 400X. **J - L** - 96 hr of gastrin / CCK 8 immunoreactivity, 400X.

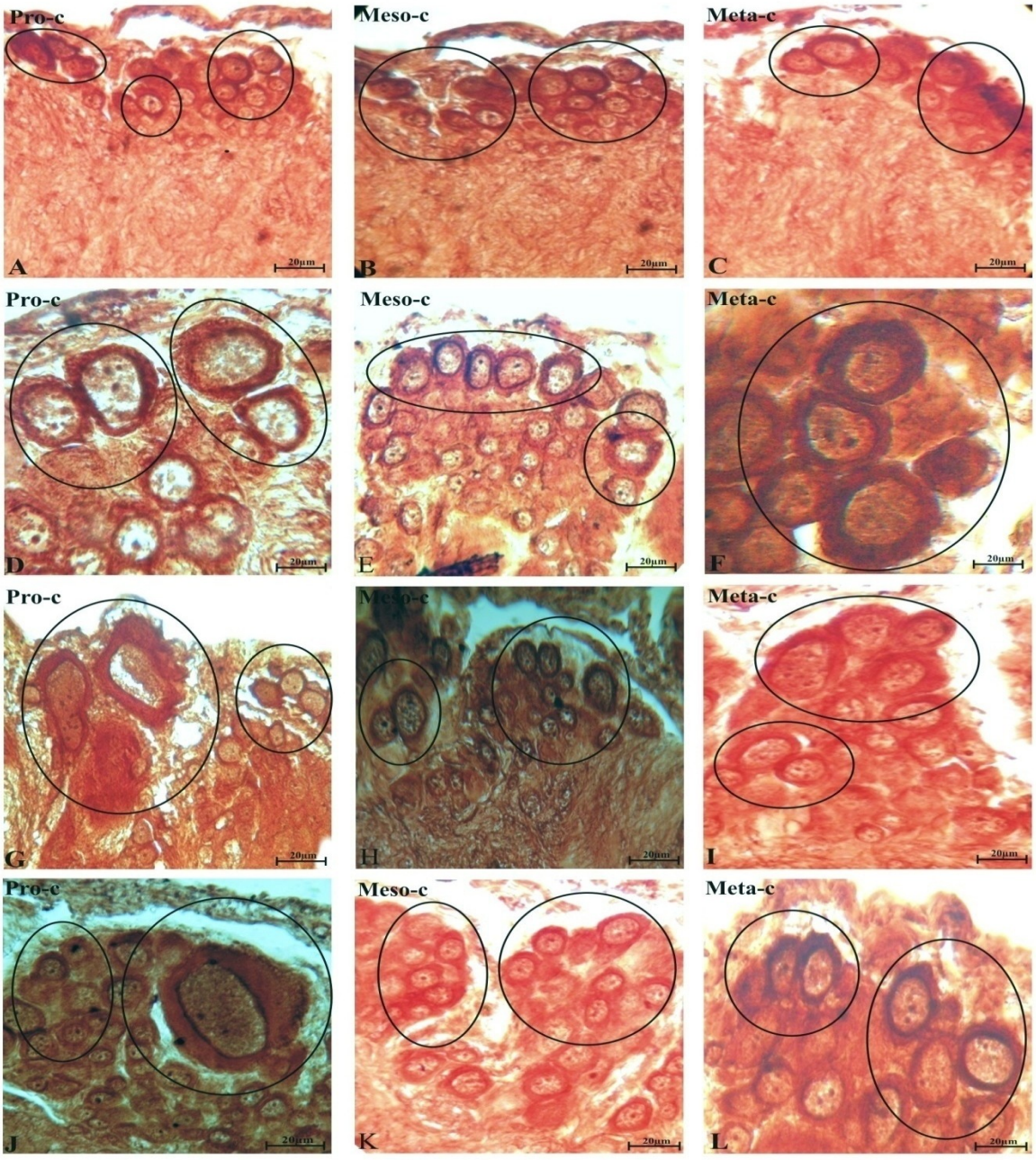

Fig.5 Localization of gastrin / CCK 8 immunoreactive neurons in the CG of *B. bengalensis* from $ZnCl_2$ control group. **A**- Diagrammatic presentation of immunoreactive neurons for gastrin / CCK 8 in different regions in CG. LCG – left cerebral ganglia, RCG- Right cerebral ganglia, Pro-c- procerebrum, Meso-c- mesocerebrum, Meta-c – metacerebrum, ○- negative neurons, ● –moderately immunoreactive neurons, ● - strongly immunoreactive neurons, **B – D** Sectional view showed no immunoreactivity in pro-c, meso-c and meta-c sections, 400X. **E - G** Gastrin / CCK 8 immunoreactivity in pro-c, meso-c and meta-c of CG in control group, 400X.

