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Interactive effect of arsenic and fluoride on cardio-respiratory disorders in male rats: possible role of reactive oxygen species

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Abstract Epidemiological evidence demonstrates positive correlation between environmental and occupational arsenic or fluoride exposure and risk to various cardio-respiratory disorders. Arsenic-exposure has been associated with atherosclerosis, hypertension, cerebrovascular diseases, ischemic heart disease, and peripheral vascular disorders, whereas Fluoride-exposure manifests cardiac irregularities and low blood pressure (BP). Present study aims to study the combined effects of these toxicants on various cardio-respiratory variables in male rats. Single intravenous (i.v.) dose of arsenic (1, 5, 10 mg/kg) or fluoride (5, 10, 20, 36.5 mg/kg) either alone or in combination were administered. Individual exposure to arsenic or fluoride led to a significant depletion of mean arterial pressure, heart rate (HR), respiration rate and neuromuscular (NM) transmission in a dose-dependent manner. These changes were accompanied by increased levels of blood reactive oxygen species (ROS) and decreased glutathione (GSH) concentrations. An increase in the blood acetyl cholinesterase (AChE) activity was observed in both arsenic or fluoride exposed rats. These changes were significantly more pronounced in arsenic-exposed animals than in fluoride. During combined exposure to arsenic (5 mg/kg) + fluoride (20 mg/kg) or arsenic (10 mg/kg) + fluoride (36.5 mg/kg) the toxic effects were more pronounced compared to individual toxicities of arsenic or fluoride alone. However, combined exposure to arsenic (5 mg/kg) + fluoride (36.5 mg/kg) resulted in antagonistic effects on variables suggestive of altered cardio-respiratory function and oxidative stress. The results from the present study suggest that arsenic or fluoride individually demonstrate cardio-respiratory failure at all doses whereas during combination exposure these toxins show variable toxicities; both synergistic and antagonistic effects depending upon the dose. Moreover, it may be concluded that arsenic and/or fluoride cardio-respiratory toxicity may be mediated via oxidative stress. However, these results are new in the discipline thus requires further exploration.

Keywords Cardio-respiration · Oxidative stress · Neuromuscular transmission · Heart rate · Respiration rate · Arterial pressure · Arsenic · Fluoride

Abbreviations

As	Arsenic
F	Fluoride
NM	Neuromuscular
ROS	Reactive oxygen species
GSH	Reduced glutathione
AChE	Acetyl cholinesterase
SBP	Systolic BP
MAP	Mean arterial pressure

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Introduction

Human exposure to arsenic and/or fluoride occurs through various sources that may be of environmental (contaminated drinking water and food), occupational or medical origin (Pandey 2010). Cardiac effects of massive acute arsenic (Westervelt et al. 2001) or fluoride (Shashi and Thapar 2001) exposure are well documented. In humans, chronic arsenic exposure has been associated with diverse health effects including cancer, hyperkeratosis, diabetes, and cardiovascular disease (Col et al. 1999; Tseng et al. 2003). Cardiovascular effects associated with high levels of arsenic in drinking water include atherosclerosis, hypertension, cerebrovascular diseases, ischemic heart disease, arrhythmia and peripheral vascular disorders such as Blackfoot disease (Navas-Acien et al. 2005; Ohnishi et al. 2002; Wang et al. 2002, 2007).

Although arsenic-exposure has been associated with cardiac dysfunction in humans (Wang et al. 2002), little is known about the possible mechanism involved (Dhar and Bhatnagar 2009; Barbier et al. 2010). Arsenic has been documented to show both cardiac and vascular effects where number of mechanism like toxicity to myocardial and vascular endothelial cells, vasoconstriction, increased susceptibility to platelets aggregation has been proposed (Shi et al. 2010; Straub et al. 2007; Balakumar and Kaur 2009; Wang et al. 2007; Lee et al. 2002). Recent reports from Inner Mongolia, China, and the United States correlating arsenic-exposure with cardiac arrhythmia strengthens possibility of its direct effects on signaling pathways of heart (Mordukhovich et al. 2009).

It is widely recognized that ingestion of fluoride not only causes dental and skeletal fluorosis but is also related to anomalies in other cellular functions (Vani and Reddy 2000). Although acute toxic effects of fluoride on heart are fairly well known, information about the exact nature of these effects is still very limited. In experimental animals given massive doses of fluoride, cardiac dysfunction and low BP have been reported (Imanishi et al. 2009; Mclvor 1990; Takamori et al. 1956). In humans, changes in the electrocardiogram and heart enlargement has been positively correlated to fluoride in the drinking water (Kant et al. 2010, Mclvor et al. 1987; Luoma and Aromaa 1983; Okushi 1954), however there is less certainty about the nature of these effects.

Combined exposure to arsenic and fluoride in ground water is well reported in various regions of India and other countries (Chouhan and Flora 2010; Farooqi et al. 2009) but there is scarcity of data to provide a mechanism of their interaction and effects on various organ systems. We earlier reported effects of arsenic-fluoride co-exposure on hematological, neurological, hepatic and renal system (Mittal and Flora 2006, 2007; Flora et al. 2009) however, so far there is hardly any published report which emphasis their combined effects on cardio-respiratory functions.

Therefore, it was of particular interest to ascertain effects of different doses of arsenic and fluoride either alone or in combination, on various cardio-respiratory variables. Since oxidative stress is supposed to be the key mechanism in arsenic or fluoride induced toxicity, we investigated possible role of oxidative stress by evaluating blood biochemical variables suggestive of the same.

Materials and methods

Chemicals and reagents

Sodium meta arsenite ($NaAsO_2$), and sodium fluoride (NaF) were procured from Sigma Chemical (USA). All other analytical laboratory chemicals and reagents were purchased from Merck (Germany), Sigma (USA) or BDH chemicals (Mumbai, India). Ultra pure water prepared by Millipore (New Delhi, India) was used through out the experiment to avoid metal contamination and for the preparation of reagents and buffers used for various biochemical assays in our study.

