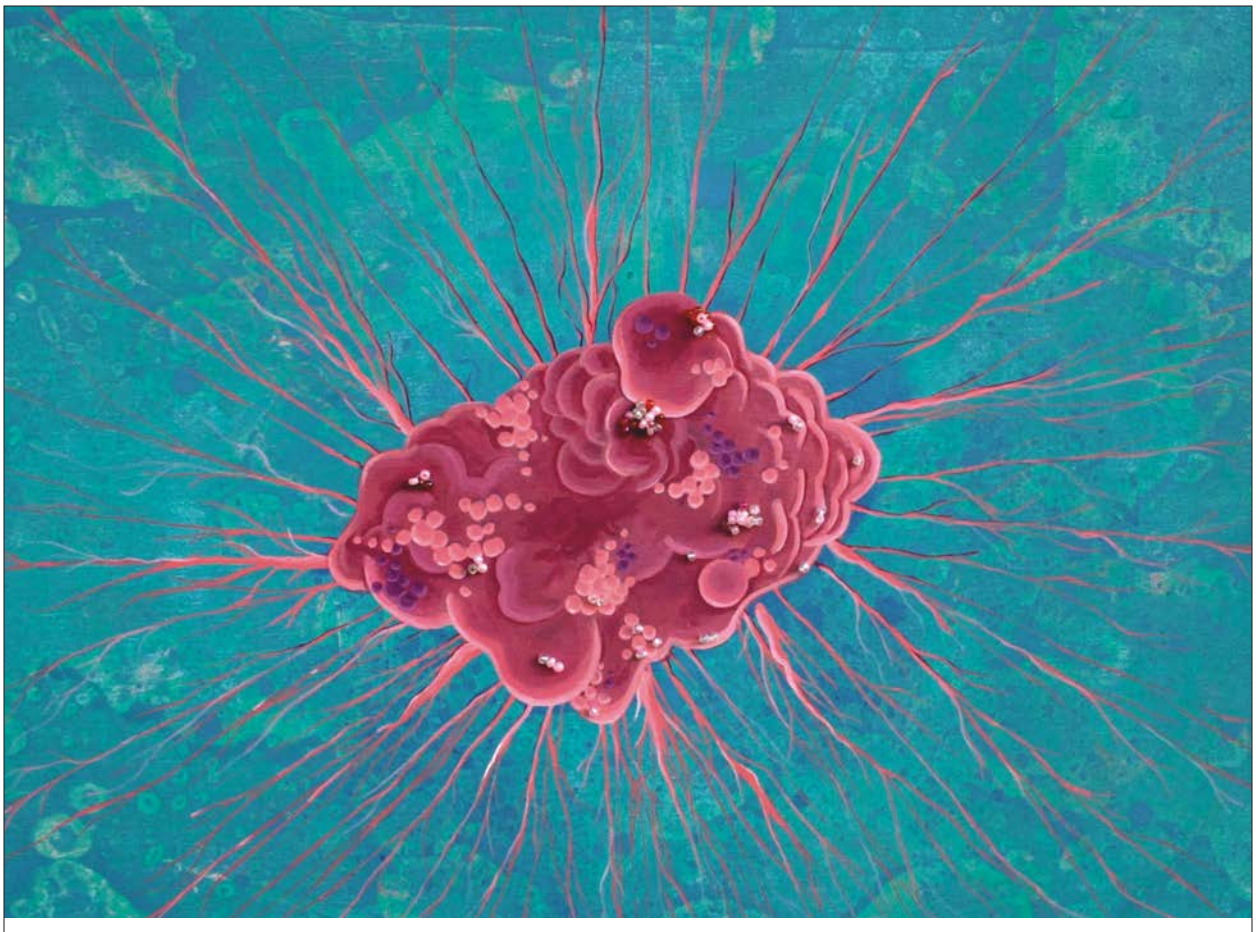

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Tissue Specific Expression of Testicular Glutathione S-transferases on β-methylcholanthrene Treatment

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Abstract: A study was made on the tissue specific expression of Glutathione S-transferases (GSTs) in testis after long and short exposure of rat to β-methylcholanthrene (MC). The separation and identification of various subunits due to the effect of β-methylcholanthrene on testicular Glutathione S-transferases is reported. Purification of rat testicular GSTs by, affinity chromatography, employing Glutathione linked Agarose-CL column and separation of individual subunits by chromatofocussing. Characterization of affinity purified GSTs by Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blot analysis. The effect of β-methylcholanthrene on testicular GSTs was observed with different doses (Short term and long term) with different intervals (24 h interval-4 mg/100 g body weight/day) and 1 week to 6 weeks. The present study reveals that majority of rat testicular GSTs are of Yb size (GST M; 60%) with molecular weight 27.5 kDa. The most predominant subunits, however, are GST Yn2 (27%), followed by GST Yc (GST A_3; 24%) and GST Yn1 (Yβ; 20%). The substrate specificity studies, purification of SDS PAGE analysis and histopathological studies of long-term treatment with low dosage of β-methylcholanthrene, reveals less effect in testis. Where as on short-term treatment with high dosage of β-methylcholanthrene, more degenerative changes were observed when compared to long-term treatment. Majority of rat testicular GSTs are Yb sized and are involved in the prevention of initiation in carcinogenesis. To encounter oxidative damage or attack and other stress conditions due to β-methylcholanthrene Yc and Yb subunits were elevated.