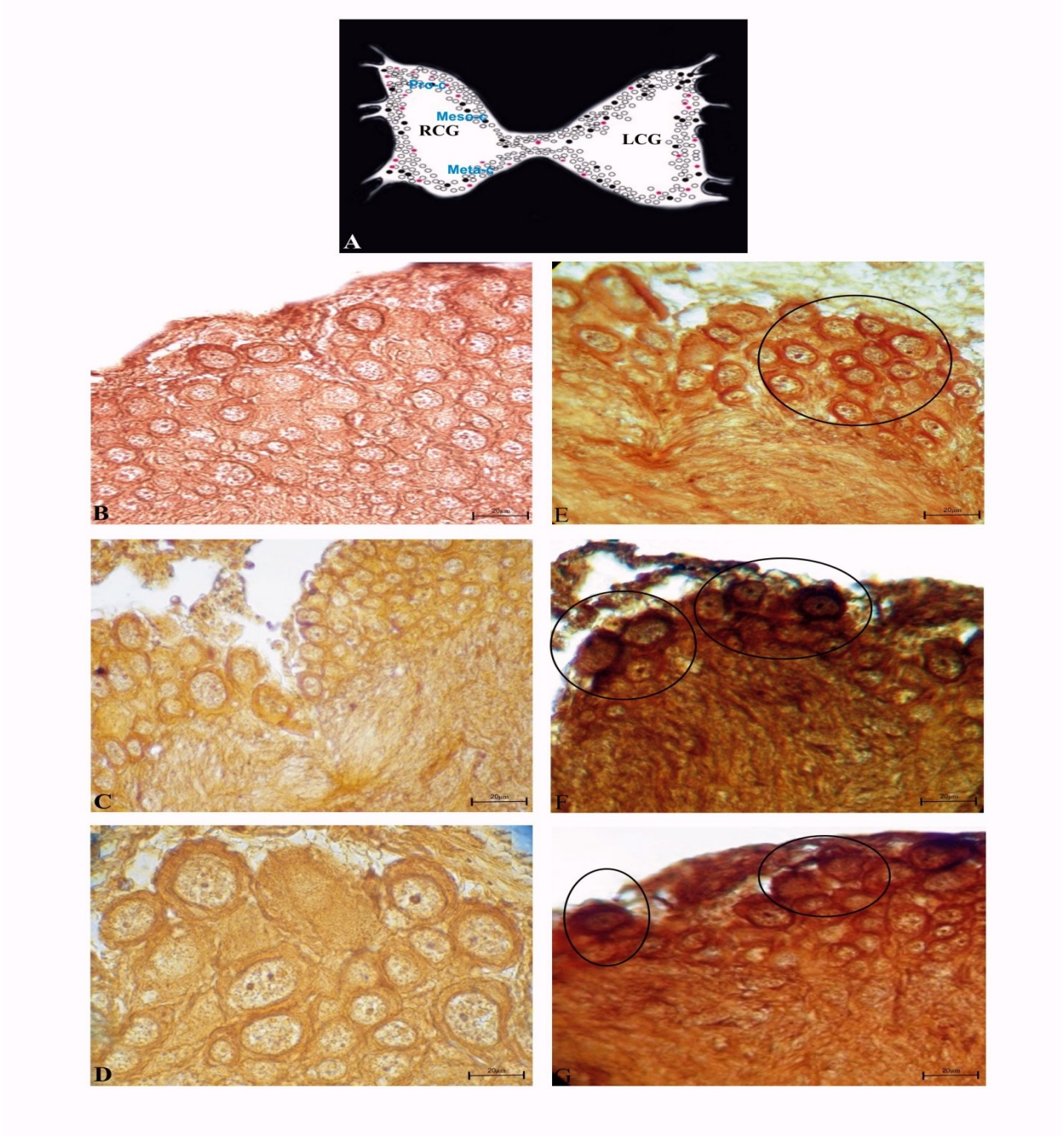

Fig.6 $ZnCl_2$ induced gastrin / CCK8 immunoreactivity in cerebral regions of *B. bengalensis*. *** - P<0.001(Significant), **- P< 0.01 (Less significant) and NS - P<0.05 (Non significant).