Animals and treatment

All experiments were performed on healthy, adult male Wistar rats, weighing approximately 180–200 g. Animals were obtained from animal house facility of Defence Research and Development Establishment (DRDE), Gwalior. Animal ethical committee of DRDE, Gwalior, India, approved the protocols for the experiments. Prior to dosing, animals were acclimatized for 7 days to alternate 12/12 h light and dark cycle. Animals were housed in stainless steel cages in

an air-conditioned room with temperature maintained at 25 ± 2°C. Rats were allowed standard chow diet (Amrut feeds, Pranav Agra, New Delhi, India; metal contents of diet, in mg/kg dry weight, Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, Fe 75.0) and water ad libitum throughout the experiment. Animals were randomized into 10 groups of 5 animals each and were treated as below:

Group I	Control animals—(normal water)
Group II	Arsenic, as sodium meta arsenite, 1 mg/kg, intravenous (i.v.)
Group III	Arsenic, 5 mg/kg, i.v
Group IV	Arsenic, 10 mg/kg, i.v
Group V	Fluoride, as sodium fluoride, 5 mg/kg, i.v
Group VI	Fluoride, 10 mg/kg, i.v
Group VII	Fluoride, 20 mg/kg, i.v
Group VIII	Arsenic, 5 mg/kg, i.v. + Fluoride, 20 mg/kg, i.v
Group IX	Arsenic, 5 mg/kg, i.v. + Fluoride, 36.5 mg/kg, i.v
Group X	Arsenic, 10 mg/kg, i.v. + Fluoride, 36.5 mg/kg, i.v

Following single i.v. dose administration BP, HR, respiratory rate and neuromuscular transmission were recorded at 15, 30, 60, 120, 180, and 240 min. Blood was collected from left carotid artery in heparinized vials for estimation of biochemical variables. Doses of arsenic and fluoride were selected on the basis of previous reports (Saralakumari and Ramakrishna Rao 1991; Guha Mazumder 2003). As fluoride has been shown to reduce arsenic toxicity during co-administration, we selected a dose equivalent to its LD_{50} (36.5 mg/kg) in combination with arsenic (Mittal and Flora 2006, 2007; Flora et al. 2009). Individual group for fluoride at 36.5 mg/kg dose was eliminated due to mortality observed at 20 mg/kg.

Cardio-respiratory variables

The rats were anaesthetized with pentobarbitone sodium (40 mg/kg, i.p.) and various physiological parameters were recorded on a Grass Polygraph (Model 7-16P-35, Quincy Mass, USA). The animals were maintained on positive pressure ventilation using a rodent ventilator (Model 7025, Ugo Basile, Comerio, Italy). The neck region was dissected and exposed. Trachea was cannulated and connected to a pneumotachograph. Right jugular vein was cannulated for intravenous administration of dose. The left carotid artery was cannulated with a thin polypropylene tube connected to a pressure transducer (Statham P23DC), filled with heparinized normal saline. The pressure transducer was connected to a preamplifier (low level DC, Grass Instruments, USA) and arterial BP was recorded on the polygraph. A bronchospasm transducer (Model 7020, Ugo Basile, Comerio, Italy) was used to measure the tracheobronchial constriction/spasm i.e. respiration rate (RR). The signals generated were fed to a low DC preamplifier (Model 7PI) to record RR. The pulse signals were also fed into the tachograph preamplifier (Type 7P4) to record the HR. The gastrocenemis muscle was opened and the sciatic nerve was stimulated with a supramaximal voltage (1–10 V) of 0.2 m sec duration at a frequency of 0.2 Hz using a Grass stimulator (Model S 88). The signals of neuromuscular transmission were fed to a polygraph integrator to record the twitch response for 1 min duration. The twitch response of the muscle was recorded using a force transducer (Model FT 03).

Biochemical assays

Amount of ROS in blood were measured using 2′,7′-dichlrofluorescin diacetate (DCF-DA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci et al. (1999). Fluorescence was determined at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Tecan Spectra Fluor Plus). Amount of ROS was expressed as ηmoles of DCF formed/min/ml of RBC. Analysis of blood GSH concentration was performed with method described by Ellman (1959) and modified by Jollow et al. (1974). The results were expressed as mg/ml. Activity of acetyl cholinesterase (AChE) in R.B.C. was determined according to the method of Ellman et al. (1961).

Statistical analysis

Data are expressed as means ± SEM. Statistical data analysis was carried out using One Way ANOVA followed by Bonferroni's test.

Results

Effect on mean arterial pressure

Dose-dependent effect of arsenic and fluoride alone or in combination on mean arterial pressure are presented in Table 1. Intravenous administration of arsenic at a dose of 1 mg/kg significantly increased BP compared to control group 15 min following administration however; it gradually returned to normal level (Fig. 1a). Interestingly administration of 5 mg/kg arsenic did not produce any significant alteration in BP till 60 min followed by significant decrease (60–120 min) and death (60–180 min) (Fig. 1b). Highest dose of arsenic (10 mg/kg) produced significant fall in BP immediately on administration and showed continued decrease till 30 min followed by animal death (Fig. 1c). Fluoride at 5 and 10 mg/kg produced a constant decrease in BP post 30 and 60 min, respectively. At the highest dose (20 mg/kg) fluoride, similar to arsenic demonstrated immediate decrease in BP following death after 60 min (Fig. 2a–c).

Arsenic was found to be toxic at the dose of 5 and 10 mg/kg whereas fluoride was most toxic at 20 mg/kg dose. Thus for combination toxicity evaluation fluoride at 20 mg/kg was administered with arsenic at 5 mg/kg. More pronounced decrease in BP was observed in combination treatment compared to arsenic or fluoride administered individually. However, increasing the dose of fluoride to 36.5 mg/kg (LD_{50}) with arsenic at 5 mg/kg rather normalized the value of BP in co-exposed animals. Combination exposed to fluoride (36.5 mg/kg) and increased dose of arsenic (10 mg/kg) resulted in sudden fall in BP on co-administration followed by animal death (Fig. 3a–c) (Table 1).

Effect on heart rate

Arsenic at 1 mg/kg dose did not produce any change in HR however; at 5 mg/kg arsenic significantly decreased HR at 120 min. At 10 mg/kg dose of arsenic, significant decrease in HR was immediately recorded post administration followed by death of animal after 30 min (Table 2). Individual exposure to fluoride also showed similar trends with lowest dose (5 mg/kg) showing no alteration in HR however, moderate dose (10 mg/kg) resulted decrease at 120 and 180 min. Highest dose of fluoride (20 mg/kg) led to decreased HR at 60 min followed by animal death.

Combined administration of arsenic (5 mg/kg) and fluoride (20 mg/kg) resulted in a sudden decrease in HR on administration that sustained low till 60 min followed by animal death. Concomitant administration of fluoride (36.5 mg/kg) and arsenic (5 mg/kg) similar to BP showed HR values comparable to normal animals. On the other hand, increasing arsenic dose to 10 mg/kg with fluoride (36.5 mg/kg) significantly decreased HR on co-administration followed by animal death (Table 2).

