

Evaluation of the Xenobiotic Effects of Various Stressors in the Hypothalamus and Testis of Laboratory Mouse with Special Reference to GST Gene

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ABSTRACT: The glutathione S-transferase (GST) family of enzymes has a vital role in biotransformation of environmental carcinogens, pollutants, drugs and other xenobiotics. GST M has been found in high concentrations in brain, muscle, liver, kidney and lung. GST P is most prevalent in mammalian cells, present at high level in many cancerous tissues. Aim of this study was to check the xenotoxicity of various stressors with reference to GST gene expression. In this experiment both hypothalamus and testes of mice receiving different stressors (water and food deprivation, BPA, CPA, PQ and *C. sativa* leaf extract) were processed for the RT-PCR of GST M1 and GST P1. RT-PCR results showed that the expression of GST P1 mRNA increased in the hypothalamus of BPA treated mice and similarly expression of GST M1 mRNA increased in the hypothalamus of CPA treated mice only. Accordingly, expression of GST M1 remained unchanged in the hypothalamus and testis of water and food deprived mice, BPA, PQ and *C. sativa* leaf extract treated mice while expression of GST P1 remained unchanged in the hypothalamus and testis of water and food deprived mice, CPA, PQ and *C. sativa* leaf extract treated mice except in the hypothalamus of BPA treated mice. From these results it can be concluded that, different stressors used in this study have no xenobiotic effects except BPA and CPA which modulated GST P1 and GST M1 mRNA respectively in the hypothalamus to some extent. However, more study is needed to confirm xenobiotic property of these compounds especially when given at higher dose level and for longer duration.

KEYWORDS: Bisphenol A, Cyclophosphamide, GST, Paraquat, Xenotoxicity.

I. INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) comprise a large family of dimeric enzymes that catalyse the nucleophilic addition of the tripeptide glutathione to a wide spectrum of compounds that have electrophilic functional groups. These isozymes can be found in most aerobic micro-organisms, plants and animals [1]. Substrates of GST-catalysed reactions include pre-carcinogens, such as polycyclic aromatic hydrocarbons, pharmacological drugs including paracetamol, chemotherapeutic agents and free radicals generated during oxidative stress [2]. GSTs have been shown to act as inhibitors of the jun kinase pathway, which is an important signaling mechanism for the activation of cytoprotective genes [3].

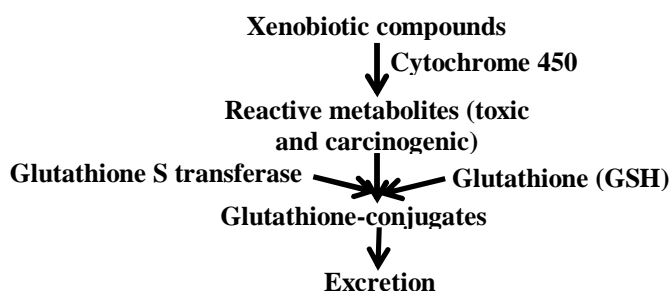
GSTs belong to a superfamily of ubiquitous, multifunctional dimeric cytosolic enzymes that catalyse several reactions with various substrates. Their essential function is detoxification of xenobiotic compounds that are conjugated to glutathione and then excreted as follows:-

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Schematic diagram of action mechanism of Glutathione S transferase (GST)

Other functions, not associated with detoxification, include repair of macromolecules oxidized by reactive oxygen species, regeneration of S-thiolated proteins, and biosynthesis of physiologically important metabolites [4, 5]. GSTs are classified on the basis of their fold, thermodynamic and kinetic properties, nature of the residues involved in catalysis, and the specific reaction catalysed [5, 6]. There are eight classes of mammalian soluble GST (alpha, mu, pi, theta, omega, zeta, sigma and kappa) and at least three membrane bound GSTs (MGST1, MGST2 and MGST3) have been described [7-9].

In general, GSTs catalyse the nucleophilic attack of the activated thiolate of GSH to electrophilic substrates and the activation of GSH to GS⁻. However, the precise mechanism of this reaction is still incompletely understood. In the GSTs belonging to the classes alpha (α), mu (μ), pi (π) and sigma (σ), glutathione activation proceeds via the interaction with a Tyr at H-bonding distance from the sulfur of GSH; in the enzymes from other classes, the catalytic residue is either Cys or Ser [4, 5]. Mammalian GSTs are expressed in discrete tissue-specific patterns. For instance livers of some species are rich in α and some μ -class GSTs but do not contain π , yet some μ subunits not present in liver are major components in certain extrahepatic tissues [10-12].

