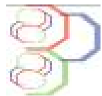

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Effect of cyclophosphamide on transcription of SOD1mRNA and GPX1 mRNA in mice liver and brain tissues

Hanaa AS Oraby*, Amal AM Hassan, and Nadia AF Aboul Maaty
Cell Biology Department, National Research Center (NRC), El Behoth Street, Dokki, Cairo, Egypt

***Corresponding author:** haoraby@gmail.com Tel: 00202 3858 0836 Fax: 00202 2390 1199; Mobile: 002011 370 1794
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ABSTRACT
Objectives: Limited information is available on the effect of chronic administration of cyclophoshamide (CP) on the transcription of genes coding for antioxidant enzymes. This work studied the effect of repeated doses of CP on transcription of genes coding for superoxide dismutases 1 (SOD1) and glutathione peroxidase 1 (GPX1) in mice liver and brain tissues.
Methodology and results: Three groups of mice were injected intraperitoneally with repeated doses of cyclophosphamide (4mg/kg) for 5, 10 or 15 consecutive days, respectively. Changes in transcription levels of SOD1 and GPX1 were assessed using reverse transcriptase polymerase chain reaction (RT- PCR) technology. Results indicated that transcription levels of GPX1 declined in liver tissue of treated animals whereas SOD1 transcription was slightly activated compared to the control. In brain cells, a dramatic depression in GPX1 transcription occurred after chronic administration of CP for five consecutive days. Exposure to CP for 10 or 15 days up-regulated the transcription of GPX1 mRNA significantly compared to the five days treatment group. Comparatively, significant decrease of transcription levels of SOD1 mRNA occurred at all CP exposure levels compared to the control.
Conclusion and application of findings: Chronic treatment of mice with cyclophosphamide affected the transcription levels of SOD1 mRNA and GPX1 mRNA in liver and brain tissues. These changes reflect the adverse effects of CP on the transcription levels of genes coding for two of the antioxidant enzymes defense system. The se results suggest that the liver is injured more by chronic treatment with CP and that an adaptive response develops in the brain cells by up regulating GPX1 gene transcription. These results further suggest there is need for precautions to be adopted among nurses, pharmacists, and workers who are occupationally exposed to cyclophosphamide during manufacturing, producing, distributing and dispensing of the drug.
Key words: Cyclophosphamide, oxidative stress, gene transcription, SOD1 and GPX1.

INTRODUCTION
Cyclophosphamide is an alkylating agent used for the treatment of malignant and non-malignant disorders (Dollery, 1999). It exerts its antitumor effects through different mechanisms which interfere with the synthesis of the genetic material of cancer cells leading to disruption of DNA function and cell death (Masta *et al.,* 1994, 1995). It also has pro-oxidant characters and its

administration is associated with the induction of oxidative stress by the formation of reactive oxygen species (ROS) (Weijl *et al.,* 1997). Such oxidative stress causes massive cellular damage associated with lipid peroxidation and alterations of proteins and nucleic acids (Mignotte & Vayssiere, 1998), consequently triggering apoptosis (Napolitano & Singh, 2002) and death of the cancerous cells. Healthy cells are also exposed to the same damage induced by CP (Stankiewicz *et al.*, 2002). CP uptake into healthy cells has been reported to be higher than its uptake into cancerous cells (Bohnenstengel *et al.,* 2000) rendering healthy cells even more susceptible to damage.

Patients are not the only group of people who are exposed to hazards from the use of CP. Significant genotoxic effects and genetic damage (e.g. increased micronuclei formation, sister chromatid exchange and chromosomal aberrations) have been reported in pharmacists and nurses who are occupationally exposed to cyclophosphamide (Baker *et al* , 1987) during its production or distribution.

All living organisms have evolved several mechanisms to suppress oxidative stress and minimize damage by ROS (Halliwell & Gutteridge, 1989). These mechanisms include the antioxidative system which is composed of free radical scavengers, endogenous antioxidant enzymes and antioxidant metabolites that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids (Sies, 1997; Vertuani *et al* ., 2004).

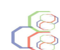
The endogenous antioxidant enzymes include catalase, superoxide dismutases (SOD) and glutathione peroxidases (GPX). There are different forms of SOD and GPX. Copper/Zinc Superoxide dismutase (SOD1) and selenium-dependent cellular glutathione peroxidase (GPX1) are the two major and most abundant intracellular antioxidant enzymes in mammals. SOD1 catalyses the conversion of superoxide anions into H_2O_2 which is further scavenged by GPX1 and converted to H_2O and thus protects biomembranes and cellular components against oxidative stress (Brigelius-Flohe, 1999). These two enzymes are perceived to function consecutively with similar roles in coping of cells with oxidative stress (Prohaska & Brokate, 2001; Brigelius-Flohe & Flohe, 2003; Comhair *et al.*, 2005) mediated by pro-oxidants that primarily induce generation of reactive oxygen species (Ho *et al* ., 1998; Cheng *et al* ., 1999).

