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Oxidative changes in brain tissue after concurrent exposure to arsenic and quinalphos in Wistar rats

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ABSTRACT

The present study was designed to assess oxidative changes occurring in brain after concurrent exposure of arsenic and quinalphos in Wistar rats. Rats of either sex were randomly allocated to nine groups of six rats each and were administered quinalphos and arsenic either alone or in-conjunction with each other for 28 days. Group I served as control, group II and III received orally quinalphos at 1/100th and 1/10th of LD_{50} respectively, whereas group IV and V received arsenic 50 and 100 ppb respectively in drinking water. Group VI and VII received low and high dose of quinalphos respectively along with arsenic (50 ppb) in drinking water. Similarly the animals comprising group VIII and IX received higher and lower doses of quinalphos respectively with arsenic (100 ppb) added in drinking water. Significant ($P<0.05$) declines in brain acetylcholinesterase (AChE), total thiols (TTH), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-s-transferase (GST), glutathione reductase (GR) along with significant elevations in ($P<0.05$) malondialdehyde (MDA) levels pointed towards the occurrence of oxidative damage in brain following repeated administrations of quinalphos at either dose levels or arsenic at the concentration of 100 ppb as compared to control. Moreover, these alterations were found to be more pronounced in groups receiving both treatments concurrently viz. decreased levels of AChE (55.4%), TTH (51.5%), CAT (38.4%), SOD (29.6%), GPx (40.9%), GST (54.9%) and GR (44.4%) with increased MDA (102.3%) as compared to control group. Histopathological changes observed in brain included neuronal degeneration and necrosis, gliosis, neuronophagia and spongiosis which correlated well with dose and co-exposure induced altered antioxidant biomarkers in brain. Hence these findings underline the subtle neurotoxic potential of arsenic and quinalphos which is enhanced with their concurrent exposure.

KEYWORDS

Acetylcholinesterase; Quinalphos; Arsenic; Malondialdehyde; Neurotoxicity; Total thiols.

INTRODUCTION

Ubiquitous contamination of environment by human activities such as indiscriminate use of agrochemicals and rampant industrialization have lead to rising ground water levels of toxicants such as metals/metalloids and organophosphorus compounds which adversely impact health of exposed human and animals. Quinalphos (O,O-diethyl O-quinoxalin-2-yl phosphorothioate), an organophosphorus compound, is used not only for the control of a wide variety of pests affecting agriculture sector but is also used as

Original Research Article

an acaricide for control of disease vectors affecting animal health[1-4]. Quinalphos has been classified as an acute, hazardous compound by the World Health Organization (WHO) and its application has either been banned or is restricted in most countries. However, in India, quinalphos is still used as a yellow-label pesticide for protection of food crops like wheat, rice, coffee, sugarcane and cotton from pests[4,5]. Quinalphos, irreversibly inhibits acetylcholinesterase (AChE) enzyme causing accumulation of acetylcholine (ACh) at neuromuscular junctions which results in a cholinergic crisis due to a continuous stimulation of cholinergic receptors ultimately causing paralysis and death[5,6].

Arsenic (As), a naturally occurring toxic metalloid in earth's crusts, is also a major toxicant posing a serious threat to animal and human health. Contaminated drinking water is the primary source of arsenic toxicity for animals and humans[7]. Exposure to lower concentrations of arsenic in drinking water has been shown to increase susceptibility to develop neurological and cognitive dysfunction in rodents and humans[8,9]. Although WHO and Environmental Protection Agency (EPA) have defined the safety limits of arsenic in drinking water but no such strict guidelines have been provided for food, beverages and air which can be the other potential sources of its exposure[10].

Accumulating experimental and clinical data suggests that increased concentration of different kinds of toxicants in the ecosystem can be linked to increased incidences of adverse health effects including neurological disorders in mammals[4,6]. However, only few prior studies have been conducted to determine the effects of co-exposure to arsenic and quinalphos on nervous tissue. Therefore, the present study was formulated to assess the impact on antioxidant status and histomorphology of brain upon concurrent exposure to low doses of arsenic and quinalphos, mimicking their exposure under natural conditions, in Wistar rats.