Key words: Glutathione S-transferases, testis, β-methylcholanthrene

Introduction

Glutathione S-transferases (GSTs; EC, 2.5.1.18) catalyze the conjugation of glutathione to numerous potentially genotoxic compounds, including aliphatic aromatic heterocyclic radicals, epoxides and arene oxides (Smith *et al*., 1995; d'Errico, 1996; Rebbeck, 1997). The enzymes detoxify carcinogenic polycyclic aromatic hydrocarbons and conjugate isothiocyanates. Six families of enzymes have been classified as a, m, p, z and Glutathione S-transferase-mu (*GSTM1*), glutathione S-transferase-theta (*GSTT1*) and glutathione S-transferase-pi (*GSTP1*) have been studied most (Rebbeck, 1997). The GSTM1 gene, which is located on chromosome 1p13.3, codes for the enzyme glutathione S-transferase-mu. The *GSTT1* gene codes for the enzyme glutathione S-transferase-theta; the *GSTT1* gene is located on chromosome 22q11.2. The *GSTP*1 gene codes for the enzyme glutathione S-transferase-pi and is located on chromosome 11q13. GSTA1 and GSTA2, the two major glutathione S-trasferase-alpha genes, are mentioned less frequently in the literature and are located on chromosome

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6p12. The *GSTZ1* gene that encodes glutathione S-transferase-zeta was mapped to chromosome 14q24 (Rebbeck, 1997; Board *et al*., 1997; Blackburn *et al*., 1998). The glutathione S-tranferase-omega (*GSTO1*) gene was recently characterized (Board, 2000). The GSTs have broad and overlapping substrate specificities that have been previously reviewed (Strange and Freyer, 1999). GST isoforms are expressed specifically in almost all the tissues, including the reproductive tissues like testes and ovaries in a discrete tissue-specific pattern (Rabahi *et al*., 1999).

The GST enzyme family includes the cytosolic isoforms GST-α, μ (GSTM), π (*GSTP*), θ (*GSTT*) and σ (GSTS). *GSTT1*, P1 and M1 are polymorphic and altered polymorphic frequency of genes encoding these proteins has been suggested as a potential risk factor for the development of hematopoietic malignancies. Over expression of GSTs has also been implicated in chemotherapeutic drug resistance. The α GSTs are highly expressed in the testis, adrenal glands and proximal tubule of the kidney. The μ class GSTs has been found in high concentrations in brain, muscle, liver, kidney and lung (Hayes and Mantel, 1986; Hayes *et al*., 1987). The π class GST is a highly acidic enzyme that is widely distributed except in adult liver, where it is localized to the biliary epithelium (Sato *et al*., 1984; Satoh *et al*., 1985). The θ class enzymes have only recently been described enzymatically in liver (Mayer *et al*., 1991; Hiratsuka *et al*., 1990).

The GSTs achieve detoxication by catalyzing the conjugation of reduced glutathione to various electrophilic substrates. Glutathione (GSH), is the chief intracellular non-protein thiol compound, functions as a cellular storage pool of reduced thiols. GSH conjugation is the first step in mercapturic acid synthesis, which aids in the protection of the cell by enhancing the excretion of toxic metabolites from both animals and humans. Therefore, the levels of GSTs have been suggested as important determinant of the susceptibility of organisms or tissues to pharmacological or physiological changes. In particular, the over expression of GSTs in tumours appear to be a factor in the development of acquired resistance towards anti-cancer drugs and hence GSTs are a therapeutic target for rational drug design (Coles and Ketterer, 1990). Several GSTs are polymorphic and some allelic variants causing impaired enzyme activity are suspected to increase susceptibility to malignancies associated with environmental PAH (Strange and Fryer, 1999).

β-methylcholanthrene (MC), a steroidal drug has been extensively studied as the prototype promoting agent (induces phase II enzyme system in rats). Tumor promotion by MC is organ specific and dose dependent (Dragan *et al*., 1996; Devi *et al*., 2002; Reynaud, 2002). The present research was aimed to study the effects of MC on GSTs in rat testis and their characterization. Therefore, measurement of the concentrations and activities of GSTs and their modulation in tissues are subjected to physiological or environmental stimuli may provide researchers with an important tool in monitoring the detoxication potential of cellular systems.

Materials and Methods

Methylcholanthrene (MC) Treatment

Short Term Treatment

Male wistar rats weighing (150-200 g) were injected intraperitoneally with 4 mg of MC in coconut oil every 24 h per each injection to a total of 12 mg. Control animals received vehicle only. The studies were carried out in 2004.

Long Term Treatment

Male wistar rats weighing (150-200 g) were injected intraperitoneally with 1 mg of MC in coconut oil with an interval of a week per each injection to a total of 6 mg. Control animals received vehicle only. The studies were carried out in 2004.

Isolation and Purification of GST

The buffers used for purification of GSTs was: buffer A: 25 mM Tris (hydroxy methyl) amino methane hydrochloric acid (Tris-HCl) pH 8, buffer B: 0.3 M phosphate buffer, pH 6.5; buffer C: 25 mM imidazole HCl, pH 7.0; buffer D: 25 mM triethanolamine- HCl, pH 10.5; buffer E: polybuffer 74 (1:8), pH 4.0; buffer F: polybuffer 96 (1:13): ampholine (1:45), 1:1 v/v, pH 7.0.