The μ class glutathione transferases (GST M) have been found in high concentrations in brain, muscle, liver, kidney and lung [13, 14]. GST π (GST P) is most prevalent in mammalian cells, present at high level in many cancerous tissues and overexpressed in drug-resistant tumors [15]. It is expressed in the Central Nervous System, and has been the only subclass implicated in protection of cells from ROS-inducing agents [16, 17].

Bisphenol A (BPA) is a high volume synthetic compound with annual production of billions of pounds worldwide. It is commonly found in consumer products and human exposure arises from inhalation, ingestion and dermal contact with such products. It is used in the manufacture of polycarbonate re-usable bottles, epoxy resins, dental composites and sealants [18]. BPA, chemically a monomer (structure given below) is the major estrogenic compound that leaches into nearby water and food supplies [19]. Cyclophosphamide (CPA), a synthetic antineoplastic drug is used widely to treat various types of cancer as well as an immunosuppressant in organ transplantation, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and other benign diseases. It binds covalently to DNA and induces DNA damage [20]. Paraquat (PQ, 1,1'-dimethyl 4,4'-bipyridylium dichloride), together with diquat and difenzoquat, belongs to the group of bipyridylium herbicides. It is a highly toxic compound for humans and animals [21]. Severe PQ poisoning is characterized by multiple-organ failure, involving mainly the lung, kidney and liver. Short-term contact leads to irritation of the skin and delays in the recovery of cuts and wounds on the skin. PQ induced oxidative stress impairs insulin-dependent mTOR activation and this impairment probably causes inhibition of insulin-dependent repression of IGF1P-1 expression [22]. *Cannabis sativa* plant is commonly used in the preparation of illicit drugs [23]. It is used in several forms such as marijuana, bhang or hashish from the same cannabis plant. In India bhang is prepared from the leaf and flower of the cannabis plant.

Yadawa and Chaturvedi [24] reported that water and food deprivation of 2 and 4 days alter the expression of stress hormones AVP and CRH in the hypothalamus and increased the level of serum corticosterone indicating the activation of hypothalamic-pituitary-adrenal (HPA) axis of laboratory mouse. It is also suggested that stressors such as BPA, CPA,

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PQ and *C. sativa* leaf extract treatment activate the HPA axis of laboratory mouse (unpublished data). Based on the previous information and role of different kind of stressors (water and food deprivation, BPA, CPA, PQ and *C. sativa* leaf extract treatment, present experiment was planned to study the xenobiotic effect of these stressors in the brain and testis of mice with reference to GST gene.

II. MATERIALS AND METHODS

Male laboratory mice weighing 25 ± 2 grams were divided in 5 groups. Group-1 mice were further divided into 5 subgroups- i) Control, provided food and water *ad libitum*, ii) water deprived for 2 days (WD 2), iii) water deprived for 4 days (WD 4), iv) food deprived for 2 days (FD 2) and v) food deprived for 4 days (FD 4) [n=5 in each subgroup]. Group-2 mice were divided into 2 subgroups- i) Control received vehicle i.p. (olive oil), ii) received BPA i.p. 20mg/kg body weight once a week for 5 weeks [n=5 in each subgroup]. Group-3 mice were divided in 2 subgroups i) control mice were given normal saline i.p., ii) mice were given CPA i.p. 100mg/kg body weight once a week for 5 weeks [n=5 in each subgroup]. Group-4 mice were divided in 2 subgroups i) control mice were given normal saline i.p., ii) mice were given PQ i.p. 10mg/kg body weight once a week for 5 weeks [n=5 in each subgroup]. Group-5 mice were also divided in to 2 subgroups i) Control mice gavaged with water and ii) *C. sativa* leaf extract mice gavaged with *C. sativa* aqueous leaf extract (bhang) 10 mg/kg body weight daily for 5 weeks. The extract was prepared from the leaves of the female cannabis plant. 1 g cannabis leaves were extensively ground in mortar and pestle with 1ml autoclaved double distilled water. From the 1 g/ml paste, 10 mg/kg body weight suspension was prepared with double distilled autoclaved water. This suspension was filtered to get a clear solution.

At the end of food and water deprivation period and BPA, CPA, PQ and *C. sativa* leaf extract treatment, mice of all the groups were weighed and sacrificed by decapitation. Hypothalamus and testis of all mice in each group were dissected out, processed for extraction of total RNA for reverse transcriptase-PCR for GST M1, GST P1 and β -actin.

The experiment was conducted in accordance with Institutional practice and within the framework of the revised Animals (scientific procedures) Act of 2002 of the Govt. of India on Animal Welfare.