MATERIAL AND METHODS

Experimental model

Adult Wistar rats (180-200g) of either sex were procured from Indian Institute of Integrative Medicine, Jammu. All animals were maintained under standard managemental conditions ($22 \pm 3^{\circ}C$, 50-60% relative humidity and 12 h light-dark cycles). Prior to the start of experiment, wistar rats were acclimatized to the laboratory conditions for a period of 15 days. The experimental protocol was dully approved by Institutional Animal Ethics Committee (IAEC) vide proposal no 7/IAEC-17/2017. All experimental rats received humane care in accordance with National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). The maximum contaminant level (MCL) of arsenic in drinking water is 50 ppb and in the present study, two dose levels *viz*., 50 and 100 ppb in drinking water were used.[11] Two different doses of quinalphos $1/100^{th}$ and $1/10^{th}$ of median lethal dose (LD_{50} - 19.9mg/kg) were used alone and in-combination with different levels of arsenic to evaluate the cumulative toxic effects on neurological tissue of rats[12].

Fifty four adult Wistar rats were randomly allocated to nine groups of six rats each and subjected to different treatments for 28 days. Group I served as control receiving only distilled water (1 ml/day/rat), group II and III received orally quinalphos at $1/100^{th}$ and $1/10^{th}$ of LD_{50} (19.9 mg/kg), respectively, whereas group IV and V received arsenic at the rate of 50 and 100 ppb, respectively, in drinking water. Group VI and VII received quinalphos at the rate of $1/100^{th}$ and $1/10^{th}$ of LD_{50} along with arsenic in drinking water at the concentration of 50 ppb. The animals comprising group VIII and IX received quinalphos at the rate of $1/100^{th}$ and $1/10^{th}$ of $LD_{50,}$ respectively, along with arsenic in drinking water at the concentration of 100 ppb. The animals received daily dosing of quinalphos orally between 9.00-10.00 AM for a period of 28 days.

Processing and estimation of parameters

After 28 days, animals were sacrificed by cervical dislocation and brain (1 g) was collected in 10 ml ice cold phosphate buffer solution (0.5 M pH-7.4) for the estimation of acetylcholinesterase (AChE) activities and antioxidant parameters. Tissue samples were homogenized using teflon coated homogenizer at 1000 rpm for 5-7 min at refrigerated temperature and 10% tissue homogenate was prepared for determination of activities of catalase (CAT)[13], superoxide dismutase

(SOD)[14], glutathione peroxidase (GPx)[15], glutathione reductase (GR)[16], glutathione-s-transferase (GST)[17] and AChE[18] in nervous tissue of Wistar rats. Concentrations of total thiols (TTH)[19] and malondialdehyde (MDA)[20] in rat brain were determined as per the standard described protocols. Level of MDA was expressed in nmol MDA produced/g of tissue/hr whereas TTH level expressed in mM using reduced glutathione as a standard. The activities of AChE were expressed in nmol thiol group formed/min using reduced glutathione as standard curve.

Histopathological studies

Processing of brain for histopathological studies was carried out according to standard protocol. Formalin fixed brains of different groups were embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) and examined under microscope. The histopathological lesions encountered in brain sections were scored as follows: no (-), mild (+), moderate (++) and severe (+++).

Statistical analysis

The antioxidant parameters were presented as mean ± standard error and analyzed by analysis of variance at 5% level of significance using the Duncan Multiple Range Test (SPSS 16.0). Results in the treatment groups were also expressed as the percentage of control values (100%) which are presented in parenthesis in the results.

RESULTS

Effect on brain AChE activities

The mean values of AChE activities in rat brain of different groups following repeated oral administration of quinalphos and arsenic alone and in combinations are presented (TABLE 1). Repeated oral administrations of quinalphos significantly ($P<0.05$) reduced AChE activities by 27.1% and 38.5% at the dose rates of 0.199 mg/kg and 1.99 mg/kg, respectively. Similarly, arsenic at 50 ppb similarly reduced AChE activity by 10.4% ($P>0.05$) whereas 100 ppb reduced activity by 35.8% ($P<0.05$) in nervous tissue of exposed rats. Repeated administrations of quinalphos (1.99 mg/kg) in rats with arsenic produced significantly ($P<0.05$) declined AChE activities in a dose dependent manner at 100 ppb (55.4%) and 50 ppb (44.5%) as compared to control.

Effect on total thiols (TTH) level

The mean values of TTH level in the brain tissue of rats of different groups following repeated oral administration of quinalphos and arsenic alone and in combination with each other are presented (TABLE 1). The repeated administrations of either quinalphos or arsenic reduced level of TTH in brain by 32.5-47.9% and 27.5-42.9%, respectively; however, decline in TTH levels was more pronounced in concurrently administered groups (47.2-53.4%).