The testes was collected from rats killed by decapitation were washed with saline to remove blood and fat debris and a 20% tissue homogenate was prepared in buffer A containing 0.2 M sucrose in a Potter Elvejhem homogenizer with a teflon pestle. This homogenate was centrifuged at for 12000 rpm 1 h at 4°C. The supernatant of this preparation was collected and dialyzed for 24 h against 10 volumes of buffer A with five changes to remove endogenous GSH. The dialysate was considered as cytosolic extract.

Affinity Column Chromatography

The Glutathione CL-Agarose affinity matrix chromatography column (5 mL) was packed into a column (2 x 7 cm) and equilibrated with 100 mL of buffer A to pH 8.0 and the flow rate was adjusted to 60 mL h^{-1}.

The dialysate was loaded into the pre-equilibrated affinity chromatography column and washed with buffer A containing 0.2 M KCl until the absorbance of protein in the eluate was below 0.005 OD at 280 nm. Bound protein was eluted with buffer A containing 5 mM S-hexyl GSH, 2.5 mM GSH and 0.2 M KCl. The eluent was collected in 3 mL fractions. Active fractions with high enzyme activity with CDNB were pooled and dialyzed against buffer A and concentrated by freeze-drying. Isoenzymes of GSTs were separated on chromatofocussing columns, PBE 94 for anionic proteins and PBE 118 for cationic proteins, according to the instructions of the manufacturer.

Enzyme Activity and Substrate Specificity Assays

Glutathione S-transferase activity was measured spectrophotometrically at 340 nm by the method of Habig *et al*. (1974) with CDNB (1-Chloro-2,4-dinitro benzene) as substrate. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of one mole of 2,4-dinitrophenol GSH product per min. Specific activity was expressed as moles of GSH conjugate formed per min per mg of protein.

GST activity with several substrates was determined with the purified proteins (Habig and Jakoby, 1974). The reactions were carried out using different substrates, buffers and initiators in a total volume of 3 mL, the blank reaction without enzyme was substracted from the test values and the activities were calculated using their respective molar extinction coefficients. All enzyme assays were carried out at 25°C. Glutathione Peroxidase (GPx) assay was carried out by monitoring the oxidation of NADPH in a recycling assay (Wendel, 1981). Total GPx activity was determined using Cumene Hydroperoxide (CHP) as substrate. Selenium-dependent GPx activity was measured using H_2O_2 as substrate.

Protein Estimation

Protein contents were determined by the method of Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as the standard and also by measuring absorbance at 260 and 280 nm (Warburg and Christian, 1941). Polyacrylamide Gel Electrophoresis (PAGE) was conducted according to the method of Laemmli (1970).

Immunological Studies

Antisera were raised against affinity purified GSTs of rat testis and liver isozymes by following the published procedure of Tu *et al*. (1985). The Ouchterlony (1966) double immunodiffusion method was followed for cross-reactivity determination of antibody and antigen. Transblot technique was performed according to Towbin *et al*. (1979).

Results

Purification and Characterization of GSTs of Rat Testis

Affinity Chromatography and Chromatofocusing

The cytosolic extracts of rat testis was loaded on to glutathione CL-Agarose affinity matrix chromatography column and on elution the bound protein was eluted as a single sharp peak. The pooled active fractions were assayed for GST activity using CDNB and the specific activity was found to be 32 μ mol^{-1} min^{-1} mg protein for testis (Table 2). The yield of GST protein was 32.9% in testis. The affinity purified testis GST proteins were resolved into six anionic peaks on PBE-94 chromatofocusing column at pH gradient 7 to 4. They were designated as anionic GSTs, t-1 to t-6 based on their elution order (Fig. 3). The unbound protein peak eluted from PBE-94 was resolved into four cationic peaks in testis on chromatofocusing on PBE-118 column at a pH gradient of 10.5 to 7. They were designated as cationic GSTs, t-7 to t-10 based on their order of elution (Fig. 4). The specific activities, total protein and PI values are given in Table 2.

Electrophoresis

GST isozymes were analysed by SDS-PAGE (Table 1). Affinity purified GSTs of rat testis contained four subunits, Yc-27.5, Yb-26.3, Yβ-26.0, Yδ-24.8 kDa (Fig. 1 and 2).

Table 1: Isozyme profile of GSTs of rat testis

		Testis	
Sample	PI	Specific activity*	Subunit composition
Crude	-	1.86	-
Affinity purified GSTs	-	32.00	Yc,Yb,Yβ, Yδ
GST t-1	6.30	6.70	Yc,Yb
GST t-2	6.17	8.40	Yc,**Yb**,Yβ
GST t-3	6.10	7.60	**Yb**,Yβ
GST t-4	5.90	7.17	Yb,Yβ
GST t-5	5.70	3.66	Yβ, Yδ
GST t-6	5.50	5.90	Yβ, Yδ
GST t-7	9.25	14.00	**Yc**,Yb
GST t-8	9.15	12.64	Yb,Yδ
GST t-9	8.80	14.50	Yc,Yb
GST t-10	8.00	13.75	Yb