Table 1 Interactive effects of arsenic, fluoride and their combination on mean arterial pressure (MAP) (% of control value) at different time interval in rats

	15 min	30 min	60 min	120 min	180 min	240 min
Control	97.6 ± 2.4	108 ± 1.8	114.7 ± 5.2	101.1 ± 1.1	113.1 ± 1.1	109.5 ± 1.9
As (1 mg/kg)	123.3 ± 1.6*	108.3 ± 1.5	100.9 ± 1.8	98.5 ± 2.7	101.6 ± 1.6	96.6 ± 3.3
As (5 mg/kg)	96.1 ± 1.5	103.2 ± 2.6	93.0 ± 0.0	60.0 ± 0.0*	Mortality	–
As (10 mg/kg)	71.5 ± 1.5*	52.6 ± 0.5*	Mortality	–	–	–
F (5 mg/kg)	99.0 ± 1.0	97.0 ± 1.9	81.8 ± 1.4*	83.1 ± 1.5*	79.1 ± 3.5*	76.6 ± 1.6*
F(10 mg/kg)	91.0 ± 1.8	98.2 ± 1.8	95.2 ± 1.6	82.1 ± 0.5*	67.8 ± 0.2*	67.8 ± 0.2*
F(20 mg/kg)	76.6 ± 1.5*	66.6 ± 2.5*	40.0 ± 0.2*	Mortality	–	–
As (5 mg/kg) + F (20 mg/kg)	48.7 ± 1.4†	45.8 ± 1.9†	60.7 ± 0.2†	Mortality	–	–
As (5 mg/kg) + F (36.5 mg/kg)	98.7 ± 2.4	99.0 ± 2.4	93.5 ± 3.0	99.3 ± 1.7	81.9 ± 1.9†	92.7 ± 2.5
As (10 mg/kg) + F (36.5 mg/kg)	69.1 ± 1.4†	Mortality	–	–	–	–

Values are mean ± SE; $n = 5$, * $P < 0.05$ are considered significant compared to normal, † $P < 0.05$ are considered significant compared to individual arsenic or fluoride groups as evaluated by One Way ANOVA followed by Bonferroni's test

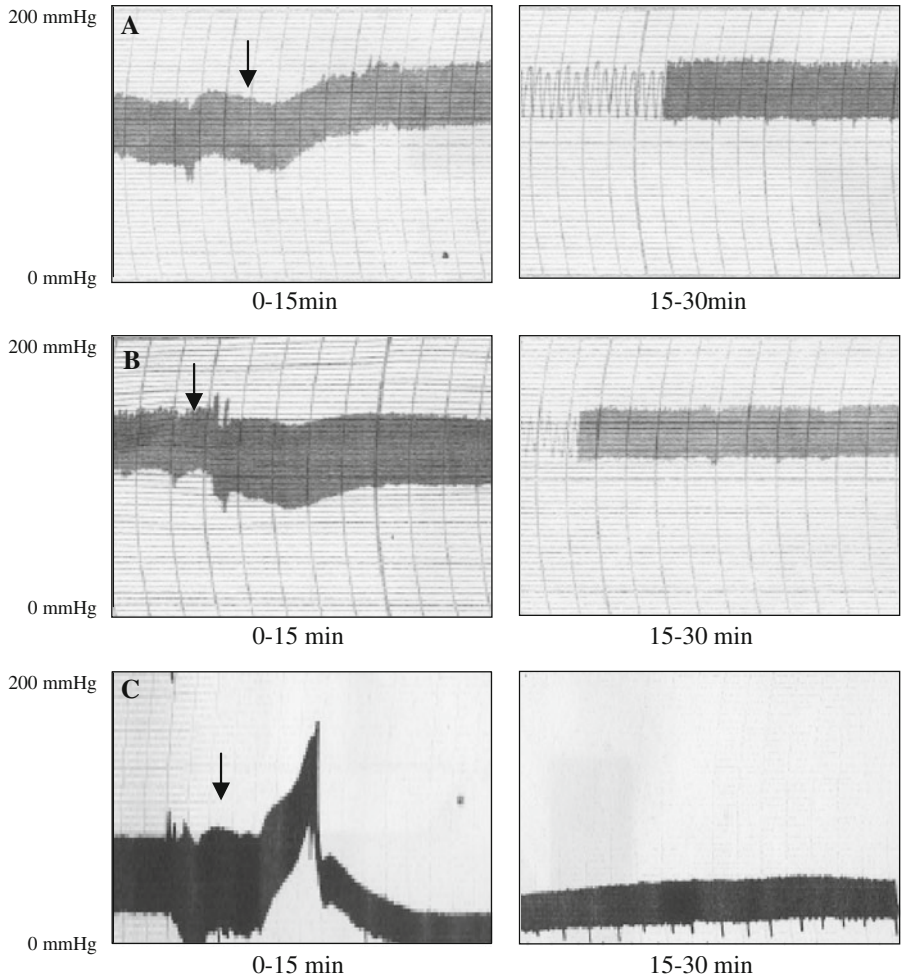

Fig. 1 Effect of intravenous administration of arsenic **a** 1 mg/kg, **b** 5 mg/kg, **c** 10 mg/kg on mean arterial pressure in anesthetized rats during the period of 0–15 min and 15–30 min

Effect on respiration rate

Respiration rate was found to be unaltered after administration of arsenic at 1 mg/kg dose however, at 5 mg/kg dose significant decrease was observed at 60 min that continued till 120 min followed by animal death. Arsenic at 10 mg/kg immediately decreased respiration rate on administration, followed by animal death after 30 min (Table 3). Fluoride at 5 and 10 mg/kg dose significantly decrease respiration rate at 240 and 180–240 min, respectively. However fluoride at 20 mg/kg decreased respiration rate at 60 min followed by death of animal (Table 3).

Combined administration of arsenic (5 mg/kg) and fluoride (20 mg/kg) resulted in a sudden reduction in respiration rate post administration that sustained low till 60 min followed by animal death. Co-administration of fluoride (36.5 mg/kg) and arsenic (5 mg/kg) showed no alteration in respiratory rate compared to normal animals. However, fluoride (36.5 mg/kg) and arsenic (10 mg/kg) co-administration suddenly decreased respiration rate followed by animal death after 15 min (Table 3).