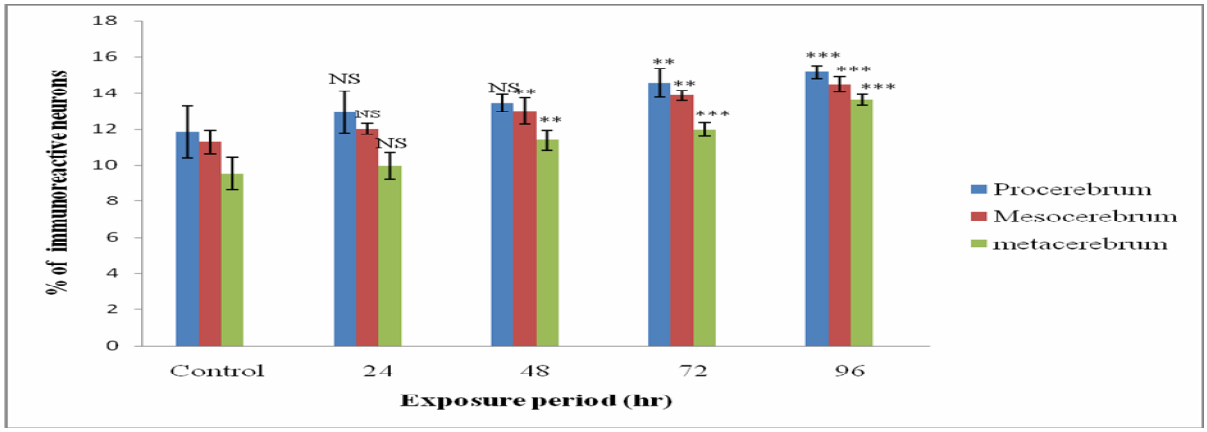

Fig.7 Distribution of gastrin / CCK 8 immunoreactive neurons in CG at 24, 48, 72 and 96 hr of $ZnCl_2$ exposure in *B. bengalensis.* A- D– Immunoreactive neurons in CG *Semperula maculata* after 24, 48, 72 and 96 hr of exposure respectively. ○ - negative neurons, ● - moderately immunoreactive neurons, ● - strongly immunoreactive neurons.