*One unit is defined as one µmole of GSH conjugate formed/min/mg protein, Bold letters indicates the dominant subunit in the gel

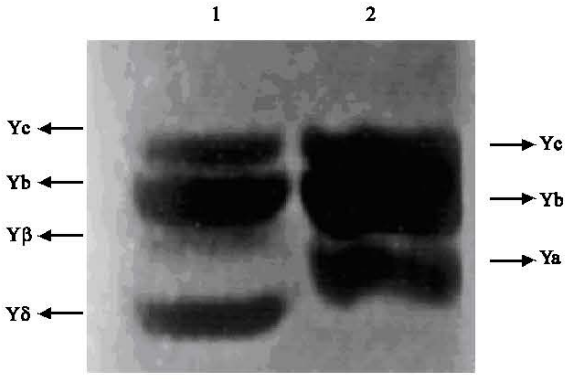

Fig. 1: Testis and liver affinity purified GSTs

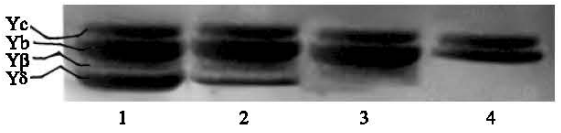

Fig. 2: Testis affinity GSTs (short term treatment)
1 Lane: Control testis; 2 Lane: MC induced testis-4 mg (24 h); 3 Lane: MC induced testis-8 mg (48 h); 4 Lane: MC induced testis-2 mg (72 h)

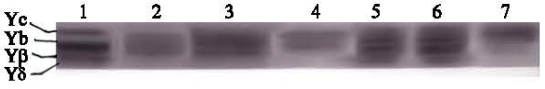

Fig. 3: Testis affinity GST Profile (Long term treatment)
1 Lane: Control testis; 2-7 lanes: MC induced testis GSTs-1-6 mg (1 mg/weekly interval)

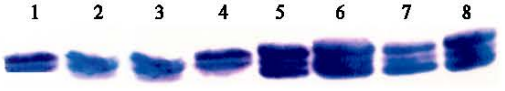

Fig. 4: Western blot with liver GST antibodies
1 Lane : Control testis GSTs; 2 Lane : MC induced testis-4 mg (24 h); 3 Lane : MC induced testis-8 mg (48 h); 4 Lane : MC induced testis-12 mg (72 h); 5 Lane : Control liver GSTs; 6 Lane : MC induced liver-4 mg (24 h); 7 Lane : MC induced liver-8 mg (48 h); 8 Lane : MC induced Liver-12 mg (72 h)

Substrate Specificity

Among all testis anionic isozymes maximum specific activity and yield was observed in t-2 and least in t-5. t-4 and t-6 containing Yβ subunits with Yb as minor component, respectively, showed identical activity towards p-NPA. The GST t-1, t-7 and t-9 containing Yc and Yb subunits showed activities towards CHP. Neither anionic nor cationic GSTs of rat testis showed activity towards p-NBC, BSP and EPNP. The PI values of rat testis GSTs were determined by separating individual

Table 2: Substrate specificities of GST isozyme of rat testis

Sample	CDNB	p-NPA	BSP	EPNP	CHP
Affinity purified GSTs	32.0	0.102	0.051	0.046	0.150
Anionic isozymes					
t-1	6.70	ND	ND	ND	0.050
t-2	8.40	0.062	0.015	0.020	0.001
t-3	7.60	0.075	0.040	0.112	ND
t-4	7.17	0.020	0.040	ND	ND
t-5	3.66	0.025	ND	ND	ND
t-6	5.90	0.070	ND	ND	ND
Cationic isozymes					
t-7	14.00	0.030	ND	0.010	0.350
t-8	12.64	0.250	0.420	0.210	ND
t-9	14.5	0.152	0.200	0.090	0.086
t-10	13.75	0.325	0.300	0.400	0.005

* specific activity: μ mol^{-1} min^{-1} mg protein, ND-Not detectable activity ($p<0.001$)

Table 3: Effect of MC treatment (Short term) on the levels of GSTs and GPx of rat testis with different substrates

MC testis (mg)	1-chloro 2,4-dinitro benzene *	p-Nitrophenyl acetate*	p-Nitrobenzyl chloride*	1,2-Epoxy-3 Bromosulfo phthalein*
Control	31.59±2.27	0.102±0.01	0.842±0.074	0.081±0.011
4	39.45±1.8[a]	0.190±0.02[a]	2.53±0.44[a]	0.165±0.01[a]
8	29.76±2.6[a]	0.032±0.004[a]	0.869±0.048[b]	0.040±0.004[a]
12	25.59±2.4[a]	0.419±0.043[a]	0.234±0.037[a]	ND
MC testis (mg)	2,4-Dichloro nitro benzene*	1,2-Epoxy-3 (p-nitro phenoxy propane*	Cumene hydroperoxide**	Hydrogen peroxide**
Control	0.2700±0.035	1.13±0.3	0.513±0.025	0.739±0.041
4	0.0350±0.008[a]	1.75±0.22[a]	0.780±0.039[a]	0.663±0.01[c]
8	0.0076±0.001[a]	0.60±0.05[a]	0.801±0.012[a]	0.373±0.025[a]
12	ND	0.45±0.15[a]	0.688±0.013[a]	0.736±0.032[ns]

*one unit is defined as micromoles of GSH conjugate formed/min/mg protein, **one unit is defined as micromoles of NADPH oxidized/min/mg protein, Values are average of three separate experiments of three samples. *Doubling the dose per day per 100 g body weight, d=$p<0.05$, NS= Not significant (t-test) Mean±SD, a=$p<0.001$, b=$p<0.01$, c=$p<0.02$

isozymes on chromatofocusing columns, PBE-94 and 118. Testis GSTs had PI between 9.2 and 5.4 (Table 2).