TABLE 1: Effects of repeated oral administrations of quinalphos and arsenic alone and in-combination on activities of acetylcholinesterase and antioxidant biomarkers in brain of Wistar rats.

Groups	Treatment given	AChE	TTH	CAT	MDA
I.	Distilled water (1 ml/day/rat)	$20901.25^{f} \pm 921.69$	$4.43^{d} \pm 0.31$	$2212.6^{b} \pm 249.0$	$38.72^{a} \pm 3.53$
II.	Quinalphos (0.199 mg/kg)	$15240.00^{cd} \pm 918.40$	$2.99^{bc} \pm 0.14$	$1764.1^{ab} \pm 260.9$	$44.44^{bc} \pm 3.36$
III.	Quinalphos (1.99 mg/kg)	$12853.75^{bc} \pm 606.21$	$2.31^{abc} \pm 0.15$	$1578.7^{ab} \pm 211.8$	$51.61^{bc} \pm 3.70$
IV.	Arsenic (50 ppb)	$18722.50^{ef} \pm 1034.54$	$3.39^{d} \pm 0.18$	$2043.6^{ab} \pm 254.0$	$42.99^{ab} \pm 2.61$
V.	Arsenic (100 ppb)	$13421.25^{bc} \pm 378.68$	$2.72^{c} \pm 0.19$	$1812.6^{ab} \pm 223.5$	$46.32^{bc} \pm 3.54$
VI.	Quinalphos (0.199 mg/kg) + As (50 ppb)	$16923.75^{de} \pm 795.74$	$2.53^{ab} \pm 0.14$	$1627.9^{ab} \pm 333.7$	$52.67^{abc} \pm 4.39$
VII.	Quinalphos (1.99 mg/kg) + As (50 ppb)	$14601.25^{bc} \pm 1000.01$	$2.49^{ab} \pm 0.16$	$1416.7^{a} \pm 170.9$	$59.22^{cd} \pm 4.71$
VIII.	Quinalphos (0.199 mg/kg) + As (100 ppb)	$12577.50^{ab} \pm 424.72$	$2.34^{a} \pm 0.11$	$1560.8^{ab} \pm 118.5$	$66.05^{bc} \pm 4.15$
IX.	Quinalphos (1.99 mg/kg) + As (100 ppb)	$10326.25^{a} \pm 749.99$	$2.16^{a} \pm 0.21$	$1363.4^{a} \pm 161.3$	$78.32^{d} \pm 5.82$

(1) Values are given as mean ± SE of 6 animals unless otherwise stated; (2) Values having different superscripts (a, b, c) in a column are statistically different from one another at 5 % level of significance; (3) Activities of acetylcholinesterase (AChE) are expressed in nmole of thiol group produced /min/ mg of tissue; (4) Values of TTH (Total thiols) are expressed in µM; (5) Values of CAT (Catalase) are expressed in µmol H_2O_2 decomposed/min/g of tissue; (6) Values of malondialdehyde (MDA) are expressed in nmol MDA formed/g in tissue/ hr

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TABLE 2: Effects of repeated oral administrations of quinalphos and arsenic alone and in-combination on activities of antioxidant enzymes in brain of control and treated rats.

Groups	Treatment given	GPx	SOD	GST	GR
I.	Distilled water (1 ml/day/rat)	$41.77^{d} \pm 3.42$	$253.67^{c} \pm 21.41$	$22.87^{e} \pm 1.05$	$28.08^{d} \pm 2.51$
II.	Quinalphos (0.199 mg/kg)	$35.47^{bcd} \pm 3.29$	$214.67^{abc} \pm 19.04$	$16.35^{bcd} \pm 1.91$	$23.28^{bcd} \pm 2.74$
III.	Quinalphos (1.99 mg/kg)	$31.74^{abc} \pm 2.73$	$194.17^{abc} \pm 16.85$	$14.37^{abc} \pm 1.77$	$21.38^{abc} \pm 1.67$
IV.	Arsenic (50 ppb)	$38.69^{cd} \pm 2.16$	$241.37^{bc} \pm 19.26$	$19.80^{de} \pm 1.43$	$25.20^{cd} \pm 2.25$
V.	Arsenic (100 ppb)	$37.45^{cd} \pm 3.55$	$233.8a^{bc} \pm 21.99$	$17.07^{cd} \pm 2.05$	$24.00^{cd} \pm 2.09$
VI.	Quinalphos (0.199 mg/kg) + As (50 ppb)	$28.73^{ab} \pm 2.09$	$201.7^{abc} \pm 15.85$	$13.72^{abc} \pm 0.98$	$20.32^{abc} \pm 0.95$
VII.	Quinalphos (1.99 mg/kg) + As (50 ppb)	$27.60^{ab} \pm 2.65$	$183.8^{ab} \pm 21.19$	$12.17^{ab} \pm 1.05$	$17.28^{ab} \pm 1.85$
VIII.	Quinalphos (0.199 mg/kg) + As (100 ppb)	$25.80^{a} \pm 2.22$	$187.2^{ab} \pm 16.95$	$12.57^{ab} \pm 1.08$	$17.42^{ab} \pm 1.89$
IX.	Quinalphos (1.99 mg/kg) + As (100 ppb)	$24.69^{a} \pm 2.50$	$178.5^{a} \pm 17.94$	$10.30^{a} \pm 1.06$	$15.60^{a} \pm 2.11$