Many studies have previously investigated the effect of chronic administration of CP (i.e. administration of repeated doses of CP over a period of time) on the activity of antioxidant enzymes in different tissues of experimental animals (Rekha,*et al* . 2001, *Selvakumar et al ., 2006;* Senthilkumar *et al* , 2006;). Wasserman and Fahl (1997) demonstrated that oxidative stress associated with cyclophosphamide treatments may alter essential cellular functions such as gene expression. CP can inhibit transcription and translation as a result of DNA damage (Masta *et al* ., 1995). It also induces the formation of cross-links which may cause a pause or arrest of the RNA polymerases, consequently resulting in inhibition of DNA replication through the formation of a second covalent bond with an adjacent DNA substituent, a protein, or RNA (Lamson & Brignall, 1999) adversely affecting transcription and gene expression (Masta *et al* , 1994).

However, limited information has been reported on the effect of chronic administration of cyclophoshamide on the level of transcription of genes coding for antioxidant enzymes especially in liver and brain tissues. Therefore, this work aimed to generate more information in this field of science.

MATERIALS AND METHODS

Experimental animals and treatments: Male albino mice of the genus Mus musculus (Swiss strain), aged 3-6 month were provided by the animal house of the National Research Center (Cairo, Egypt). Animals, weighing 20 -30 g, were maintained under standard laboratory ambient



conditions, housed in cages (6 mice each) at a constant temperature approximately 22◦C with a 12 h light/dark cycle. Food and water were given *ad libitum*. The study was approved by the animal ethics committee of the National Research Center in Egypt. Mice were randomly divided into four groups (6 mice each). One group was injected intraperitoneally (i.p) with sterile water as a vehicle and considered as a control. The other three groups were injected intraperitoneally with repeated doses of cyclophosphamide (4 mg/kg) for three different lengths of time, i.e. group 1 was injected repeatedly for five consecutive days, group 2 for ten days and group 3 for 15 consecutive days. Mice were sacrificed by cervical dislocation at the end of each treatment.

RNA extraction and RT-PCR assay: Liver and brain tissues were excised from each animal of the control and treated groups. Total RNA was isolated from liver and brain samples using Pure Script RNA Isolation Kit (Gentra, USA) according to the protocol of the manufacturer.

The quality and concentration of total RNA were measured at 260 nm using Ultraviolet Spectrophotometer (Pharmacia LKB .Utrospec 111, McAllen, TX, USA). Equal concentrations of RNA from each sample were prepared for the subsequent reverse transcription and polymerase chain reaction technique (RT-PCR). Reverse transcription was carried out using Ready–To-Go You-Primer First-Strand Beads (Amersham Biosciences UK) at 37°C for 1 h using Pd(N)6 primer to generate the first strand cDNA.

The cDNA samples were subjected to PCR amplification using the MJ Research PTC-100 thermocycler (MJ Research Inc, MA, USA) under optimum conditions for each pair of primers of the investigated genes. Table 1 shows the sequences of the used primers, annealing temperatures (ta) and the length of the amplified segment (amplicon) for each of the investigated genes. β-actin is a housekeeping gene that is used as a reference gene. Polymerase chain reactions for each gene were performed in 25 µl reaction mixture consisting of 100 ng of cDNA, 5 pmoles each oligonucleotide primer, 200 µM of each dNTP, and 1.25 units of Taq Polymerase (Sigma-Aldrich, Oakville, ON, Canada). The PCR program initially started with a 95 °C denaturation for 5 min, followed by 30 cycles of 95 °C /2 min, ta °C /1 min, 72 °C /2 min. A final extension step for 10 min at 72ºC was performed in order to complete the PCR reaction. The PCR products were stained with 10 µg/ml Ethidium Bromide (EB) then separated on 1.5% agarose gel electrophoresis.

The ethidium bromide-stained gel bands were documented using gel electronic documentation system BioDocAnalyze (Biometra biomedizinische Analytik GmbH). The signal intensities of the gel bands were semi-quantified using the computerized Gel-Pro image software analyzer (Version 3.1 for Windows).