Effect of β-methylcholanthrene on GSTs

Effect of MC on Short Term Treatment

The effect of MC administration as single dose (4 mg) in rats resulted in an increase in GST activity in testis by 1.25 folds compared to control rats (n = 6). The activities of GPx in testis was increased by 1.52 folds compared to control rat testis. Multiple dose treatment with MC showed decreased GST activity by 1.06 folds in 8 mg treatment compared to control and 1.23 folds in 12 mg treatment (Table 3). Where as, GPx levels were elevated by 1.56 folds upon 8 mg treatment and 1.34 fold in testis upon 12 mg treatment.

Effect of MC on GSTs on Long Term Treatment

The effect of MC administration as multiple doses (1-6 mg) in rats resulted in an increase in GST activity in testis by 1.08 folds compared to control rats (n = 6). The GPx activities in testis were increased by 1.25 times when compared to controls. Decreased GST activities were observed in 3 and 4 mg MC treatments (Table 4).

Table 4: Effect of MC treatment (Long term) on the levels of GSTs and GPx of rat testis with different substrates

MC testis (mg)	1-chloro 2,4-dinitro benzene *	p-Nitrophenyl acetate*	p-Nitrobenzyl chloride*	1,2-Epoxy-3 Bromosulfo phthalein*
Control	10.8±0.271	0.299±0.053	0.820±0.19	0.8100±0.011
1	10.2±1.3[d]	0.238±0.024[a]	0.713±0.167[b]	0.5350±0.01[a]
2	5.58±1.5[a]	0.151±0.022[a]	0.789±0.001[a]	0.6800±0.002[a]
3	6.69±0.41[a]	0.830±0.052[a]	0.405±0.045[d]	0.0220±0.0004[a]
4	8.55±0.72[b]	1.661±0.196[a]	0.361±0.033[a]	0.2300±0.0035[a]
5	11.36±0.65[d]	2.175±0.519[a]	0.332±0.114[a]	0.0135±0.0005[a]
6	11.70±2.23[d]	1.617±0.431[a]	0.163±0.03[a]	0.0200±0.001[a]
MC testis (mg)	2,4-Dichloro nitro benzene*	1,2-Epoxy-3 (p-nitro phenoxy propane*	Cumene hydroperoxide**	Hydrogen peroxide**
Control	0.085±0.017	0.791±0.31	0.529±0.135	0.483±0.145
1	ND	1.838±0.50[a]	0.688±0.12[a]	0.592±0.315[a]
2	ND	0.713±0.51[c]	0.270±0.042[a]	0.315±0.002[a]
3	0.016±0.006[a]	0.895±0.091[a]	0.162±0.005[a]	0.174±0.038[a]
4	0.160±0.048[a]	0.416±0.058[a]	0.263±0.05[a]	0.108±0.047[a]
5	0.008±0.001[a]	1.218±0.472[a]	0.403±0.057[b]	0.215±0.09[a]
6	0.031±0.002[a]	0.349±0.048[a]	0.659±0.198[a]	0.150±0.002[a]

*one unit is defined as micromoles of GSH conjugate formed/min/mg protein, **one unit is defined as micromoles of NADPH oxidized/min/mg protein. Values are average of three separate experiments of three samples. *Doubling the dose per day per 100 g body weight, d=p<0.05
Mean±SD, a=p<0.001, b=p<0.01, c=p<0.02

Substrate Specificity on MC Treatment

The specific activity of GSTs of rat testis with the substrates CDNB, DCNB, p-NPA, p-NBC, EPNP and BSP and GPx with CHP and H_2O_2 was determined (Table 4). In MC treated testis samples on short-term treatment with 4 mg MC, higher activities were observed with CDNB, p-NBC and EPNP, p-NPA and BSP. GPx levels were increased with CHP in testis samples on short-term treatment. On exposure to MC, the largest activity variation was found towards BSP and EPNP. The testis GST activities with substrates CDNB, p-NBC, EPNP, p-NPA and BSP found to be increased by 1.25, 3.0, 1.54, 1.86 and 2.03 fold of control, respectively. The GPx levels with substrate CHP increased by 1.52 fold in testis with respect to controls.

In MC treated testis on short term treatment (8 mg MC), decreased activities were observed with CDNB, BSP, EPNP, p-NPA in the order of 2.52, 2.02, 1.88 and 3.18 folds, respectively when compared to controls. The GPx levels with substrate H_2O_2 decreased by 1.98 folds in testis with respect to controls, but with CHP non-differential change was observed. The highest activity was observed with p-NBC by 1.03 folds compared control testis.