RNA extraction

The total RNA from hypothalami of the mice of various groups was extracted using TRIzol Reagent (Ambion, life technologies) according to its user manual, dissolved in DEPC - treated water and quantified using spectrophotometer.

cDNA synthesis

cDNA was synthesized from 10 μ g of total RNA using random hexamer primer reverse transcriptase. In brief, 10 μ g of RNA was mixed with 200 ng of random hexamer in DEPC - treated water in the reaction volume of 11 μ l. It was incubated at 70°C for 5 min. Thereafter the following components were added in the order indicated: 5x Reaction buffer-4.0 μ l, dNTP mix (2.5mM each) - 2.0 μ l, RNase inhibitor - 0.5 μ l, DEPC treated water - 1.5 μ l.

The tubes containing reaction mixture were incubated for 5 min at 25°C and 1.0 μ l of M-MuLV reverse transcriptase (RevertAid, 200 units) was added. Further, the tubes were incubated for 10 min at 25°C and then at 42°C for 1 h. The reaction was terminated by heating at 70°C for 10 min, and after chilling, the tubes were stored at -20°C to be used directly for the PCR reaction.

Polymerase chain reaction

Specific oligonucleotides used for the analysis of GST M1 (forward primer 5'-CCTATGATACTGGGATACTGGAACG- 3', Reverse primer 5'-GGAGCGTCACCCATGGTG- 3'), GST P1 (Forward primer 5'-GCAAATATGTCACCCTCATCTACACC-3', Reverse primer 5'-GCAGGGTCTCAAAAGGCTTCA-3') and β -actin (Forward primer 5'-ATC GTG GGC CGC TCT AGG CAC C-3' Reverse primer 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3') were synthesized by mwg - operon, Bangalore, India. The PCR were performed in 25 μ l reaction mixture containing 2 μ l cDNA, 10x Taq polymerase buffer, 2.5 mM of each dNTP (Biobharati, India), 1.0 unit of Taq DNA polymerase (Biobharati, India), and 10 pmol of appropriate primers. The reactions were carried out in T 100™ BioRad Thermal cycler. The samples were denatured at 94°C for 5

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min and amplified using different conditions for GST M1 (denaturation at 94°C for 1 min, primer annealing at 61°C for 1 min elongation at 72°C for 1 min and no. of cycles – 35); GST P1 (denaturation at 94°C for 1 min, primer annealing at 61°C for 50 sec elongation at 72°C for 1 min and no. of cycles – 35) and β -actin (denaturation at 94°C for 30 sec, primer annealing at 57°C for 30 sec elongation at 72°C for 30 sec and no. of cycles – 30). The resultant PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. The resulting gel bands were visualized using UV-transilluminator Alpha Digi doc from Alpha Innotech (India) and photographed by camera Olympus (USA). The expression of GST M1 and GST P1 were expressed as percent (%) band intensity relative to that of β -actin.

Statistical analysis

All the data were analysed by Student's t test for the comparison of BPA treated group from control. Difference was considered significant at the level of $P < 0.05$.

III. EXPERIMENTAL RESULTS

Effect of water and food deprivation on the expression of GST M1 and GST P1 in the hypothalamus and testis

The expression of GST M1 mRNA in the hypothalamus and testis of water and food deprived mice did not change compared to their respective controls (Fig. 1A).

