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Duration of exposure modulates combined effects of arsenic and fluoride on oxidative stress in the liver of rat

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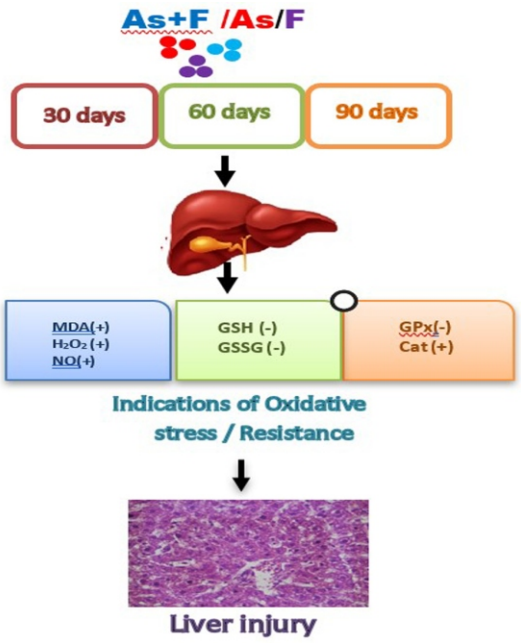
Abstract

Aim: Exposure time dependent combined effects of arsenic (sodium arsenate) and fluoride (sodium fluoride), two major ground water pollutants, have been studied on oxidative stress and antioxidant mechanisms in the liver of female Wistar rats.

Methodology: Healthy rats were treated with sodium arsenate (4mg kg^{-1} b.wt.) and sodium fluoride (4mg kg^{-1} b.wt.) for three-time intervals, *i.e.,* 30, 60 and 90 days through gavage. Liver tissues of exposed rats were analysed for malonaldehyde (MDA), hydrogen peroxide (H_2O_2), nitric oxide (NO), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase (GPx) and catalase (CAT).

Results: Combined exposure of rats to arsenic and fluoride for selected period of exposure viz. 30, 60 and 90 days yielded different results on parameters of oxidative stress in liver. The highest values for malondialdehyde (0.610 nmoles mg^{-1} protein), hydrogen peroxide (2.31µ moles) and nitric oxide (2.57µ moles) were recorded after 60 days of exposure. Maximum decline in GSH (0.154µ mol g^{-1} liver) after 30 days of exposure, in GSSG (110µ mol g^{-1} liver) after 90 days of exposure, GPx (1.03 nmoles NADPH min mg^{-1} protein) after 30 days of exposure and catalse (74.96 units) after 30 days of exposure were registered. Results showed resistance against oxidative stress after 90 days of combined exposure to arsenic and fluoride.

Interpretation: It is hypothesized that internalization of arsenic and fluoride in the hepatic parenchyma leads to the formation of a compound, arsenic trifluoride (AsF3). It was found less toxic than arsenic but more toxic than fluoride. Arsenic-protein interaction is also modulated in the presence of fluoride in the hepatic parenchymal cell.



Key words: Antioxidant enzymes, Arsenic, Fluoride, Liver, Oxidative stress, Rat

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Introduction

Arsenic and fluoride both are major contaminants of ground water. Available reports confirm their presence in drinking water of several countries in Latin America viz., Argentina, Bolivia, Chile, Columbia, Mexico and Peru (Alarcon-Herrera *et al.,* 2013). Asian countries like, China, Japan, India, Korea, Malaysia and Pakistan are also known to contain their higher concentration in ground water (NRC, 2006). Similarly, in Africa, ground water of Ethiopia, Ghana, Nigeria and Tanzania too, are contaminated with high concentrations of arsenic and fluoride (Fatoki *et al.,* 2013). Agency for Toxic Substances and Disease Registry (ATSDR, 2007) and International Agency for Research on Cancer (IARC, 2015) have classified arsenic as the highest priority environmental contaminant and Group I human carcinogen. Persistent drinking of water contaminated with arsenic by human population has led to a specific toxic syndrome-arsenicosis.