Effect on neuromuscular transmission

Among all physiological variables studied, most prominent changes were observed in NM transmission by arsenic and/or fluoride administration. Arsenic at dose of 1 and 5 mg/kg demonstrated significant reduction in NM transmission at 120 and 60 min, respectively. High dose (10 mg/kg) of

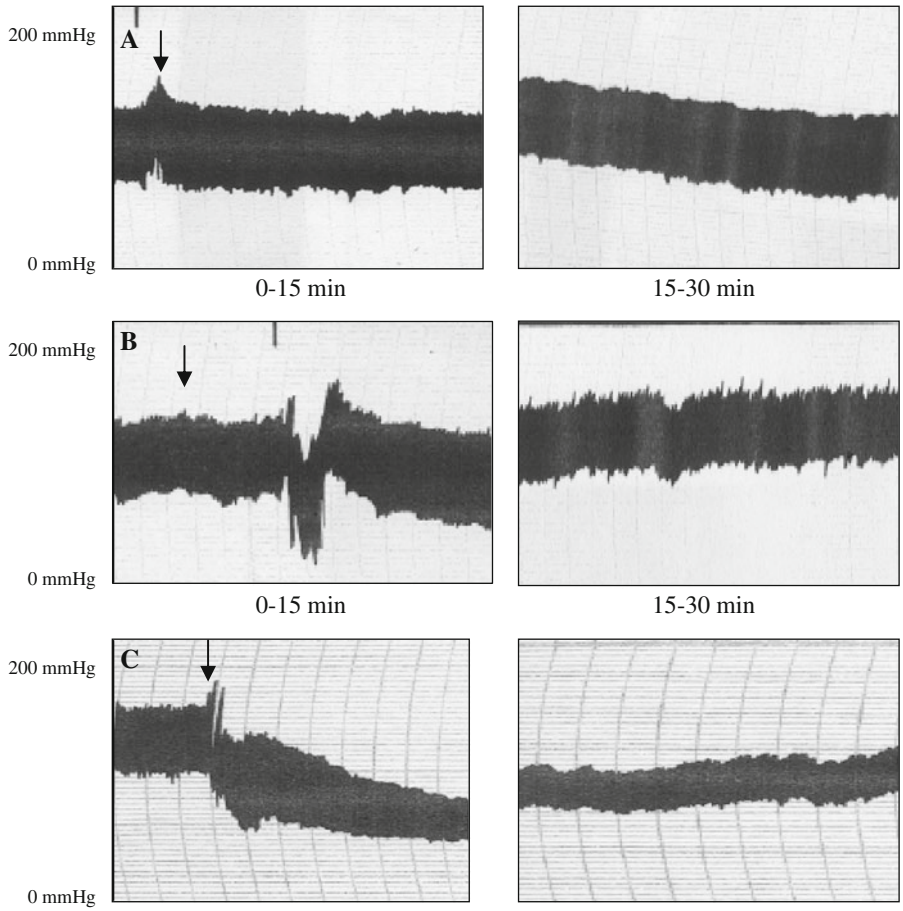

Fig. 2 Effect of intravenous administration of fluoride **a** 5 mg/kg, **b** 10 mg/kg, **c** 20 mg/kg on mean arterial pressure in anesthetized rats during the period of 0–15 min and 15–30 min

arsenic produced completely inhibited NM transmission post administration that followed mortality in animals (Table 4).

Administration of fluoride alone, at 5 and 10 mg/kg dose significantly decreased NM transmission after 180 and 60 min, respectively. Highest dose of 20 mg/kg fluoride produced decrease in NM transmission immediately after 15 min that continued till 60 min followed by animal death (Table 4).

Combined administration of arsenic (5 mg/kg) and fluoride (20 mg/kg) showed decreased NM transmission after 15 min whereas, fluoride (36.5 mg/kg) and arsenic (5 mg/kg) did not produced any change in NM transmission compared to normal animals. Co-administration of fluoride (36.5 mg/kg) and arsenic (10 mg/kg) decreased NM transmission followed by death of animal at 30 min (Table 4).

Effect on biochemical variables indicative of oxidative stress

Figures 4 and 5 shows concentration of blood ROS and GSH, respectively. Lower doses of arsenic (1 mg/kg and 5 mg/kg) did not alter ROS and GSH level significantly; however arsenic at 10 mg/kg significantly increased ROS and decreased GSH concentration in comparison to normal group. Fluoride at the dose of 5 and 20 mg/kg significantly increased ROS level however middle dose of 10 mg/kg fluoride, did not alter ROS level compared to normal animals. GSH concentration was unaltered at fluoride 5 and 10 mg/kg dose but significantly decreased at 20 mg/kg fluoride compared to normal group.

Combined administration of arsenic (5 mg/kg) and fluoride (20 mg/kg) elevated ROS level and

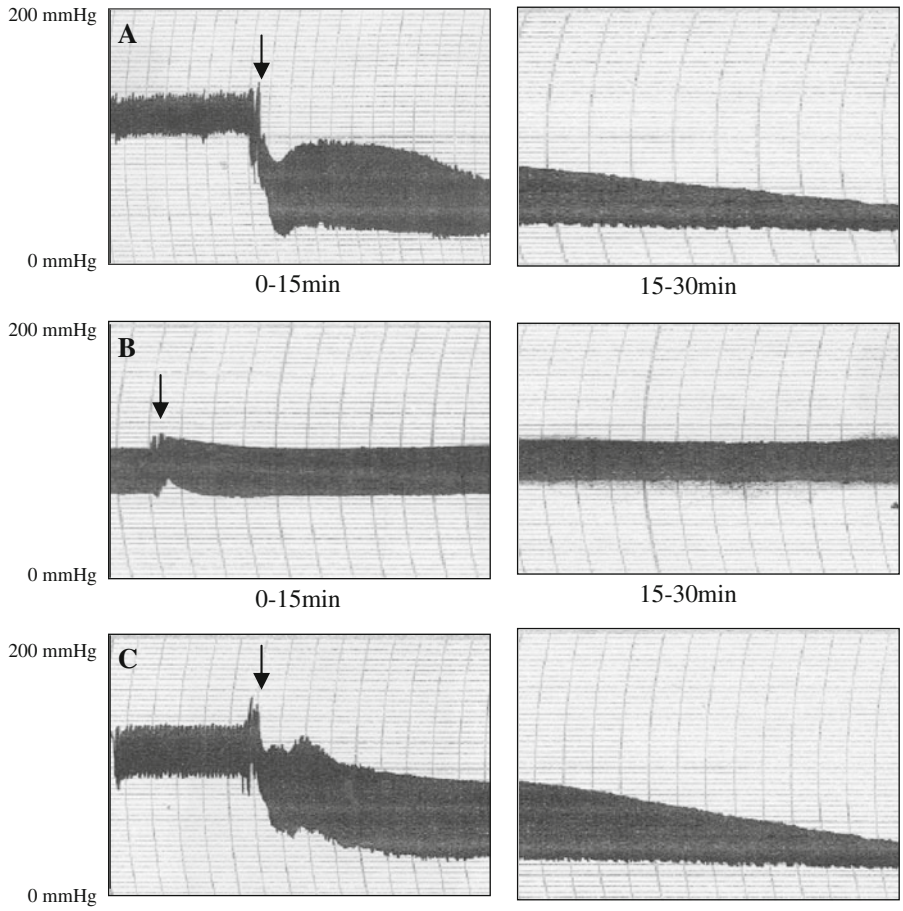

Fig. 3 Effect of intravenous administration of **a** arsenic (5 mg/kg) + fluoride (20 mg/kg), **b** arsenic (5 mg/kg) + fluoride (36.5 mg/kg), **c** arsenic (10 mg/kg) + fluoride (36.5 mg/kg) on mean arterial pressure in anesthetized rats during the period of 0–15 min and 15–30 min