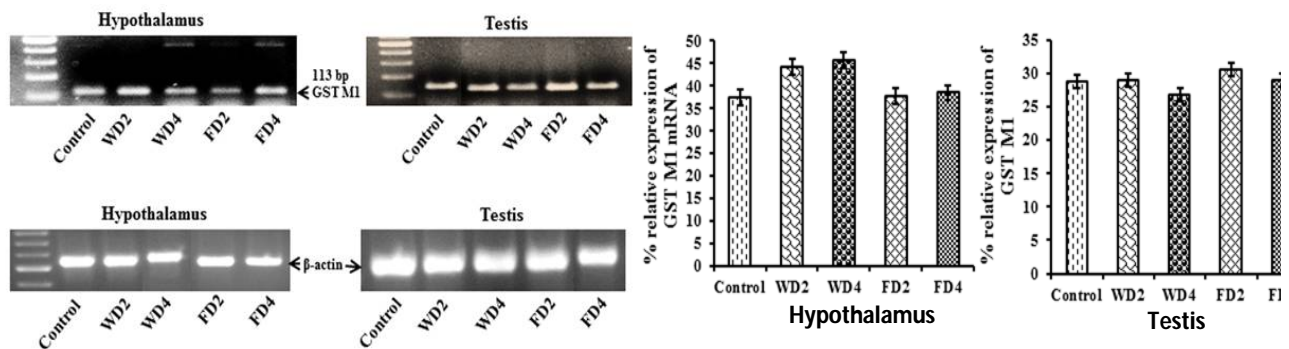


Fig. 1A - RT-PCR products of GST M1 and β -actin in the hypothalamus and testis of WD2, WD4, FD2 and FD4 mice analyzed on 2% agarose gel and densitometric analysis of RT-PCR products of GST M1 normalized by β -actin (bar graphs). Note GST M1 mRNA expressions were not changed in water and food deprived mice hypothalamus and testis compare to their respective controls

The expression of GST P1 mRNA in the hypothalamus and testis of water and food deprived mice also did not change compared to their respective controls (Fig. 1B).

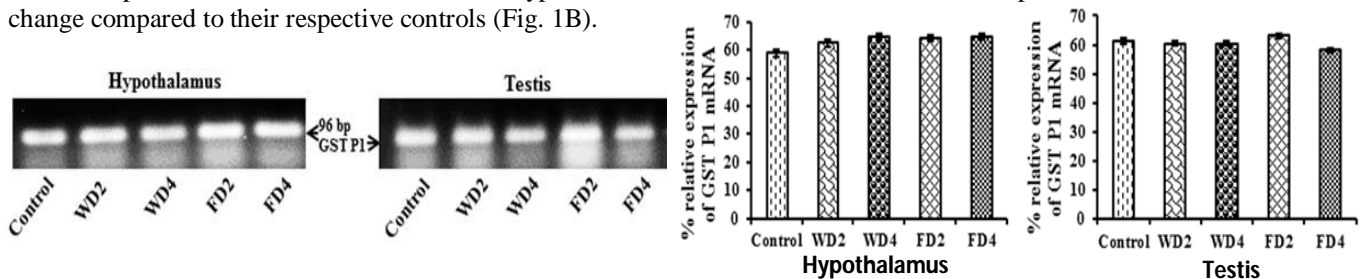


Fig. 1B- RT-PCR products of GST P1 in the hypothalamus and testis of WD2, WD4, FD2 and FD4 mice analyzed on 2% agarose gel and densitometric analysis of RT-PCR products of GST P1 normalized by β -actin (bar graphs). Note GST P1 mRNA expressions were not changed in water and food deprived mice hypothalamus and testis compare to their respective control

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Effect of BPA on the expression of GST M1 and GST P1 in the hypothalamus and testis

The expression of GST M1 mRNA in the hypothalamus and testis of BPA treated mice was not different from the control (Fig. 2A).

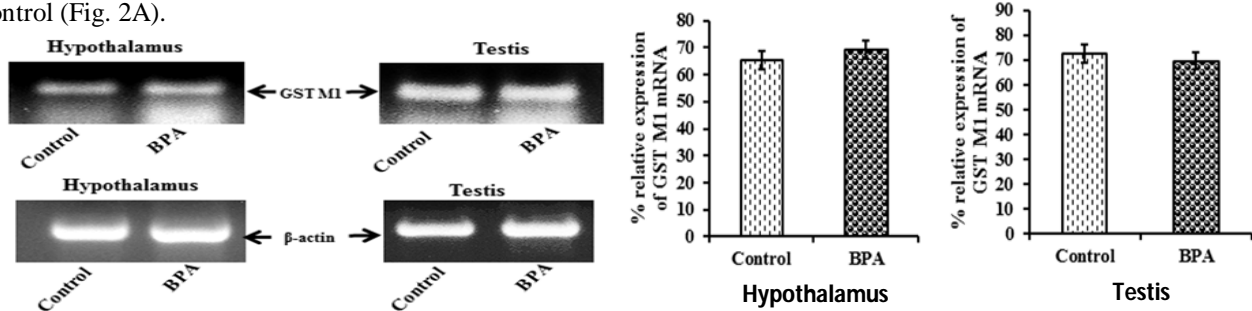


Fig. 2A - RT-PCR products of GST M1 and β -actin in the hypothalamus and testis of BPA treated mice analyzed on 2% agarose gel and densitometric analysis of RT-PCR products of GST M1 normalized by β -actin (bar graphs). Note GST M1 mRNA expressions were not changed in BPA treated mice hypothalamus and testis compare to their respective controls

The RT-PCR results showed that GST P1 mRNA expression in the hypothalamus of BPA treated mice increased however; it did not change in testis of BPA treated mice (Fig. 2B).