Similarly, continued consumption of fluoride contaminated water by humans and animals can lead to dental and skeletal fluorosis. Health effects of combined exposure to arsenic and fluoride have also been reported. They can cause adverse effects on liver, kidney, nervous (Flora *et al.,* 2009) and reproductive systems (Zhang *et al.,* 2006). Intriguingly, very few attempts have been made to delineate molecular/ biochemical mechanisms involved in their combined toxicity. They can bioaccumulate in soft tissues and generate reactive oxygen species. Co-exposure to arsenic and fluoride may induce oxidative (Flora *et al.,* 2009) and endoplasmic reticulum stress (Dong *et al.,* 2020). Their effects on inflammation, apoptosis and DNA damage have also been demonstrated (Mondal *et al.,* 2021). There appears a strong possibility that chemical interaction between these two elements at cellular level may manifest into different but poorly understood patterns of cytotoxicity.

Experimental evidence gathered so far, however, remains inconclusive. Combined exposure to arsenic and fluoride may cause oxidative stress in target organs like liver, kidney and brain by inhibiting antioxidant enzymes and enhancing lipid peroxidation (Yao and Wang, 1988). Nevertheless, their combined effects on antioxidant enzymes also remain controversial (Chlubek, 2003). A few studies suggest an antagonistic relationship between arsenic and fluoride (Li *et al.,* 1996), while other reports present contradictory results (Liu *et al.,* 1994). Therefore, it seems prudent to investigate further, the mechanism(s) of their combined toxicity in different organs. A recent study made in our laboratory showed dose and time dependent variations in their combined effects on liver function of rat (Huma *et al.,* 2021). Duration of combined exposure may be a confounding factor in orchestrating their toxicokinetics/ toxicodynamics. The objective of present study, therefore, was to record the combined effects of arsenic and fluoride on lipid peroxidation, glutathione cycle and antioxidant enzymes in the liver of Wistar rat after exposure to three different time intervals *i.e.,* 30, 60 and 90 days.

Materials and Methods

Animals and treatments schedule: Three months old (140±20g) female Wistar rats were purchased from the animal facility of All India Institute of Medical Sciences, New Delhi. These rats were acclimatized for two weeks and maintained under standard laboratory conditions (room temperature-25±5^{0}C; relative humidity -50±10% and 12hr light/dark cycle). Each rat was housed individually in a suitable polypropylene cage and offered commercial rat feed and tap water *ad libitum*. The study/experiment was undertaken after obtaining approval (No.IAEC/01/06/2018) from the Institutional Animal Ethical Committee (IAEC) of Chaudhary Charan Singh University, Meerut.

After acclimatization, rats were divided into twelve groups, each containing five rats. An alternative arrangement of the administration of arsenic and fluoride was followed. Rats of group 1were administered arsenic (4mg kg^{-1} b.wt.) and fluoride (4mg kg^{-1} b.wt.) on alternate days through gavage for 30 days. The dose was selected after determining LD_{50} of each element as described earlier (Huma *et al.,* 2021). Rats of Group 2 were administered arsenic only and Group 3, fluoride only, in the same dose and manner as the rats of Group 1on each alternate day for 30 days. The rats of Groups 4, 5, 6were similarly treated with arsenic and fluoride, as the rats of Groups 1 to 3, but for 60 days. The rats of group 7, 8, 9 contained rats offered the same dose regimen of arsenic + fluoride, but for 90 days. Rats of group 10,11,12 were administered saline only on each alternate day and served as controls against respective experimental groups.

Collection and preparation of sample: On termination of each exposure schedule, animals were fasted overnight and euthanized next morning by ether anaesthesia. Rats were carefully operated to remove the liver. Small pieces of liver (15-20 mm) were then removed from each rat and subjected to further analyses.After homogenization in prescribed media, the samples were centrifuged and analyzed following standard methods.