Table 2 Interactive effects of arsenic, fluoride and their combination on HR (% of control value) at different time intervals in rats

	15 min	30 min	60 min	120 min	180 min	240 min
Control	101.6 ± 4.7	100.2 ± 3.3	101.9 ± 2.1	98.6 ± 3.7	100.1 ± 3.2	104.8 ± 3.8
As (1 mg/kg)	110.0 ± 1.7	107.9 ± 3.2	112.2 ± 3.9	101.3 ± 2.4	105.6 ± 2.6	109.0 ± 3.5
As (5 mg/kg)	92.7 ± 2.7	96.3 ± 3.7	92.6 ± 0.0	85.3 ± 0.0*	Mortality	–
As (10 mg/kg)	75.8 ± 2.6*	67.5 ± 0.5*	Mortality	–	–	–
F (5 mg/kg)	100.2 ± 1.7	98.4 ± 2.5	83.3 ± 3.6*	95.2 ± 1.8	90.2 ± 2.3	97.9 ± 3.1
F(10 mg/kg)	87.3 ± 3.5	90.6 ± 0.3	96.2 ± 2.0	96.0 ± 0.5	64.5 ± 0.2*	59.6 ± 0.2*
F(20 mg/kg)	94.4 ± 2.3	106.8 ± 2.8	73.3 ± 0.5*	Mortality	–	–
As(5 mg/kg) + F(20 mg/kg)	58.1 ± 2.4†	60.1 ± 2.3†	74.1 ± 0.5†	Mortality	–	–
As(5 mg/kg) + F(36.5 mg/kg)	93.4 ± 3.7	103.3 ± 2.9	106.1 ± 2.8	100.8 ± 2.7	82.4 ± 2.0	80.1 ± 2.0
As(10 mg/kg) + F(36.5 mg/kg)	68.3 ± 1.7†	Mortality	–	–	–	–

Values are mean ± SE; $n = 5$, * $P < 0.05$ are considered significant compared to normal, † $P < 0.05$ are considered significant compared to individual arsenic or fluoride groups as evaluated by One Way ANOVA followed by Bonferroni's test

decreased GSH concentration significantly compared to normal group. However co-administration of highest dose of fluoride (36.5 mg/kg) and arsenic (5 mg/kg) did not alter ROS and GSH level. ROS level were increased once again at high doses of fluoride (36.5 mg/kg) and arsenic (10 mg kg) co-exposure compared to normal animals, GSH level remaining unaltered.

Table 3 Interactive effects of arsenic, fluoride and their combination on respiration rate (% of control value) at different time interval in rats

	15 min	30 min	60 min	120 min	180 min	240 min
Control	95.8 ± 4.2	90.8 ± 0.8	85.8 ± 3.8	85.8 ± 3.2	84.2 ± 2.8	85.6 ± 2.4
As (1 mg/kg)	95.8 ± 4.1	95.8 ± 3.4	91.0 ± 1.4	87.3 ± 0.89	83.1 ± 1.4	79.0 ± 2.2
As (5 mg/kg)	78.3 ± 1.6	87.5 ± 2.5	62.5 ± 0.0*	50.0 ± 0.0*	Mortality	–
As (10 mg/kg)	70.8 ± 2.2*	50.0 ± 0.5*	Mortality	–	–	–
F (5 mg/kg)	105.8 ± 2.5	97.7 ± 2.2	89.1 ± 1.6	79.1 ± 1.9	78.5 ± 1.7	64.9 ± 1.8*
F (10 mg/kg)	86.6 ± 0.9	86.6 ± 1.2	105.5 ± 1.3	75.0 ± 0.5	62.5 ± 0.4*	62.0 ± 0.2*
F (20 mg/kg)	78.1 ± 1.7	81.2 ± 2.2	50.0 ± 0.2*	Mortality	–	–
As (5 mg/kg) + F (20 mg/kg)	48.1 ± 1.5†	38.8 ± 2.6†	33.3 ± 0.5†	Mortality	–	–
As (5 mg/kg) + F (36.5 mg/kg)	88.6 ± 2.5	88.4 ± 2.4	85.3 ± 2.7	84.9 ± 2.4	83.1 ± 1.4	83.0 ± 1.2
As (10 mg/kg) + F (36.5 mg/kg)	46.4 ± 3.6†	Mortality	–	–	–	–

Values are mean ± SE; $n = 5$, * $P < 0.05$ are considered significant compared to normal, † $P < 0.05$ are considered significant compared to individual arsenic or fluoride groups as evaluated by One Way ANOVA followed by Bonferroni's test

Table 4 Interactive effects of arsenic, fluoride and their combination on neuromuscular transmission (% of control value) at different time interval in rats

	15 min	30 min	60 min	120 min	180 min	240 min
Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	110.0 ± 1.0	104.0 ± 1.6	108.0 ± 1.2
As (1 mg/kg)	94.6 ± 1.3	93.3 ± 2.5	86.6 ± 2.8	58.6 ± 1.8*	52.0 ± 2.8*	40.0 ± 0.5*
As (5 mg/kg)	90.6 ± 3.5	82.0 ± 2.6	56.0 ± 0.5*	12.0 ± 0.5*	Mortality	–
As (10 mg/kg)	5.5 ± 1.3*	2.0 ± 0.5*	Mortality	–	–	–
F (5 mg/kg)	97.0 ± 1.9	95.0 ± 1.8	88.0 ± 2.3	84.0 ± 2.5	77.3 ± 2.3*	64.0 ± 2.6*
F (10 mg/kg)	98.0 ± 2.0	94.0 ± 2.1	79.1 ± 0.5*	80.0 ± 0.5*	48.0 ± 0.2*	48.2 ± 0.2*
F (20 mg/kg)	74.0 ± 2.5*	58.0 ± 1.8*	16.0 ± 0.2*	Mortality	–	–
As (5 mg/kg) + F (20 mg/kg)	70.1 ± 2.0†	20.0 ± 2.8†	2.0 ± 0.5†	Mortality	–	–
As (5 mg/kg) + F (36.5 mg/kg)	92.0 ± 2.5	86.0 ± 2.6	82.0 ± 2.9	75.0 ± 3.0	88.0 ± 2.5	84.0 ± 2.5
As (10 mg/kg) + F (36.5 mg/kg)	78.0 ± 1.4†	Mortality	–	–	–	–

Values are mean ± SE; $n = 5$, * $P < 0.05$ are considered significant compared to normal, † $P < 0.05$ are considered significant compared to individual arsenic or fluoride groups as evaluated by One Way ANOVA followed by Bonferroni's test

Effect on acetyl cholinesterase (AChE) activity

AChE activity is depicted in Fig. 6, arsenic at 5 and 10 mg/kg or fluoride at 10 and 20 mg/kg individually increased AChE activity compared to normal. Combined administration of arsenic and fluoride also significantly increased AChE activity but to variable degrees. Arsenic at 5 and fluoride at 20 mg/kg in combination showed the highest increase in AChE activity followed by arsenic at 10 and fluoride at 36.5 mg/kg, and compared to their individual administration. Co-administration of fluoride (36.5 mg/kg) and arsenic either at 5 or 10 mg/kg increased AChE activity significantly however; it was less pronounced compared to co-administration of arsenic at 5 and fluoride at 20 mg/kg.