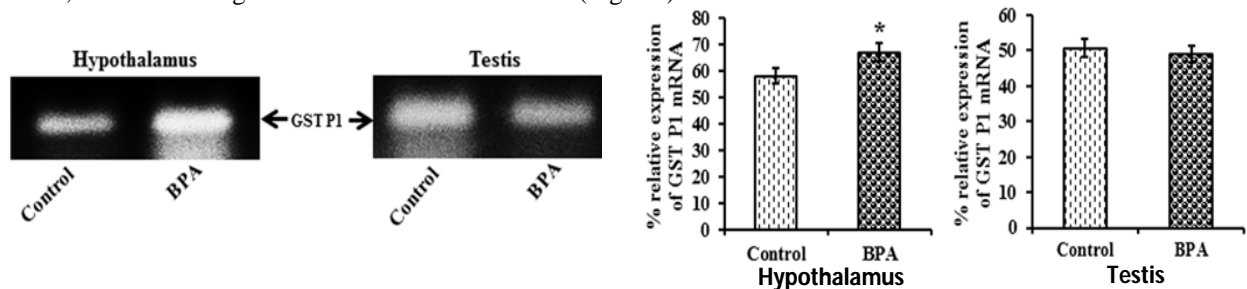


Fig. 2B - RT-PCR products of GST P1 in the hypothalamus and testis of BPA treated mice analyzed on 2% agarose gel and densitometric analysis of RT-PCR products of GST P1 normalized by β -actin (bar graph). Note GST P1 mRNA expression increased in the hypothalamus but not changed in the testis of BPA treated mice compare to their respective controls.* $P < 0.05$, significance of difference from control

Effect of CPA on the expression of GST M1 and GST P1 in the hypothalamus and testis

The expression of GST M1 mRNA in the hypothalamus of CPA treated mice upregulated while in testis of CPA treated mice it was not different from the control (Fig. 3A).

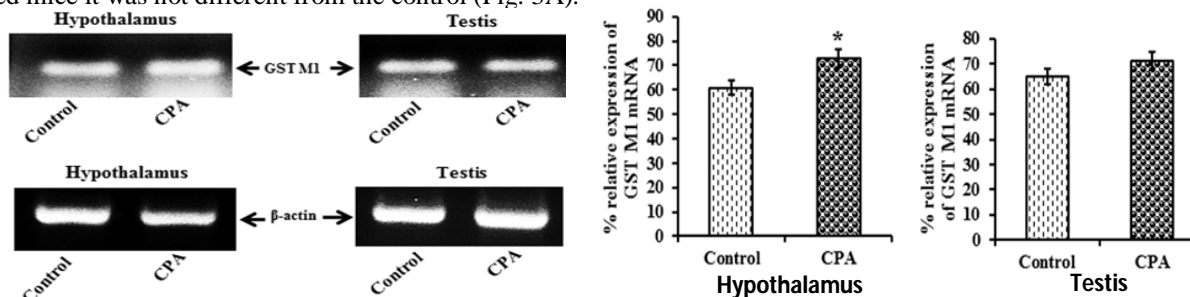


Fig. 3A - RT-PCR products of GST M1 and β -actin in the hypothalamus and testis of CPA treated mice analyzed on 2% agarose gel and densitometric analysis of RT-PCR products of GST M1 normalized by β -actin (bar graphs). Note GST M1 mRNA expressions increased in the hypothalamus but not changed in testis of CPA treated mice compare to their respective controls.* $P < 0.05$, significance of difference from control

The RT-PCR results demonstrated that GST P1 mRNA expression in the hypothalamus and testis of CPA treated mice did not change compared to control (Fig. 3B).

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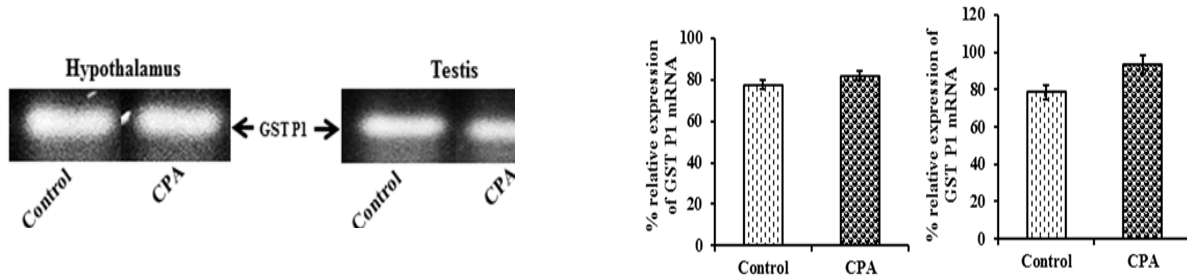