Estimation of malondialdehyde: Small pieces of liver were homogenized in 0.25 M sucrose and centrifuged at 7000xg. Supernatant was de-proteininzed by trichloroacetic acid and used for the estimation of malondialdehyde using thiobarbituric acid following the method of Jordan and Schenkman (1982). The absorbance was read at 532 nm on a spectrophotometer (Systronics, India).

Estimation of hydrogen peroxide: Liver homogenates were prepared in 0.25 M sucrose. The concentration of H_2O_2 was determined using ferrithiocynate following the method of Thurman *et al.* (1972). The absorbance was read at 480 nm using a spectrophotometer (Systronics, India).

Estimation of nitric oxide: Nitric oxide concentration in the post mitochondrial fraction of liver samples was estimated following the method of Cortas and Wakid (1990). The absorbance was read at 550 nm on a spectrophotometer (Systronics, India).

Estimation of GSH and GSSG: GSH in liver samples was determined using Ellmans' reagent (Ellman,1959). The absorbance was read at 412nm. GSSG was estimated following the method of Ohmori *et al.* (1981) and the absorbance was read at 458 nm on a spectrophotometer (Systronics,India).

Estimation of glutathione peroxidase: Liver samples were prepared in 0.25M sucrose. Enzyme was assayed following the method of Paglia and Valantine (1967). The method is based on the reduction of glutathione peroxidase by excess of glutathione reductase. The consumption of NADPH by glutathione reductase for catalytic conversion of GSSG to GSH was read at 340 nm on a spectrophotometer (Systronics, India).

Estimation of catalase: The enzyme catalase was assayed following the titration method of Aebi (1984). Hydrogen peroxide and potassium permanganate were used for titration.

Statistical analyses: Students' "t" test was used to compare the observations amongst different groups. P value < 0.05 was considered significant. Intergroup comparisons of the results were made applying one way analysis of variance (ANOVA) followed by Duncans' post hoc test.

Results and Discussion

Amongst three treatment groups, arsenic was found to be the most potent inducer of malondialdehyde (0.88 nmoles mg^{-1} protein) in liver followed by arsenic + fluoride (0.61 nmoles mg^{-1} protein) and fluoride (0.51 n moles mg^{-1} protein) treated rats. Maximum surge in the generation of MDA occurred after 60 days of treatment amongst all the groups. However, a significant decline in its value was recorded after 90 days of treatments. These results conclude that period of exposure affected generation of MDA in the liver of arsenic, fluoride and arsenic + fluoride treated rats (Table.1). Arsenic and fluoride both are known to manifest cytotoxicity through ROS, the products of phase-I metabolism. Attributes of LPO in heavy metal toxicity have been confirmed by several reports (Rana and Kumar, 1984; Sunderman, 1986). Arsenic in particular is known to mediate the formation of super oxide anion (O^{-}•), singlet oxygen (O•), the peroxy radical (ROO•), nitric oxide (NO•) and hydrogen peroxide (H_2O_2)(Yamanaka and Okada, 1994). During present study, three reactive species viz. MDA, NO and H_2O_2 were monitored in the liver of rats after exposure to single and combined treatment of arsenic and fluoride for 30, 60 and 90 days. It was observed that period of exposure affected the generation of these reactive species in the liver. Exposure for 60 days produced pronounced effects in comparison to 30 or 90 days of treatments.

Effects of fluoride alone or in combination with arsenic were less severe than arsenic treated rats. Allen *et al.* (2004) suggested that longer period of exposure to arsenic develops resistance against lipid peroxidation in liver and kidney of a fresh water fish. Similar trends were observed in mercury treated fish as well. Resistance noted as reduced values of malondialdehyde in the liver of rat after 90 days of exposure to arsenic/fluoride/arsenic + fluoride thus finds support from these observations. Fluoride is also known to generate MDA in the liver of rats (Lu *et al.,* 2017). A few studies on the combined effects of arsenic + fluoride on LPO are also available in literature. Increased generation of MDA was observed in the brain (Flora *et al.,* 2009), liver and kidney of rats after a selected period of exposure. However, the present results on MDA after their combined treatment (s) suggest a physiological antagonism between arsenic and fluoride.