In MC treated testis on short term treatment (12 mg MC), decreased activities were observed with CDNB, EPNP and p-NBC in the order of 1.23. 2.51 and 3.59 folds, respectively when compared to controls. Where as P-NPA and BSP elevated the specific activities by 4.10 and 1.53, folds, respectively. The Gpx levels with substrate CHP was increased by 1.34 fold over control. Non-significant change was observed with H_2O.

In MC treated testis on long term treatment (1-6 mg MC), substrate specificity with CDNB GST activities decreased gradually from 1 to 4 mg MC treated testis, then increased gradually from 5 and 6 mg MC treatments compared to control. The decreased activities were observed with BSP and p-NBC on MC treatment (1-6 mg) when compared to control. The substrates p-NPA and EPNP elevated the GST activities by 7.2 and 1.53 folds upon 5 mg MC treatment over control. The substrates DCNB and p-NPA elevated the GST activities by 1.88 and 5.5 folds upon 4 mg MC

Table 5: Effect of MC on subunit composition of GSTs of testis cytosols as evidenced by immunoprecipitation with rat liver and testis GST antibodies

	Subunit composition precipitated with anti-GST of	
Samples	Rat liver	Rat testis
Control liver	Yc, Yb, Ya	Yc, Yb
Control testis	Yc, Yb, Yβ	Yc, Yb, Yβ, Yδ
MC testis 4 mg	**Yc**, Yb,**Y**β	**Yc**, Yb, **Y**β,
testis 8 mg	**Yc**, Yb, Yβ	**Yc**, Yb, Yβ, Yδ
testis 12 mg	**Yc**,Yb	**Yc**, Yb,Yβ
testis 1 mg	**Yc, Yb**,Yβ	**Yc, Yb**,Yβ, Yδ
testis 2 mg	**Yc, Yb**,Yβ	**Yc, Yb**,Yβ, Yδ
testis 3 mg	**Yc, Yb**,Yβ	**Yc, Yb**,Yβ, Yδ
testis 4 mg	**Yc, Yb**	**Yc, Yb**,Yβ
testis 5 mg	**Yc, Yb**,Yβ	**Yc, Yb**,**Y**β, Yδ
testis 6 mg	**Yc, Yb**,Yβ	**Yc, Yb**,Yβ, Yδ

Bold letters indicates the dominant precipitated bands in the gel

treatment over control. GPxlevels with CHP were decreased in sequential order from 1 to 5 mg, again increased in 6 mg MC treatment by 1.24 folds, respectively. Upon 6 mg MC treatment, GST and GPx levels both increased preferentially GPx-II (non-selenium dependent) over control.

Immunology

Antisera against affinity purified GSTs of rat testis, liver (raised in our laboratory) GSTs, showed immunoprecipitin bands with affinity purified GST proteins of the respective tissues of rat. Cross-reactivities of (i) control (ii) MC (iii) induced GST proteins of testis (A) liver (B) as antigens with the antisera of affinity purified GSTs of testis was given. In treated proteins differential expression of cross reactivity was observed in both liver and testis (Table 5). Both dot and protein transblot immunoprecipitation analyses showed different results for long term and short term treatment when compared to controls. In short term treatment (12 mg MC) Yc of α-class and Yb μ class were expressed, whereas on long term treatment (1-6 mg MC), Yc of α-class and Yb and Yβ of μ class were induced (Fig. 4-8).

Discussion

Male infertility and abnormal progeny outcome are some of the consequences resulting from the exposure of germ cells to stressors such as environmental chemicals and drugs (Hales and Robaire, 1997). Testicular cancer is a disease in which cells become malignant in one or both testicles. Testicles produce and store sperm and are also the body's main source of male hormones. These hormones control the development of reproductive organs and male characteristics. An estimated 7,400 men in US will be diagnosed with testicular cancer in 1999. During the male germ cell development, cells have different abilities to cope with diverse types of stress such as oxidative stress and protein and DNA damage. A hypothesis tested that sustained induction of GST enzymes in testis by 3-MC is mediated by mechanisms due to long-term retention of the carcinogen in the body. Persistent induction may enhance metabolism of PAHs, leading to generation through redox cycling of increasing amounts of reactive species which may cause oxidative DNA damage (Morrthy, 2000; Ichinose *et al*., 1997).

Rat GSTs are encoded by a multigene family and are differentially regulated in a tissue specific manner (Abramovitz *et al*., 1988) to meet the special detoxication needs of various organs

(Awasthi and Singh, 1985). The isoforms may be distinguished on the basis of differences in binding specificities and catalytic properties toward various molecules (Tahir and Mannervik, 1986). The testis and brain cytosols have high GST activity but their isozyme pattern differs from each other with respect to the species as well as organ (Guthenberg *et al*., 1983).

In sheep lung cationic and anionic isozyme patterns were observed (Thyagaraju *et al*., 1994) in the same way as the affinity purified rat testis GSTs were separated into six anionic and four cationic isozymes and as brain consists of four anionic and three cationic isozymes. Maximum (68%) GST activity was associated with the anionic isozymes and less (32%) was found with the cationic isozymes in brain. The testis GST showed 65% of cationic isozymes and 35% of the anionic isozymes. These results on rats indicate that the cationic isozymes in testis and anionic isozymes in brain are abundant.