(1) Values are given as mean ± SE of 6 animals unless otherwise stated; (2) Values having different superscripts (a, b, c) in a column are statistically different from one another at 5 % level of significance; (3) Values of SOD (Superoxide dismutase) and GPx (glutathione peroxidase) are expressed in Unit/gof tissue; (4) Values of GST (glutathione-s-transferase) are expressed in µmol of CDNB conjugate formed/ min/gof tissue; (5) Values of GR (glutathione reductase) are expressed nmol of NADPH/min

Effect on activities of SOD and CAT

Significant ($P<0.05$) decline in CAT (28.7%) and SOD (23.5%) was observed after quinalphos administration at higher dose ($1/10^{th}$ of LD_{50}) whereas only non-significant alterations in the activities were observed in groups administered low dose of quinalphos and either doses of arsenic. But concurrent exposure of quinalphos and arsenic reduced activities of CAT (38.4%) and SOD (29.6%) significantly ($P<0.05$) (TABLE 2).

Effect on activities of thiols containing enzymes

Activities of GPx, GST and GR were significantly reduced by subacute administrations of quinalphos (1.99mg/kg), whereas quinalphos at 0.199mg/kg and either dose of arsenic didn't alter the activities significantly. However, concurrent exposure of quinalphos and arsenic significantly ($P<0.05$) reduced the activities of GPx (31.2-40.9%), GST (40.0-54.9%) and GR (27.6-44.4%) in rats (TABLE 2).

Effect on MDA levels

The significantly ($P<0.05$) increased level of malondialdehyde (MDA) in brain indicates increased oxidative damage in nervous tissue on exposure of toxicants. MDA levels in quinalphos (14.8-33.3%) and arsenic (11.0-19.6%) treated animals were significantly increased as compared to control. More pronouncedly altered levels (36.0-102.3%) were observed in groups of animals receiving toxicants concurrently.

Histopathological alterations

Treatment with toxicants individually or in combination produced notable lesions in brain tissue and the lesion scores as recorded among various treatment groups are given in TABLE 3. Histology of brain in control rats showed presence of healthy neurons possessing a nucleus and pink cytoplasm, neuronal processes and glial cells (Figure 1a). Varying degree of neuronal degeneration was seen in different treatment groups after repeated administration of arsenic and quinalphos which became more severe as the dose of these toxicants was increased or when they were co-administered. Neuronal necrosis wherein the nerve cells became shrunken, intensely eosinophilic with pyknotic or absent nuclei was seen in group III to IX except group IV rats (Figure 1b). However, neuronal necrosis was most severe in rats of group IX (Figure 2a). In many cases accumulation of glial cells (gliosis) and neuronophagia was often seen wherein these necrotic neurons were being devoured by surrounding glial cells and this was particularly striking and most severe in group IX animals. Lesions such as axonal degeneration, edema and resultant spongiosis as evidenced by presence of vacuolation in brain sections, albeit of a milder degree, were seen in group III, VI and VII rats. While a moderate degree of vacuolation occurred in group VIII, a very severe degree of spongiosis was present in group IX animals (Figure 2b). Overall, most severe alterations in brain architecture were found in group IX rats.