Another set of observations on nitric oxide also showed a similar trend. Though its values were significantly high in all experimental groups than the control rats, the liver samples of group treated for 60 days exhibited maximum generation (3.48 µm) of this reactive species. Minimum values were registered in arsenic + fluoride (2.01 µm) treated rats after all the three exposure periods. In this group alone, non significant results in comparison to controls were obtained in rats treated for 60 and 90 days (Table 1). Individually, fluoride is known to enhance generation in target organs (Zhou *et al.,* 2015). Similarly, arsenic treatments do enhance the generation of NO (Kumagai and

Table 1: Effect of single and combined treatments of arsenic and fluoride on lipid peroxidation in the liver of female rats

Parameters	Exposure	Arsenic	Fluoride	Arsenic+Fluoride	Control
Malondialdehyde (n moles mg^{-1} protein)	30	0.71±0.06*(+)	0.49±0.03(+)	0.58±0.02*@#(+)	0.12±0.04
	60	0.88±0.02*(+)	0.51±0.03*(+)	0.61±0.030*@#(+)	0.22±0.03
	90	0.61±0.02*(+)	0.46±0.01*(+)	0.54±0.02*@NS(+)	0.19±0.05
H_2O_2 (µm)	30	1.18±0.25*(+)	1.14±0.12*(+)	0.952±0.10*@#(-)	1.02±0.25
	60	3.42±0.13*(+)	2.94±0.11*(+)	2.31±0.08*@#(+)	1.15±0.12
	90	3.04±0.09*(+)	2.58±0.27*(+)	2.44±0.42*@#(+)	1.56±0.22
NO (µm)	30	3.09±0.1*(+)	2.36±0.18*(+)	2.01±0.09*@#(+)	1.19±0.02
	60	3.48±0.04*(+)	2.94±0.16*(+)	2.57±0.11*@#(+)	1.045±0.01
	90	3.06±0.3*(+)	1.692±0.25*(+)	2.44±0.42*@#(+)	1.038±0.08

Results are expressed as mean of five replicates ±SE; *values significantly different from control rats (p<0.05); @values significantly different from As treated rats (p<0.05); #values significantly different from F treated rats (p<0.05) and NS-Non significant values

Table 2: Effect of single and combined treatment of arsenic and fluoride on glutathione and antioxidant enzymes in the liver of female rats

Parameters	Exposure	Arsenic	Fluoride	Arsenic+Fluoride	Control
GSH (µ mol g^{-1} liver)	30	0.124±0.02*(-)	0.112±0.03*(-)	0.154±0.03*@#(-)	0.20±0.04
	60	0.248±0.01*(-)	0.208±0.03*(-)	0.270±0.03*@#(-)	0.33±0.02
	90	0.196±0.02*(-)	0.360±0.06NS(-)	0.240±0.04*@#(-)	0.39±0.05
GSSG (µ mol)	30	143 ± 32.8*(-)	230 ± 40*(+)	322 ±52*@#(+)	223±6.4
	60	218 ±71.2*(-)	273 ±56*(-)	265 ±68*@#(-)	292 ± 3.3
	90	271±70*(+)	146± 40*(-)	110 ±11.6*@#(-)	232 ±7.3
Glutathione	30	1.92±0.09*(+)	0.97±0.10*(-)	1.03±0.2*@#(-)	1.57±0.05
peroxidase	60	1.99±0.09*(+)	1.10±4.6*(-)	1.34±11.1*@#(-)	1.72±0.15
(n moles	90	1.73±12.1*(+)	0.93±3.8*(-)	1.06±16.2*@#(-)	1.52±0.08
NADPH min mg^{-1} protein					
Catalase (K units)	30	68.38±3.0*(+)	65.26±5.5*(+)	74.96±3.90*@#(+)	27.2±4.6
	60	79.19±4.2*(+)	80.36±5.5*(+)	84.25±7.8*@#(+)	46.0±7.7
	90	78.3±5.05*(+)	87.22±2.22*(+)	93.2±5.5*@#(+)	36.6±4.5