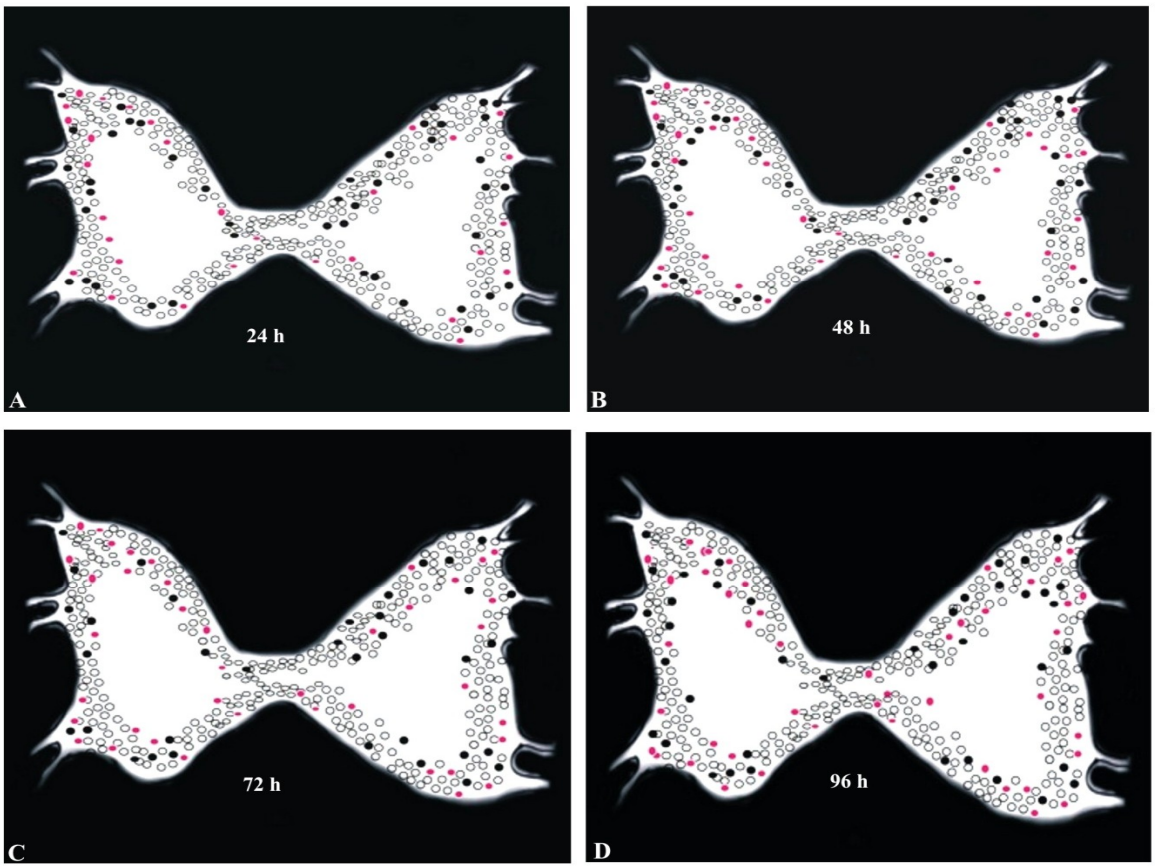

Fig.8 $ZnCl_2$ induced gastrin / CCK 8 immunoreactivity in CG of snail *B. bengalensis* at different exposure period**. A - C** Showed 24 hr of gastrin / CCK 8 immunoreactivity, 400X. **D- F** 48 hr of gastrin / CCK 8 immunoreactivity, 400X. **G- I –** 72 hr of gastrin / CCK 8 mmunoreactivity, 400X. **J - L** - 96 hr of gastrin / CCK 8 immunoreactivity, 400X.