Statistical analysis: The results of transcription of SOD1, GPX1 and β-actin as expressed by maximum optical densities (Max.OD) in the control and different groups of mice treated with cyclophosphamide (4mg/kg) for five, 10 or 15 consecutive days were recorded for each sample in both liver and brain tissues. Levels of transcription of GPX1 mRNA and SOD1 mRNA were then normalized for each animal in comparison with the levels of transcription of β-actin mRNA as a reference control. Results were expressed as mean values of the maximum optical density (max. OD) ± Standard error (SE.) for each group. Analysis of data was performed with one-way ANOVA (Analysis of Variance) using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was regarded as statistically significant.

The multiple comparisons for the transcription levels of β-actin data (table 2) revealed no statistically significant differences between levels of expression of β-actin mRNA in all the experimental groups of mice.

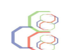


Table 1: DNA sequences, annealing temperatures (ta) and amplicon size of the primers tested.

Tested genes	Primer Sequence 5´-------3´	Annealing temp.	Amplicon Size (bp)
GPX1	Forward: GGGCTCCCTGCGGGGCAAGGT Reverse: ATGTACTTGGGGTCGGTCATG	64C°	354 bp
SOD1	Forward: AAGGCCGTGTGCGTGCTGAA. Reverse: CAGGTCTCCAACATGCCTCT.	60C°	246 bp
β-actin	Forward: CGTATTAAGGAGAAGCTGTGC Reverse: CTCAGGAGGAGCAATGATCTTGAT	64C°	374 bp

Table 2: Transcription levels of β-actin mRNA in liver and brain cells of control and mice treated with cyclophosphamide (4 mg/kg. B.W.) for 5, 10 and 15 consecutive days as expressed by maximum Optical Density (Max. OD) ± SE.

Treatments	Liver β-actin Mean Max.OD ± SE	Brain β-actin Mean Max. OD ± SE
Control	115.29±0.6893	207.88±21.1083
CP 5 days	92.29±4.6211	198.08 ±20.3817
CP 10 days	103.41±8.2781	197.02±29.7808
CP 15 days	106.65±10.6461	195.92±31.9572

Note: No statistically significant differences were obtained in each tissue.

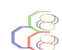
RESULTS

In liver tissue, insignificant increases in the transcription levels of SOD1 mRNA were recorded after CP treatment for five days (1.38±0.0624), 10 days (1.16±0.2404) and 15 days (1.41±0.4284) (Table 3) compared to the control (1.09±0.0685). CP treatments for five consecutive days did not, significantly affect the transcription levels of GPX1 but after administration for 10 and 15 days the transcription levels declined significantly in liver to 0.66±0.1063 and 0.19±0.0400, respectively, compared to 1.18±0.1572 for the control and 1.13±0.0232 for the five day treatment groups.

In brain cells, a dramatic depression in the levels of transcription of GPX1 occurred after chronic administration of CP for five consecutive days (0.33±0.0304) compared to the control (0.79±0.0210). Further exposure to CP for 10 days and 15 days up-regulated the transcription levels of GPX1 mRNA significantly to 0.66±0.0706 and 0.85±0.036, respectively, compared to the five days treatment group. On the other hand, statistically significant decrease of transcription levels of SOD1 mRNA occurred in the brain cells after chronic administration of CP for five (0.12±0.0188), 10 (0.17±0.0276) and 15 consecutive days (0.19±0.0293) compared to the control (0.48±0.0107).

DISCUSSION

Cyclophosphamide treatment is associated with induction of oxidative stress that may alter essential cellular functions such as gene expression (Wasserman & Fahl, 1997). Chronic CP treatment has been previously reported to decrease the transcription of all antioxidant genes in rat male germ cells (Aguilar *et al* , 2002). However, limited studies have been reported on the effect of CP on the transcriptional levels of genes coding for antioxidant enzymes especially in liver and brain tissues. Most of the available reports investigated the effect of single doses of CP on the activities of antioxidant enzymes



(Sie,1997; Ventuani *et al* ., 2004) rather than on the transcriptional levels of genes coding for these enzymes. In the present study, chronic treatment of mice with cyclophosphamide resulted in a substantial variation in the transcription levels of SOD1 mRNA and GPX1 mRNA that are involved in the antioxidant defence mechanism in liver and brain cells.

Table 3: Normalized values of transcription levels of SOD1 and GPX1 relative to β-actin in liver and brain tissues of mice treated with water (control) and CP (4 mg/kg. B.W.) for 5, 10 or 15 consecutive days.