Results are expressed as mean of five replicates ±SE; *values significantly different from control rats ($p<0.05$); @values significantly different from As treated rats($p<0.05$); #values significantly different from F treatedrats ($p<0.05$); NS-Non significant values

JIngbo, 2004). Present study on their combined toxicity, however, suggests exposure period dependent effects on NO in liver. H_2O_2 was also estimated in the liver of selected groups of rats. Its values ranged from 1.02 µm in control rats to 3.42 µm in arsenic treated rats. In arsenic and fluoride treated rats, the highest concentration *i.e.,* 2.31 µm was recorded after 60 days of exposure. However, the maximum concentration of H_2O_2 (3.42 µm) was observed in the liver of rats treated with arsenic + fluoride for 60 days (Table 1). Participation of H_2O_2 in arsenic induced human diseases has been discussed by Jomova *et al.* (2011). It is agreeable that H_2O_2 significantly contributes to arsenic and fluoride toxicity. (Liu *et al.,* 2001). However, the present results suggest severe effects of H_2O_2 in the liver after 90 days of treatment combined of arsenic and fluoride. It makes their physiological relationship controversial again. ROS generation may result from thiol chelation by arsenic or complexation with dithiol containing enzymes. Glutathione system of liver constitutes central protective mechanism in the toxicity of xenobiotics. In principle, enhanced production of free radicals depletes GSH stores in the liver. Present results on GSH in different groups reciprocated with observations on LPO. Amongst arsenic treated rats, minimum values for GSH (0.124 µmol g^{-1} liver) were noticed after 30 days of treatment. Similar were the observations on fluoride (0.112 µmol $^{-1}$ g liver) and arsenic + fluoride (0.154 µmol g^{-1} liver) treated rats.

A significant increase in GSH content was observed in the liver of all treated groups after 60 days of treatment. GSH content declined again in arsenic (0.196 µmol g^{-1} liver) and arsenic + fluoride (0.240 µmol g^{-1} liver) treated rats but increased in fluoride (0.360 µmol g^{-1} liver) treated rats (Table 2). Significant variation in GSSG content of liver was noticed in different groups of rats. A non-significant increase in the liver of arsenic (143 µmol) treated and significant increase in fluoride (230 µmol) and arsenic + fluoride (322 µmol) treated rats, in comparison to control rats, was observed after 30 days of exposure. Amongst fluoride (273 µmol) treated rats, maximum values were obtained after 60 days of exposure whereas 90 days of exposure yielded minimum values. Similar trend was recorded in arsenic + fluoride (110 µmol) treated group of rats (Table 2). Disturbances caused due to the combined treatment of arsenic and fluoride in redox homeostasis of liver were studied by estimating GSH and GSSG. GSH is an effective cellular antioxidant and thus makes a good marker of oxidative stress (Halliwell and Gutteridge, 2007). Previous studies have reported decreased GSH content in the liver of arsenic treated rats (Maiti and Chatterjee, 2001; Allen and Rana, 2003). Similarly, fluoride has also been found to deplete GSH stores of liver (Lu *et al.,* 2017). During present investigations, a reciprocal relationship between increased levels of ROS and decreased levels of GSH was observed in all treated groups. Further in combined arsenic and fluoride treated rats, an increase in GSH values was observed in the liver after 30 and 60 days of treatment. Its values decreased again after 90 days of co-exposure. Thus, antagonistic behaviour between these two elements appears to be time dependent.