Fig. 3B - RT-PCR products of GST P1 in the hypothalamus and testis of CPA treated mice analyzed on 2% agarose gel and densitometric analysis of RT- PCR products of GST P1 normalized by β -actin (bar graphs). Note GST P1 mRNA expressions were not changed in CPA treated mice hypothalamus and testis compare to their respective controls

Effect of PQ on the expression of GST M1 and GST P1 in the hypothalamus and testis

The expression of GST M1 mRNA in the hypothalamus and testis of PQ treated mice was not different from the control (Fig. 4A).

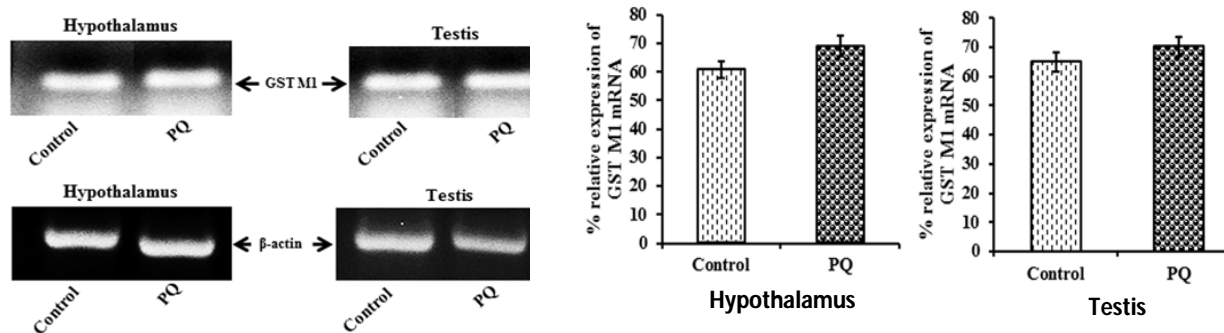


Fig.4A - RT-PCR products of β -actin and GST M1 in the hypothalamus and testis of PQ treated mice analyzed on 2% agarose gel and densitometric analysis of RT- PCR products of GST M1 normalized by β -actin (bar graphs). Note GST M1 mRNA expressions were not changed in PQ treated mice hypothalamus and testis compare to their respective controls

The expression of GST P1 mRNA in the hypothalamus and testis of PQ treated mice also did not change compared to control (Fig. 4B).

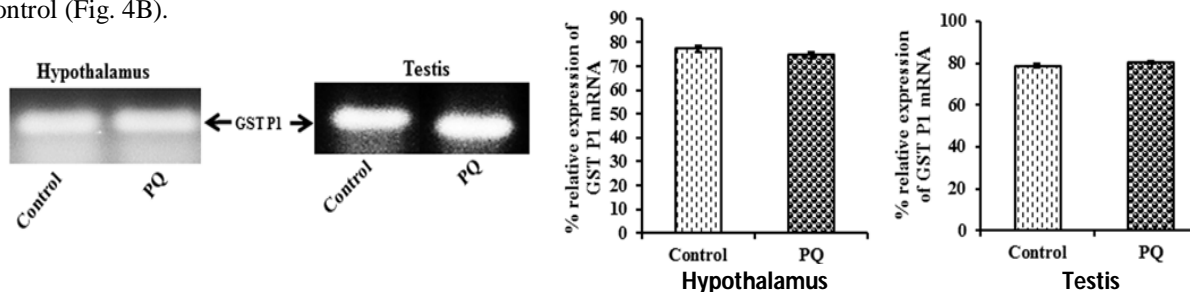


Fig. 4B - RT-PCR products of GST P1 in the hypothalamus and testis of PQ treated mice analyzed on 2% agarose gel and densitometric analysis of RT-PCR products of GST P1 normalized by β -actin (bar graphs). Note GST P1 mRNA expressions were not changed in PQ treated mice hypothalamus and testis compare to their respective controls

Effect of *C. sativa* leaf extract on the expression of GST M1 and GST P1 in the hypothalamus and testis

The expression of GST M1 mRNA in the hypothalamus and testis of *C. sativa* leaf extract administered mice was not different from the control (Fig. 5A).