Discussion

Individual exposure to arsenic or fluoride has been reported to cause hypertension, ischemic heart disease, cerebrovascular disease, and carotid atherosclerosis with little known about the exact mechanism involved (Tseng et al. 2003; Wang et al. 2002; Rahman et al. 1999; Takamori et al. 1956; Donmez and Cinar 2003). In the present study we evaluated the effect of different doses of arsenic and fluoride

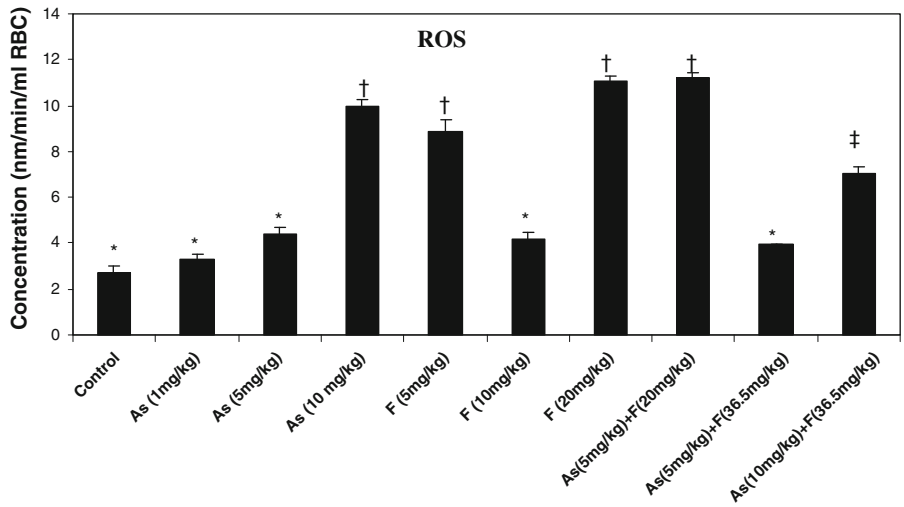

Fig. 4 Effect of different concentration of arsenic and/or fluoride on reactive oxygen species level in blood of rats. Abbreviation used and unit: ROS-reactive oxygen species as nm/min/ml of RBC. Values are mean ± SEM $n = 5$. $*,\dagger,\ddagger$ $P < 0.05$ are considered significant by One Way ANOVA followed by Bonferroni's test

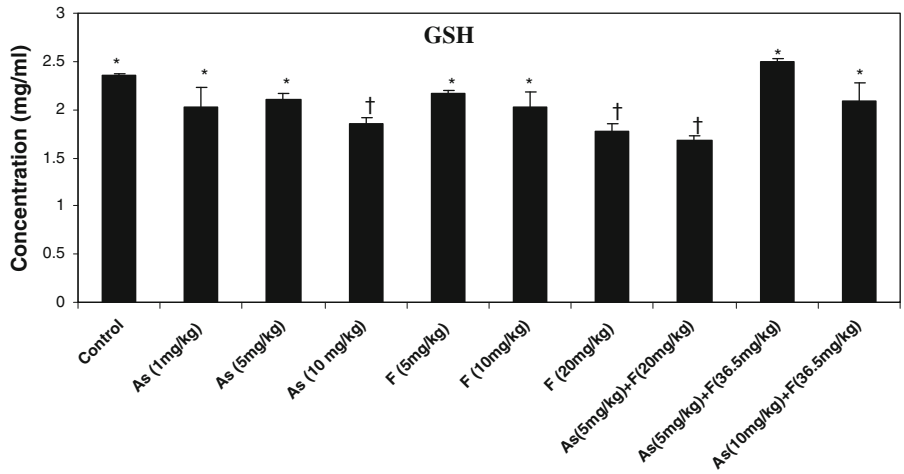

Fig. 5 Effect of different concentration of arsenic and/or fluoride on glutathione level in blood of rats. Abbreviation used and unit: GSH-reduced glutathione as mg/ml of RBC. Values are mean ± SEM, $n = 5$. $*,\dagger$ $P < 0.05$ are considered significant by One Way ANOVA followed by Bonferroni's test

either alone or in combination on cardio-respiratory variables indicative of cardiac dysfunction. In addition, during acute exposure, the role of oxidative stress in arsenic and/or fluoride induced cardio-respiratory toxicity was investigated based on generation of ROS and the effect on GSH level. The results led us to suggest that arsenic or fluoride individually produced cardio-respiratory toxicity in a fairly dose related manner, whereas combination exposure to both the toxicants demonstrated both synergistic and antagonistic effects depending upon toxin dose combinations administered.

In the cases of high-dose acute arsenic toxicity that may presumably be accidental or suicidal, death occurs due to cardiac or respiratory arrest which is rarely reported in time to save patient (Lai et al. 2005; Martínez-Barbeito et al. 2007). Thus, rationale of choosing close to lethal dose was to analyze effect of arsenic and/or fluoride at high doses especially in combination. Further, understanding the effects of