TABLE 3: Scoring of histopathological alterations in brain tissue of rats on subacute exposure of quinalphos and arsenic alone and in-combination in Wistar rats

Brain alterations	Treatment groups (n=6)								
	I	II	III	IV	V	VI	VII	VIII	IX
Neuronal degeneration	-	+ (3)	+ (4)	+ (4)	+ (4)	+ (5)	++ (5)	++ (4)	+++ (6)
Neuronal necrosis	-	-	+ (3)	-	+ (4)	+ (5)	++ (5)	++ (5)	+++ (6)
Gliosis	-	-	++ (2)	-	+ (2)	+ (2)	+ (3)	++ (3)	+++ (4)
Axonal degeneration and spongiosis	-	-	+ (2)	-	-	+ (3)	+ (3)	++ (4)	+++ (6)

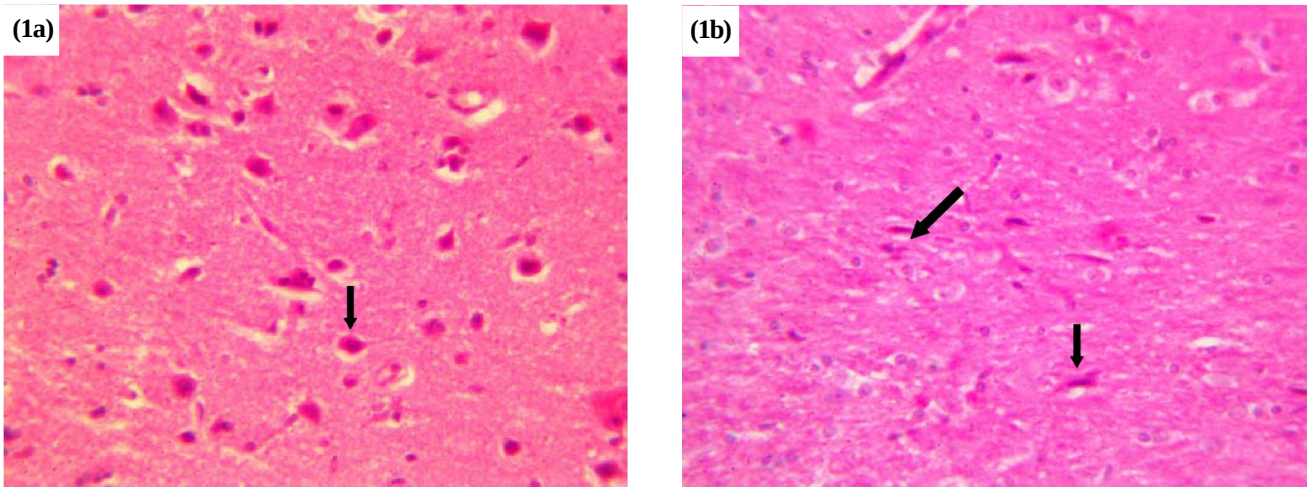

Figure 1: Brain section with normal neurons (arrow) having a well defined cytoplasmic boundary and a distinct nucleus in brain of group I rats (1a) and deeply eosinophilic and shrunken neurons (arrows) in brain of group VI rats (1b). (H&E 400X)