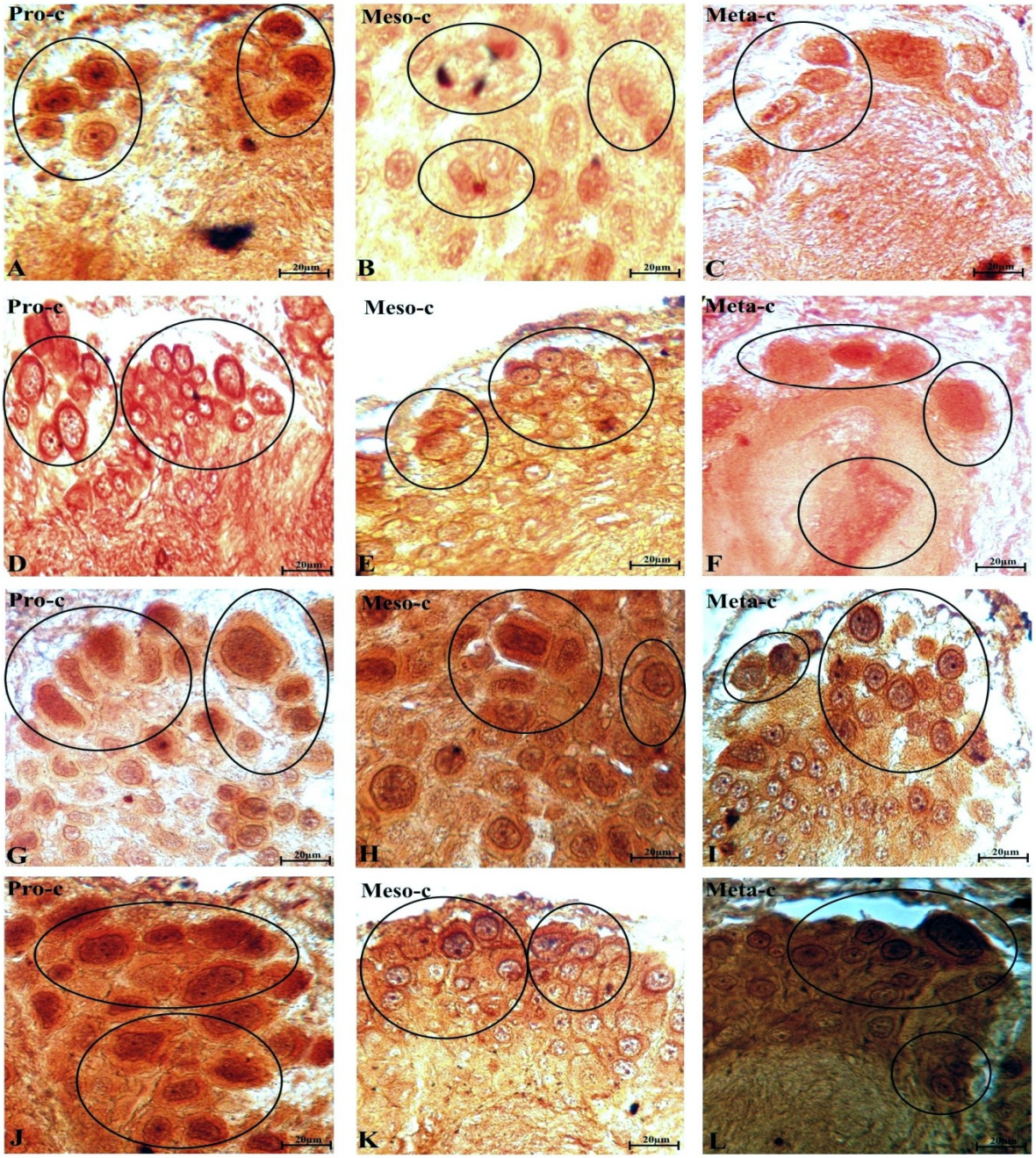

Table.1 Staining intensity of Gastrin / CCK8 immunoreactivity in neurons of control and $HgCl_2$, ZnCl2 and Co $(NO_3)_2$ induced CG of *B. bengalensis*. ++++ very strong intensity, +++ strong intensity, ++ moderate intensity, + light intensity, CG- cerebral ganglia, Pro-c- procerebrum, Meso-c- mesocerebrum, Meta-c- metacerebrum

Heavy metals	Regions of CG	Gastrin / CCK 8 immunoreactivity				
		Control	24 hr	48 hr	72 hr	96 hr
HgCl2 induction	Pro-c	+	++	+++	++++	++++
	Meso-c	+	+	++	+++	++++
	Meta-c	+	+	++	+++	++++
ZnCl2 induction	Pro-c	+	+	++	++	++
	Meso-c	+	+	+	++	++
	Meta-c	+	+	+	++	++

and meta-c neurons were moderately immunostained to gastrin / CCK 8. Chase and Li (1994), Li and Chase (1995), documented generic role of gastrin / CCK 8 in neuromodulatary molecules of meso-c in courtship behaviour. Pisu et al (2000) found of gastrin / CCK 8 reactivity in relation to integrated functions as feeding behavior. Shevelikin et al (1994), documented behavioral dependent metabolic activities as peptide formation in snail *Helix aspersa*.

After intoxication of $HgCl_2$, very strong gastrin / CCK 8 immunostaining was observed in the cerebral neurons in all experimental animals as compared to intoxication of $ZnCl_2$ and Co $(NO_3)_2$. Similarly we observed the immunostaining activity of gastrin / CCK 8 was increased in cerebral ganglia of terrestrial slug *Semperula maculata* after intoxication of $HgCl_2$ (Londhe and Kamble 2013). Acute and chronic exposure of mercury therefore known to cause neuropathic condition in organism (Mutter et al.,2005). According to Ferraro et al (2009) CNS seems to be vulnerable organ affected by mercury toxicity, cerebral part as major targeted organ in experimental animals. Shenkar et al (1992), documented effect of heavy metal toxicity in general, mercury in particular related to disturbances of immunity in experimental animals. They have also documented that, mercury has dose dependent effect on reduction of T-cell proliferation and functioning of monocytes in vertebrate organisms. They also observed that, after 24 h the T-cell expressions reduced leading to suppressed immunity in animals. Result proved immunotoxic effect of mercury even at low concentration. We found that, immunostaining reactions were increased as per increased exposure period this may be because toxicity of Hg in cells, as s result sectional view showed more immunostained reactivity.

Sager (1988), studied acute intoxication of methyl mercury over cell cytokinesis and documented arrested proliferation in cultured cells, but excess induction of Hg showed highly impaired cellular immunity. Similarly, we found that, acute doses of $HgCl_2$, where the gastrin / CCK 8 immunoreactivity of neurons has blocked immunoreactions as result, animals lost their normal movement and it was directly related to time of exposure.

Mercury intoxication at different exposure has elevated bioconcentration and biomagnifications in neurons. As a result, neuronal content was disturbed. As membrane cytoskeleton found disturbed within the intoxicated neurons, their cellular communication found impaired, resulting to reduced neurotransmission. Pekel et al (1993), has documented decreased ionic current with respect to increased concentration of $HgCl_2$ which has arrested neurotransmission in abdominal ganglia of *Aplysia california*, they have also reported irreversible neurotoxic effect of mercury for alteration in the cellular activity. Kamble and Londhe (2012), reported histopathological alteration in the neurons of CG in slug *S. maculata* due to acute exposure of $HgCl_2$. Scientists have reported that, pesticide carbamate provide to have neurotoxic impact and has altered feeding behavior of experimental animals (Wright and Williams, 1980; Bailey, 1989).

From obtained result we predicted that, pathology of neuronal cells hamper over the communication to each other. Due to that the gastrin / CCK 8 found highly accumulated in cytoplasm of neuronal cells after toxicity of $HgCl_2$. This arrested gastrin / CCK 8 was responsible for alterations in the behavior of experimental animal. Finally it concludes that, mercuric chloride has proved its neurotoxic effect over experimental snail *B. bengalensis* then the $ZnCl_2$.

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