Antioxidant enzymes work to protect the cell from oxidative stress in general. Amongst them, the function of glutathione peroxidase is to reduce hydrogen peroxide to water and lipid hydroperoxides to corresponding alcohols. Present study showed a decreased activity of enzyme corresponding to the increase in LPO in the liver of arsenic and fluoride treated. However, after cotreatment, increased enzyme activity in comparison to arsenic treated rats and decreased activity in comparison to fluoride treated rats was witnessed. A number of studies have elucidated the role of glutathione peroxidase in fluoride (Zhou *et al.,* 2015) and arsenic (Allen and Rana, 2004; Bashir *et al.,* 2006) induced cell injury. The combined effects of arsenic and fluoride on phase – II enzymes in zebrafish (Mondal *et al.,* 2019); mice (Mittal and Flora, 2006) and mouse models

(Flora *et al.,* 2009) have also been studied. The enzyme is used in the catalysis of GSH and H_2O_2 and as a result GSSG and H_2O are formed. Glutathione reductase in turn reduces GSSG to GSH to complete the redox cycle. Significantly decreased activity of glutathione peroxidase was registered in the liver of rats of all the groups vis a vis control rats. In arsenic (1.99 nmoles/ NADPH/ min/mg protein) and fluoride (1.10 nmoles/NADPH/min/mg protein) treated group maximum decline was recorded after 60 days of respective treatments. However, in rats treated with arsenic + fluoride both (1.06 nmoles/ NADPH/ min/ mg protein), minimum values were obtained in the liver after 90 days of exposure (Table 2). Catalase scavenges peroxy radicals including H_2O_2. It catalyzes the reaction by which H_2O_2 is decomposed to water and oxygen. Its activity increased in the liver of rats amongst all the groups. Although minimum values were recorded in the liver of rats after 30 days of exposure to arsenic (68.38 K unit), fluoride (65.26 K unit), and arsenic + fluoride (74.96 K unit), significant surge in its activity was noticed after 60 (84.25 K units) and 90 days (93.2 K units) of respective treatments (Table 2). Its activity decreased in the liver of rats treated individually with arsenic and fluoride for 30, 60,90 days. No similar study seems to have been made after different time periods of exposure, however, arsenic (Altikat *et al.,* 2015) and fluoride (Goschorska *et al.,* 2015) both are known to inhibit catalase activity in experimental models. Present results show that catalase activity improved in the liver of rats after cotreatment with arsenic and fluoride. Different values were recorded after different exposure periods.

It is assumed that As or F ions may interact with di or trivalent metals located on the active site of antioxidant enzymes culminating in their inhibition. Present results showed that the combined treatment of arsenic and fluoride together induce greater oxidative injury than individual exposures. Duration of exposure served as an important confounding factor. Finally, plausible interactions between arsenic and fluoride in the cell needs to be appraised. Fluoride inhibits the bioaccumulation of arsenic in liver and kidney (Huma *et al.*, 2021). Secondly arsenic binds with fluoride due to their electronegativity. In trivalent state it can hybridize to form arsenic trifluoride (AsF_3) while in pentavalent state, it can form arsenic pentafluoride (AsF5). Thus, sodium fluoride can suppress the ionization of sodium arsenate reducing its toxicity (Chauhan and Flora, 2010). Moreover, arsenic-protein interactions that are known to determine cellular responses to arsenic may be modulated in the presence of fluoride. Specific and stable interaction between arsenic and fluoride and biomolecules is necessary for its biological impact (Shen *et al.,* 2012). It is concluded that identification of As and F binding domains is essential to understand physiological relationship between them. Dose and exposure period will continue to be the determinants of all arsenic + fluoride interactions in diverse cellular systems.

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Add-on Information

Authors' contribution: Huma: Performed the experiments; **Y. Verma:** Analysed the data; **S.V.S. Rana:** Supervised the work and prepared the report.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: The authors have followed and complied with the national Guidelines of appropriate for the use of plants or animals used in the experimental study.

Conflict of interest: Authors declare no conflict of interest in present investigations.

Availability of data and materials: All data generated and analyzed during our study are included in manuscript.

Consent for publication: Not applicable.

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