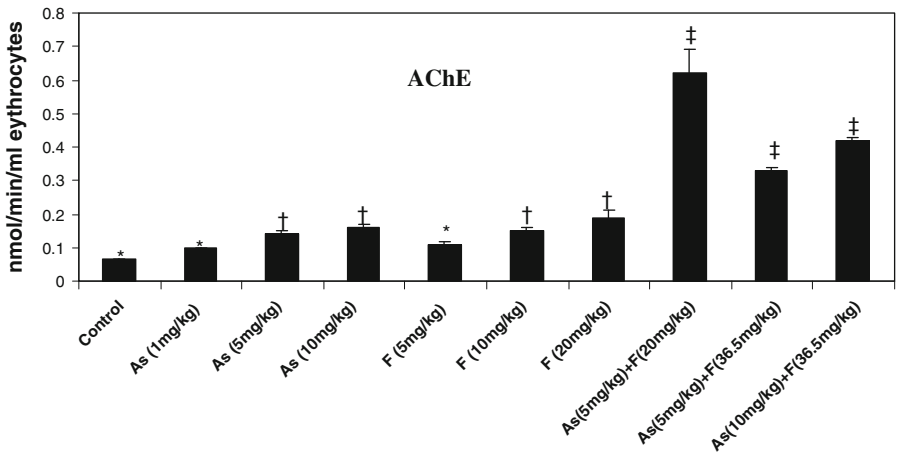

Fig. 6 Effect of different concentration of arsenic and/or fluoride on AChE activity in blood of rats. Abbreviation used and unit: AChE—acetyl cholinesterase as nmol/min/ml of erythrocytes. Values are mean ± SEM, $n = 5$. $*,\dagger,\ddagger$ $P < 0.05$ are considered significant by One Way ANOVA followed by Bonferroni's test

multi-metal exposure where they may mask or potentiate the toxicity of the each other is of current research interest (Yanez et al. 1991). Arsenic at 1 mg/kg did not significantly alter the cardio-respiratory variables at most of the measured time points and 10 mg/kg dose was found lethal to most animals per group therefore, 5 mg/kg dose of arsenic was selected for combination exposure with fluoride. In contrast, highest toxic dose of fluoride (20 mg/kg) was selected based on our previous findings that fluoride may mask arsenic-induced toxic manifestations (Mittal and Flora 2006, 2007; Flora et al. 2009). Although 36.5 mg/kg, which is the reported LD_{50} of fluoride in rat, has been used in combination to evaluate the above mentioned hypothesis, the individual group was excluded due to mortality observed at 20 mg/kg dose. Further, since results from the present study demonstrate lowered toxicity during concomitant exposure to arsenic (5 mg/kg) and fluoride (36.5 mg/kg) compared to the individual exposures, a second co-exposure with increased arsenic dose (10 mg/kg) was tested with fluoride at 36.5 mg/kg which was found synergistically toxic (lethal).

Mean arterial pressure or BP was lowered by individual exposure to arsenic or fluoride except for low arsenic dose (1 mg/kg) where immediate increase was followed by recovery to normal BP with time. Hypertension is generally associated with chronic arsenic exposure by oral route in animals and humans (Balakumar and Kaur 2009; Wang et al. 2007). However, acute arsenic toxicity via parenteral route couples with immediate elevation in BP may have been the results of increased vascular resistance and enhanced pressor responses to either preganglionic nerve stimulation or vasoactive agents such as vasopressin and phenylephrine (Balakumar and Kaur 2009). Moreover, low-level arsenic exposure has also been associated with increased risk of high BP (Zierold et al. 2004; Chen et al. 1995). The exact mechanism by which low-level arsenic exposure induces high BP is not clear however, it may be due to the inflammatory mediators (Tseng et al. 2003). Acute arsenic poisoning is characterized with sudden onset of profound hypotension which was in co-ordinance with our findings as evident at 5 and 10 mg/kg arsenic (Lai et al. 2005). Co-exposure to arsenic and fluoride on the other hand, demonstrated variable results. Arsenic (5 mg/kg) + fluoride (20 mg/kg) was found synergistically toxic causing significant decrease in BP that ended with mortality, while increased fluoride dose to 36.5 mg/kg resulted in antagonistic response with no change in BP that was comparable to normal. Thus, arsenic and fluoride interaction was dose related since high rate of mortality was observed on increasing arsenic (10 mg/kg) with fluoride at the same dose; demonstrating a non-toxic combination profile at doses close to there respective LD_{50} only at optimal combination doses. Decrease in mean arterial pressure is related to ischemic condition. In the present study, the decreased arterial pressure following arsenic or fluoride administration may be due to arterial blockage, which results in insufficient supply of oxygen to brain. Lee et al. (2002) also demonstrated that arsenic increased the susceptibility of platelets to aggregate, resulting in enhanced risk of arterial thrombosis, which could be a causal factor in the development of cardiovascular disease. Ischemic condition following arsenic or

fluoride administration is also supported by decreased respiration rate.

Heart rate (HR) is the average resting heartbeat per minute (Opie 2004). Animals exposed to arsenic at the dose of 1 mg/kg showed slight increase (non-significant) in resting HR (tachycardia) whereas high doses of arsenic (5 and 10 mg/kg) slowed down HR (bradycardia). There are several reports of serious life-threatening ventricular tachycardia on acute arsenic exposure (Lai et al. 2005; Ohnishi et al. 2000). Normally low blood pressure is regulated by increase in force of contraction of heart, to increase the stroke volume or the heart rate as a compensatory mechanism to maintain normal cardiac output that may be true in most reported cases of acute arsenic poisonings characterized with profound hypotension and tachycardia (Tortora and Grabowski 1993). However, in the present study high dose arsenic (5 and 10 mg/kg) may be producing sever acute cardiac arrhythmia progressing towards cardiac arrest due to cardiac conduction systems largely affected and low HR progressing to mortality in animal results. Arrhythmia is a series of sudden irregular heartbeats that can cause the heart to function incorrectly, or in the most severe cases to stop completely. Significant arrhythmias causing irregular heart function may also inhibit oxygen flow depriving the entire body of oxygen (Tortora and Grabowski 1993). Arsenic has been associated with cardiac conduction system abnormalities mainly due to prolongation of action potential duration that may be by blocking of potassium currents IKr and IKs in human (Barbey et al. 2003; Raghu and Cherian 2009). Conduction abnormalities like QT prolongation are reported even at the lowest arsenic doses ranging from 0.15 to 1.5 mg/kg via i.v. route without change in HR thus supportive of present findings (Chiang et al. 2002). In all the three doses of fluoride (5, 10, 20 mg/kg) reduced HR was recorded that was in co-ordinance with previous studies in fluorotic sheep (Donmez and Cinar 2003). Acute fluoride poisoning is reported to manifest severe hypocalcaemia, ventricular arrhythmias, and respiratory failure (Yolken et al. 1976). Thus, at high doses of fluoride decreased respiration rate leading to mortality may be contributed by combination of its effect on heart and respiratory failure. Moreover, reduced HR at high dose fluoride may be due to sudden cardiac death previously reported during acute fluoride intoxication by virtue of calcium fluoride salt precipitation leading to profound hypocalcaemia. Fluoride induced lethal ventricular arrhythmia has also been correlated with elevated serum potassium followed by drop in serum calcium (Mclvor et al. 1987). Combination exposure to both arsenic and fluoride showed results as those of BP, with arsenic (5 mg/kg) + fluoride (20 mg/kg) causing reduced HR following mortality whereas fluoride at LD_{50} (36.5 mg/kg) dose with arsenic (5 mg/kg) rendering the mixture nontoxic, supported by antagonistic interaction reported in our previous studies in other organ systems (Flora et al. 2009).