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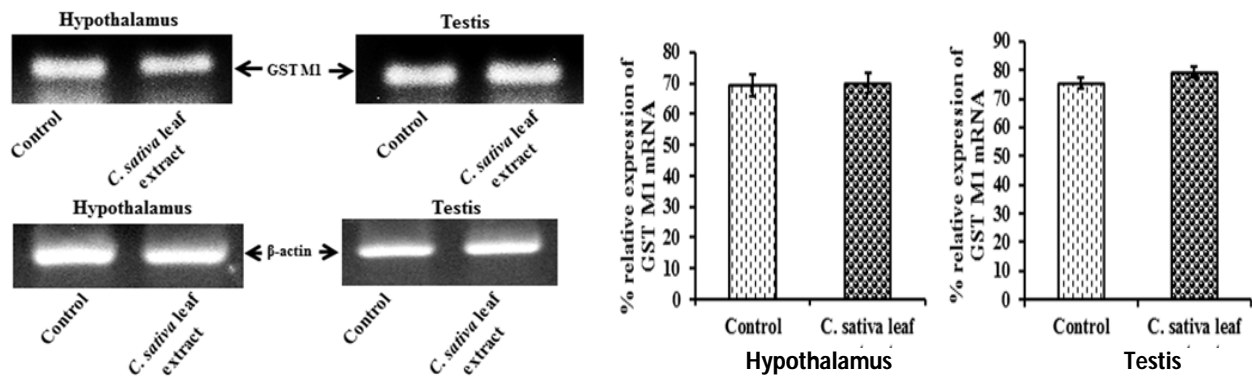


Fig. 5A - RT-PCR products of GST M1 and β -actin in the hypothalamus and testis of *C. sativa* leaf extract treated mice analyzed on 2% agarose gel and densitometric analysis of RT-PCR products of GST M1 normalized by β -actin (bar graphs). Note GST M1 mRNA expressions were not changed in *C. sativa* leaf extract treated mice hypothalamus and testis compare to their respective controls

The RT-PCR results showed that GST P1 mRNA expression in the hypothalamus and testis of *C. sativa* leaf extract administered mice did not change compared to control (Fig. 5B).

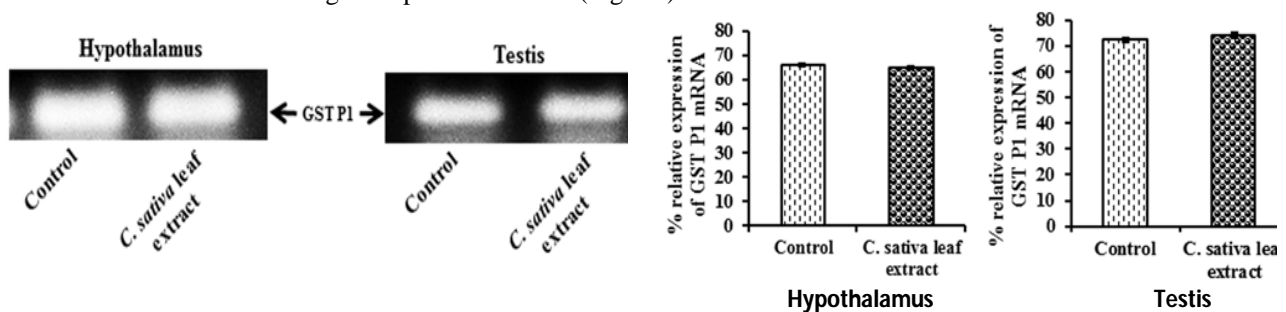


Fig. 5B - RT-PCR products of GST P1 in the hypothalamus and testis of *C. sativa* leaf extract treated mice analyzed on 2% agarose gel and densitometric analysis of RT-PCR products of GST P1 normalized by β -actin (bar graphs). Note GST P1 mRNA expressions were not changed in *C. sativa* leaf extract treated mice hypothalamus and testis compare to their respective controls

IV. DISCUSSION

The aim of this study was to evaluate the xenobiotic effect of different stressors on the brain and testis of mice with reference to GST M1 and P1 gene. RT-PCR results indicate that, in CPA treated mice, the expression of only GST M1 and in BPA treated mice only GST P1 mRNA was upregulated in the hypothalamus but not in the testis of either CPA or BPA groups. On the other hand, the expressions of GST M1 and GST P1 genes changed neither in the hypothalamus nor in the testis of water and food deprived mice or PQ or *C. sativa* leaf extract treated mice compared to their respective controls. Since GSTs are reported to be involved in the detoxification of xenobiotic compounds. However, stressors (water and food deprived, PQ and *C. sativa* leaf extract treatment except BPA and CPA) used in the present study were unable to induce the expression of GST M1 and GST P1 mRNA in the hypothalamus or in the testis. Since all these stressors were able to activate the HPA axis of mice, it is suggested that present dose (of PQ and *C. sativa* leaf extract) and duration of treatment (water and food deprivation) might be unable to induce any toxicity in the brain and testis, and thereby the expression of GST M1 and GST P1 genes remained unaltered except of BPA and CPA.