In rat testis the basic GSTs, showed very high activity towards CHP. The three rat testis isozymes 9.2 (t-7), 8.6 (t-9) and 6.2 (t-1), which are hetero dimers of Yc subunits showed more non-selenium dependent GPx activity. This GPx II activity associated with cationic GSTs of testis might protect the testis tissues from oxidative stress (Chang *et al*., 1987; Thyagaraju *et al*., 1996). The rat testis isozymes having the PI values of 5.8 (t-4) and 5.4 (t-6), showed activity towards p-NPA. The p-NPA studies demonstrated that some of the isozymes at this PI values are having identical proteins.

The existence of cytosolic GSTs as homo, hetero, di and trimers was reported in rat tissues by Smith *et al*.(1995) and Thyagaraju *et al*. (1997). This type of combination of subunits in GST may occur due to the exchange of gene product at the level of transcription and translation and may offer protection from various xenobiotics (Kaplowitz *et al*., 1975) and even with slightly modified xenobiotics. Gene conversion may be an important mechanism for generating sequence diversity in the GST multigene family. The generation of sequence divergence between members of related genes allows the expression of protein with broad overlapping substrate specificities (Morton *et al*., 1990). These mechanisms should influence the GST proteins to have either different or identical functions with testis GST proteins at a single PI value.

Western blot analysis of β-methylcholanthrene treated testis cytosol with rat liver GST antibodies showed induction of Yc on 12 mg MC treatment (Vasundhara *et al*., 2002). However with testicular GST antibodies analysis showed induction of Yc, Yb and Yβ. The Ya subunit of liver was absent in rat brain and testis (Li *et al*., 1986; Devi *et al*., 2002). The induction of specific isozymes in rat testis was also confirmed by dot blot analysis using a GST subunit specific antiserum. The testis GSTs contained more of Yb subunits, which are separated at near neutral points on chromatofocusing. Both brain and testis GSTs comprised more of Yb and Yβ subunits, which may indeed play a pivotal role in the detoxication process. The testis cationic GSTs has more of GPx activity rather than the brain isozymes. The existence of extra Yβ and Yδ subunits in testis are in accordance with the earlier reports of Li *et al*. (1986).

Many detoxication enzymes exist as isozymes with multiple genes and exhibit differential expression with a wide variety of xenobiotics to prevent chemical lesions such as mutagenesis, carcinogenesis and tissue necrosis (Guthenberg *et al*., 1980). Purified GST μ and θ have high activities against polycyclic aromatic hydrocarbon epoxide metabolites that may be generated from constituents of tobacco smoke (Hussey and Hayes, 1992; Robersson *et al*., 1986). Over expression of α GSTs in enhances their protection from necrotic toxicity produced by carcinogens.

GSTs exhibit differential expression to a wide variety of xenobiotics. In this study the activity levels of GST and the GSH-mediated peroxidases, were increased significantly in testis on exposure

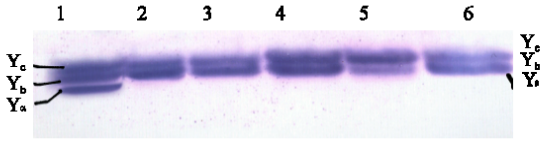

Fig. 5: Western blot with liver GST antibodies
1 Lane: Control liver GSTs; 2-6 Lanes : MC induced testis : 2-6 mg (1 mg/weekly) (2-6 week)

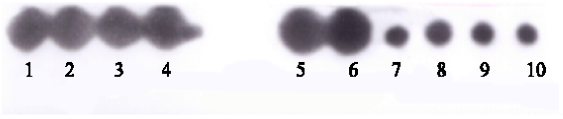

Fig. 6: Dot blot with rat GST-Yd antibody
Dot 1: Control testis (15 μg); Dot 2-4: MC treated 4 mg (24 h), 8 mg (48 h), 12 mg (72 h) Dot 5-10: MC treated 1-6 mg (1 mg/week)

MC on multiple doses. This result is in accordance with the earlier reports that the GST levels were induced in liver, small intestine and kidney by intraperitoneal administration of PAH (Clifton and Kaplowitz, 1978). PAH represents a major group of chemical carcinogens, including β-methylcholanthrene, many of which are substrates for class μ and π GST (Philips, 1983). Therefore the induction of GST serves to overcome the deleterious effects caused by MC. This was further confirmed by immunological studies.

Variation in activity levels of GST in testis are in agreement with the earlier studies of Kaplowitz *et al.* (1975) and further showed that the GST activity was variable towards CDNB MC treatment. The classes of GST isozymes differed in their specificity toward xenobiotic or endogenous substrates such as Acrylamide, PB and MC. The CDNB is the main substrate, which undergoes nucleophilic displacement of the chloro moiety by GSH. All classes (α, μ, π) of GSTs except θ catalyze this reaction. The most characteristic feature of testicular GSTs is in association of approximately 40% of total cytosolic activity with proteins having isoelectric points below pH 7 (Chang *et al.*, 1987). These results are further confirmed by Western blot analysis. Cytosol of MC treated testis showed cross reactivity with Yc, Yb and Yβ subunits.