Insufficient supply of oxygen to different parts of the body due to ischemic condition may be responsible for decreased neuromuscular transmission (Whetzel et al. 1997). Neuromuscular transmissions were severely affected by arsenic or fluoride exposure in dose dependent manner. The signal passes through the neuromuscular junction via the neurotransmitter acetylcholine and its action is terminated by enzyme acetylcholinesterase which hydrolyses acetylcholine (Tortora and Grabowski 1993). In the present experiment we observed increased activity of AChE following arsenic or fluoride administration which suggests more pronounced hydrolysis of ACh at synapse. Rao et al. (2005) also reported increased activity of AChE on arsenic administration. Various reports suggest retardation in intellectual development of individuals exposed to arsenic or fluoride (Rocha-Amador et al. 2007; Li et al. 1995) that was attributed to decreased neuromuscular transmission. Impaired neuromuscular transmission also leads to respiratory failure. Fluoride causes retardation of the development of nerve cells in the cerebral cortex, with relatively poor differentiation, and fewer mitochondria, microtubules, and vesicles in the synapses as well as fewer synapses in general, possibly leading to less connection between neurons and abnormal synapse function, resulting in decreased transmission of signals (Shan et al. 2004). Disorders of neuromuscular transmission affect the neuromuscular junction. Presynaptic release of acetylcholine, breakdown of acetylcholine within the synapse may be one of the significant causes for such manifestation. Drugs or toxic chemicals, cholinergic drugs, organophosphate insecticides, and most nerve gases (arsine) block neuromuscular transmission by excessive acetylcholine action that depolarizes postsynaptic receptors which is in accordance with the present results (Munro et al. 1990).

Increased oxygen radical generation and lipid peroxidation have been implicated in the pathogenesis of arsenic and fluoride (Yanez et al. 1991; Smith and Steinmaus 2000; Shivarajashankara et al. 2001). Arsenic is reported to stimulate the formation of ROS in vascular endothelial and smooth muscle cells, mainly via NADH/NADPH oxidase (Lynn et al. 2000). Although there are many reports that arsenic and fluoride stimulates ROS production and induces oxidative stress in vitro and in vivo, little is known about the relationship between oxidative stress and physiological variables indicative of cardio-respiratory dysfunction. Oxidative stress produced by free radicals is greater if arsenic or fluoride impairs the production of free radical scavengers (Shivarajashankara et al. 2001; Flora et al. 2009). In the present experiment increased ROS levels and decreased concentration of GSH in arsenic or fluoride exposed animals may be involved in the pathogenesis of cardiac disorders. We find significant correlation between arsenic concentration and ROS level ($r^2 = 0.972$) (Fig. 7a), however, there was no correlation between increased concentration of fluoride and ROS level ($r^2 = 0.0961$) (Fig. 7b) and combined exposure to arsenic and fluoride with ROS ($r^2 = 0.3224$) (Fig. 7c). There are various reports which suggest controversial findings related to fluoride induced oxidative stress at different doses (Chouhan and Flora 2008; Chlubek 2003), however, none of them provide appropriate justification regarding more pronounced toxicity at lower dose and less toxicity at higher dose.

One of the interesting finding of the present experiment is synergism and antagonism at different doses of arsenic and fluoride. Reports are available that suggest toxic effects of the combined exposure to arsenic and fluoride are more pronounced than their individual exposure (Rao and Tiwari 2006), however other reports indicate an antagonistic behavior between arsenic and fluoride at certain dose level (Yao and Wang 1988a, b). In the present experiment we also observed that administration of higher dose of fluoride (36.5 mg/kg) with arsenic (5 mg/kg) provided significant protection against oxidative stress and altered cardio-respiratory variables. Chevrier and Brownstein (1980) have reported complexation between arsenic tri fluoride and fluoride ion in solution. The formation of AsF_5OH^- (Kolditz and Nussbuk 1965), $AsF_4(OH)_2^-$ and $AsF_4(OCH_3)_2^-$ species have also been reported (Kolditz 1967). Mazej and Zemva

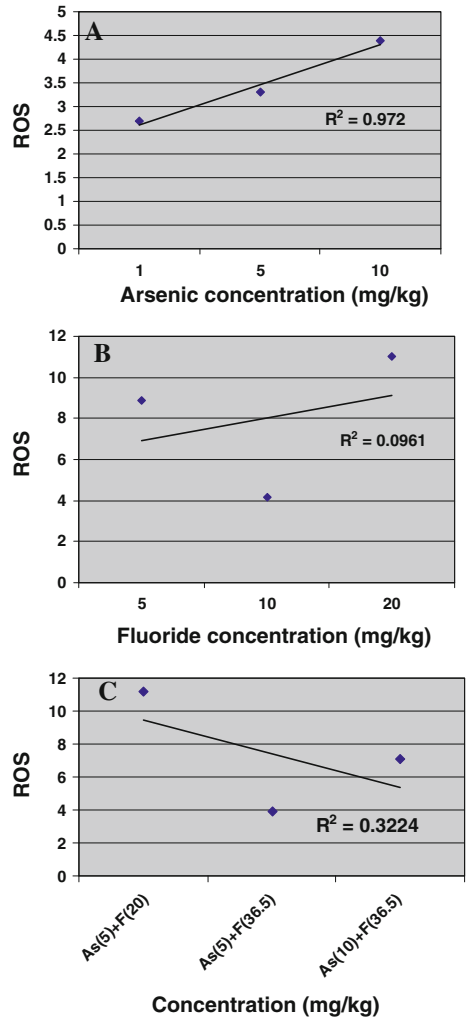

Fig. 7 Correlation between ROS level and **a** different concentration of arsenic, **b** different concentration of fluoride, **c** combination of arsenic and fluoride at different doses

(2005) reported oxidation of arsenic-trioxide to arsenic-penta fluoride by elemental fluoride. Although it can be suggested that arsenic and fluoride may interact with each other to form a stable complex thereby antagonizing toxic effects of each other but this interaction depend on the concentration of both the toxicant, which is evident from the present study.

In conclusion, arsenic and fluoride produce deleterious effects on heart and produce ischemic condition at high doses. The results also suggest that toxic effects of arsenic and fluoride may be mediated via generation of ROS and depleting antioxidant defense system. At certain concentration, arsenic and fluoride shows antagonistic behavior, probably due to neutralization

of active ions of arsenic and fluoride which have high affinity for bio-molecules however, it needs further exploration especially with higher doses of arsenic and fluoride.

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Conflict of interest None.

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