A study conducted by Kim et al. [25] reported that water deprivation in rats has no effect on the expression of GST mRNA in the liver; however this status was not reported in the brain. On the other hand, in the liver of rockbream fish, significant increase in GST mRNA level was detected at day 4 of starvation with further increase until day 10 [26].

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The toxic compound BPA is reported to have no remarkable effect on the mRNA level of GST in rat mid stage embryos but upregulates dose dependently in late stage embryos [27]. In the present study, in the mice treated with BPA (20 mg/kg body weight for 5 weeks, once a week), the expression of GST P1 mRNA in the hypothalamus was upregulated while in the testis it was not affected. On the other hand, GST M1 mRNA remained unchanged in both hypothalamus and testis of BPA treated mice. It is possible that, expression of GST mRNA might be BPA dose and duration dependent and hence at the present dose and duration level was not sufficient to induce the expression of GST M1 mRNA in the hypothalamus and testis. This possibility is supported by the report that different GST isoenzymes are involved in detoxification of different xenobiotic compounds [28]. In another study on rats, treatment with various doses of BPA (0.1, 1, 10, and 50mg/kg), led to a decrease in the expression of GST gene in liver with increasing dose of BPA [29]. A study conducted on TM4 cells also reported the decreased expression of GST P protein after 10^{-8} M BPA exposure [30] while BPA exposure in juvenile Atlantic cod had no effect on GST P gene expression [31].

CPA treatment (100 mg/kg for 5 weeks, once a week) upregulated the expression of GST M1 mRNA only that too only in the hypothalamus but not in the testis. After CPA chemotherapy in the ovarian carcinoma patients, GST M gene expression increased [32]. Acrolein is formed during cyclophosphamide metabolism in the liver which reacts with glutathione and forms Michael adducts and this reaction is catalysed by GST [33]. GSTP protects against CPA-induced urotoxicity, perhaps in part by detoxifying acrolein in the bladder [34]. GST A, GST M and GST P isoenzymes increase the conjugation of phosphoramidate mustard (another CPA metabolite) and glutathione detoxifying the effect of CPA cytotoxicity [35]. Possibly in the present case no effect on GST P1 mRNA might be due to insufficient dose and duration of CPA treatment which was unable to induce xenotoxicity.

Paraquat (PQ) is Known to be highly toxic herbicide for humans and animals [21] which causes multiple organ failure. A study conducted on rats showed increased expression of GST gene in the kidney after 3 h of 20 mg/kg PQ treatment [36]. This treatment also results in the increased expression of GSTP1, GSTA3 and GSTA4; however no change in the expression of GSTM1 or the microsomal GST's mGST1, mGST2 and mGST3 was observed in response to paraquat [37]. Generation of oxidative stress [38, 39] and increased oxidative load in cells is associated with altered expression of antioxidant enzymes which function to limit cytotoxicity [40-42]. Further, treatment of paraquat did not exhibit any significant alteration in GSTA4-4 expression in the brain up to 6 weeks; however, an augmentation in its expression was observed in the animals exposed to paraquat for 9 weeks[43]. Dose dependent decrease in the expression of GST gene has been also reported in the lungs of paraquat treated mice [44].

Based on these reports, it may be suggested that present dose and duration of PQ treatment may not be enough to induce any effect on the expression of GST (M1 and P1) mRNA in the hypothalamus and testis of treated mice compared to control. This suggestion gets support by the findings of Tomita et al. [38]and Patel et al. [43] where only increased doses and longer duration of PQ treatments increased GST expression.

C. sativa consumption in any form is known to be neuromodulatory and cannabidiol and tetrahydrocannabinol are reported to induce the enhanced expression of GST M gene in BV-2 microglial cells[45]. But in this study, *C. sativa* leaf extract did not induce the expression of GST (M1 and GST P1) mRNAs in the hypothalamus and testis of mice.

V. CONCLUSION

Overall, from our findings it is concluded that, dose and duration of different stressors used in this study are not sufficient to induce any xenobiotic effect except in BPA and CPA treated mice to some extent. Taken together, previous reports and these findings also suggest that, a particular stressor at a particular dose or duration might modulate one system but at the same time it may be non-effective for other system (GST defence system).

VI. ACKNOWLEDGEMENT

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