On short term treatment with MC in testis, in single dose treatment with 4 mg MC, there is an increase in the GST activity, then a gradual decrease was observed with 8 and 12 mg MC treatments. In contrast, GPx II levels were elevated gradually from 4 to 12 mg treatments. This shows that to prevent oxidative damage, Yc subunit increased to counter testicular damage (Fig. 5). In addition to GPx II levels (non-selenium dependent), the substrate specificity studies indicated expression of Yb family of μ class with 12 mg MC (Fig. 6). In long term treatment there was a gradual increase in GST specific activities from 1-6 mg treatment and also a gradual increase in GPx II levels (non-Se dependent) with MC were observed in rat testis with CDNB, p-NPA and CHP. Where as EPNP, DCNB, p-NBC given different GST activities, because GSTs having overlapping substrate specificity.

The MC treated testis on short-term treatment, testis samples showed the induction of Yc and Yb prominently (Fig. 2) while on long term treatment samples showed Yc, Yb and Yβ subunits (Fig. 3). The induction of specific isozymes in rat testis was also confirmed by dot blot analysis using a GST subunit specific antiserum (Yc, Yb1, Yb2 and Ya). These results indicated that Yc, Yb1 and

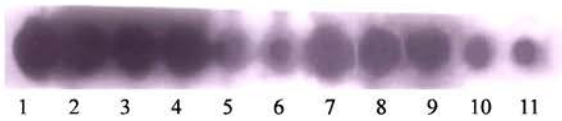

Fig. 7: Dot blot with rat GST-Yc antibody
Dot 1: Control testis (15 μg); Dot 2-4: MC treated 4 mg (24 h), 8 mg (48 h), 12 mg (72 h) Dot 5-10: MC treated 1-6 mg (1 mg/week)

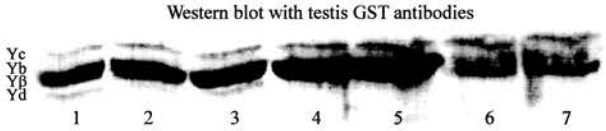

Fig. 8: Western blot with testis GST antibodies
Lane 1: Control testis
Lane 2-4: MC induced testis (4 mg (24 h) 8 mg (48 h) 12 mg (72 h))
Lane 5: 4 mg MC testis lane
Lane 6: 5 mg MC testis
Lane 7: 6 mg MC testis
Lane 5-7: Mc induced testis (1 mg/weekly interval)

Yb2 subunits of rat testis have sequence homology with rat liver respective Yc and Yb GSTs, because these proteins showed immunoprecipitation with rat liver antisera (Fig. 4 and 5). Whereas testis GST antisera showed immunoprecipitation of Yδ subunit in control, but Yδ is not related to any one of the subunits of rat liver GSTs (Fig. 8). Therefore testis contains more of μ subunits (Yb1-b1, Yb2-b2), they are preferentially induced by β-methylcholanthrene because it is a good substrate for μ class GSTs (Philips, 1983).

The over expression of α (Yc) and μ (Yb, Yβ) class GSTs in MC treated testis in rapidly multiplying cells indicates that they can act as a marker of neoplasia and thereby play a role as chemoprotector. Additionally, over expression of α GSTs in testis cells enhances their protection from necrotic toxicity produced by MC. On short term treatment, with doubling dosage (4-12 mg for 24-72 h), male rat reproductive system affected with degenerative changes. But in long term treatment with low dosage of MC and prolonging time (1-6 mg for 1-6 weeks), there was mild degenerative changes in testis by 4 mg MC treatment and there was some regenerative changes were observed with 5 and 6 mg dosages of MC compared to control rat testis. So, MC might be acting as a chemoprotector on small doses provided substantial protection against the generation of tumors by inducing testicular GST subunits on long term treatment (Talalay, 1992). Elevated α GSTs have been found in plasma of patients with hepatocellular damage caused by hypoglycemia (Aliya *et al*., 2003), birth asphyxia (Beckett *et al*., 1989), or autoimmune chronic hepatitis (Hayes *et al*., 1986). These studies have been related to acute or chronic liver disease and renal damage (Beckett and Hayes, 1993; Feinfeld *et al*., 1981). The α GSTs were also a better measure for successful intervention to alleviate rejection following transplantation, thereby demonstrating the important clinical use of monitoring GST levels in biological tissues (Trull *et al*., 1994). The presence of *GSTM3(Mu)* allele (Loktionov *et al*., 2001) has been associated with both increased risk for basal cell skin

(Yengi *et al*., 1996) and laryngeal (Jourenkova-Mironova *et al*., 1999; Jourenkova-Mironova *et al*., 2000) cancers and a protective effect for lung (To-Figueras *et al*., 2000) and oral (Park *et al*., 2000) cancers. It is also possible that cancer risk may be affected by other combinations of particular genetic variants of the polymorphic GST enzymes; however, interactions between different members of the GST family in terms of affecting pathogenesis of tumours are still poorly understood.

The α (Yc) and μ (Yb, Yβ) classes induced by MC should inhibit the initiation of chemical carcinogenesis by detoxication process and thereby acts as a chemo protector. Elevated levels of α, μ and π GSTs have been associated with protection of tissues from cytotoxicity produced by MC. Therefore, measurement of GST α and μ class expression have clinical benefit for monitoring therapeutic progression of cancerous disease or identification of populations susceptible to chemotherapeutic interventions.

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