	Liver		Brain	
CP treatments	SOD1	GPX1	SOD1	GPX1
Control	1.09±0.0685	1.18±0.1572	0.48±0.0165	0.79±0.0210
CP (5 days)	1.39±0.0624	1.13±0.0233	0.12±0.0188*	0.33±0.0304*
CP (10 days)	1.16±0.2404	0.66±0.1063*[a]	0.17±0.0276*	0.66±0.0706*[a]
CP (15 days)	1.41±0.4248	0.19±0.0400*[a]	0.19±0.0293*	0.85±0.0360[ab]

Value are mean ± SE; * P<0.05 compared to control; [a] P<0.05 compared to the 5 days treated group;
[b] P<0.05 compared to the 10 days treated group

In liver cells, slight insignificant increases in the transcription levels of SOD1 mRNA occurred in treated mice compared to the control. On the enzymatic level, it is known that SOD is the first line of defence against deleterious effects of oxyradicals in the cells. It catalyses the dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 which is further converted to H_2O by GPX (Mruk *et al* ., 2002). Transcription levels of GPX1 mRNA in the present investigation declined in liver cells after chronic administration of CP for 15 days. This reduction was significant when compared to the control and to mice treated for five days. It is known that when GPX is inadequate , there is reduced degradation of H_2O_2, which implies that more H_2O_2 is converted to toxic hydroxyl radicals that may contribute to the oxidative stress due to CP toxicity (Selvakumar *et al* ., 2005).

Michiels *et al* . (1994) and Muchova *et al* . (2001) suggested that the sensitivity of cells to free radicals depends on the equilibrium between the formation of hydrogen peroxide by SOD and its degradation by GPX or CAT, rather than on the activities of individual antioxidant enzymes. The balance of this enzyme system has been reported to be essential in the disposal of the superoxide anion and peroxides generated in the cells (Selvakumar *et al.,* 2005). Therefore, the reduction in GPX1 mRNA transcription in liver cells after chronic treatment with CP for 15 consecutive days reflects the adverse effects of CP on this finely balanced antioxidant system. This may be attributed to the fact that the liver is the first site for drug metabolism (Arumugam *et al.,* 1997) where local bioactivation of the parent compound CP, rather than transport of the active metabolites, may contribute to the effect of CP on levels of transcription of GPX1 mRNA and SOD1 mRNA.

In brain cells dramatic depression occurred in the levels of transcription of SOD1 and GPX1 after chronic administration of CP for five days. This decrease may contribute to a redox imbalance, since exposure to CP was reported previously to induce the formation of ROS (Sulkowska *et al* ., 1998; Adams & Klaidman, 1993) and lipid peroxidation (Berrigan *et al* ., 1987), which leads to cellular injury (Hamilton *et al* ., 1998). Decline in the activities of these enzymes (GPX and SOD) has been previously attributed to their inactivation caused by excess ROS production (Pigeolet *et al.,* 1990). A decrease in SOD activity has been related to increased levels of superoxide anion, which is known to inactivate GPX enzyme (Pigeolet *et al* ., 1990).

Although, the brain is relatively more susceptible to free radical damage than other tissues because it is rich in lipids and iron, and both are known to be important in generating reactive oxygen species (Halliwell & Gutteridage, 1989), the results of the present work showed that further exposure to CP caused a significant increase in the transcription levels of GPX1 mRNA in the brain cells after CP administration for ten and 15 days compared to application for five days. These up-regulations in the GPX1 mRNA transcription may be attributed to an adaptive phenomenon in mice brain cells to cope with CP-induced increase of oxidative stress (Sanchez-Reus *et al* ., 2007). An adaptive response to oxidative stress induced by CP via up-regulation of GPX activity was also suggested by Zhang *et al* . (2006).

CONCLUSION AND RECOMMENDATIONS

This study has shown that chronic treatment with cyclophosphamide changes the transcription levels of SOD1 mRNA and GPX1 mRNA in liver and brain tissues of treated mice. These changes reflect the adverse effects of CP on the transcription levels of genes coding for two of the antioxidant enzymes defense system. The results of this study also suggest that the liver is more injured by chronic treatment with CP and that an adaptive response develops in the brain cells by up regulating GPX1 gene transcription. Since healthy cells are more susceptible to the hazards associated with exposure to cyclophosphamide, more precautions should be adopted among nurses, pharmacists, and workers occupationally exposed to cyclophosphamide during manufacturing, packaging, distribution and dispensing of the drug.

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