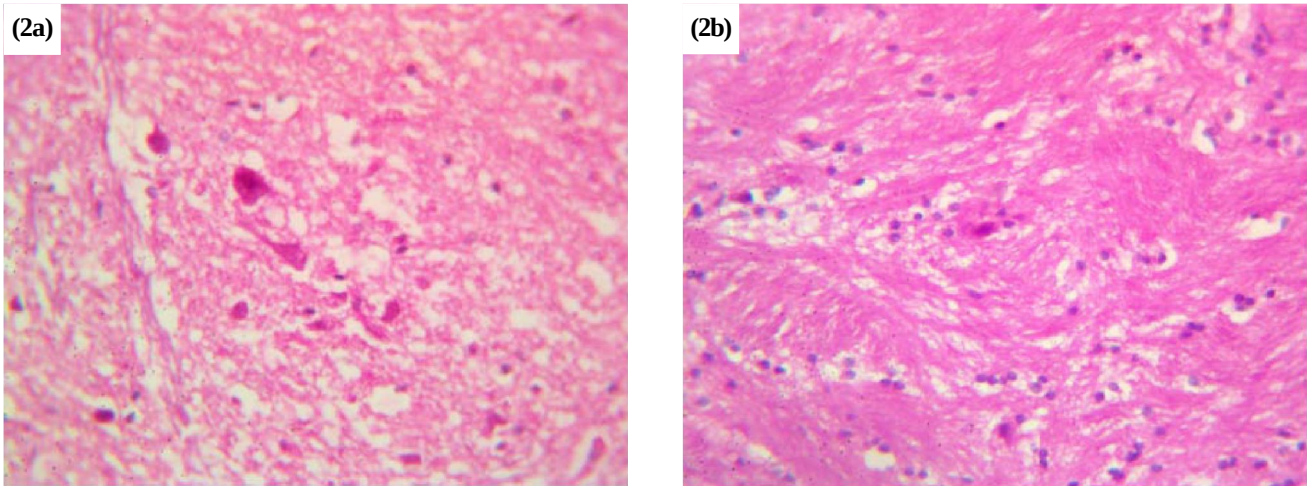

Figure 2: Vacuolation due to edema (arrow) and severe neuronal necrosis (arrowhead) in brain of group IX rats (2a) and gliosis (arrow) and neuronophagia of necrotic neuron (arrow heads) by glial cells in brain of Group IX rats (2b). (H&E 400X)

DISCUSSION

Acetylcholine is major neurotransmitter in central nervous system and inhibition of AChE activities leads to accumulation of acetylcholine in brain inducing neurotoxicity which depends on intensity of enzyme inhibition[5,6]. These findings are in accordance with the reported AChE inhibitory activities of quinalphos in buffalo[21] as well as that of chlorpyrifos[22], bifenthrin[23] and deltamethrin[24] in Wistar rats. High incidence of neurological disorders has also been reported in populations inhabiting areas where concentrations of arsenic in drinking water is high, which has been

postulated to be due to altered levels of neurotransmitters, particularly acetylcholine, in brain[6,25,26]. Our findings, in fact, illustrated that inhibitory effect on AChE activity was significantly more pronounced in rats exposed to both the toxicants. The AChE activity inhibition has also been observed in rats exposed to fluoride and chlorpyrifos[22], fluoride and deltamethrin[24] and bifenthrin[6,23].

Decline in TTH level in a dose dependent manner indicates oxidative damage induced by arsenic and quinalphos. Binding of arsenic and other reactive intermediate molecules to thiol (-SH) molecules decreases direct free radical scavenging potential of cell[29,30]. Reduction in level of thiols leads to arsenic accumulation in tissues and depletion of endogenous antioxidant activity and this oxidative damage is believed to accelerate arsenic induced tissue damage[29,31]. Besides tendency of arsenicals to react with thiol-containing antioxidants, it also down-regulates the expression of nuclear factor 2-antioxidant response element (Nrf2-ARE) components[29,32,33].

Nrf2-ARE is the primary pathway involved in stimulating several cellular proteins like GPx, GST, GR, SOD, CAT, etc which have a central regulatory role in cellular defense against cellular oxidative stress[29,31,32]. Activities of CAT and SOD are important for scavenging the peroxide and superoxide radicals formed during metabolic reactions. Significant reduction in the activities on administration of quinalphos and non-significant reduction in arsenic indicate either increased production of these radicals or declined turnover of CAT and SOD. Studies have suggested that quinalphos does not dispose reactive groups capable of inducing oxidative stress directly, but rather inflicts a secondary oxidative damage by the 2-hydroxyquinoxaline metabolite which easily undergoes redox reaction[34,35]. Declined activities of Nrf2-ARE components increase cellular accumulation of peroxides, superoxide and other free radicals which initiate chain reactions causing cellular damage. Activities of GST and GPx require thiols for catalysis and reduced thiol levels (declined GR activities) as occurred in the present study may contribute to reduction in turn over of these enzymes. Alterations in thiol homeostasis have also been reported in subacute toxic interaction of arsenic and imidacloprid[36], and fluoride and deltamethrin[24].

Reduced antioxidant components and increased levels of toxic intermediates induce damage to proteins, nucleic acids and lipids leading to various cellular dysfunctions including apoptosis and necrosis[37,38]. The significantly increased level of MDA upon co-exposure as compared to the individual treatments indicates the enhancement in oxidative damage in nervous tissue after co-exposure. A number of other experimental studies have also shown that quinalphos and its metabolites can induce oxidative insults on nervous tissue due to the depletion of protective antioxidant system[2,12,39-41]. Increased MDA levels have been reported on exposure of arsenic and quinalphos alone and also after concurrent administration of toxicants like arsenic and imidacloprid[36], and fluoride and deltamethrin[24].

Histopathologically, mild, moderate or severe changes in brain induced by toxicants were in proportion to the dose level of individual toxicant administered and toxicant co-administration. The microscopic lesions observed included neuronal degeneration and necrosis, neuronophagia, gliosis, edema and vacuolation. Similar changes in brain were also observed upon exposing brown trout to cadmium and humic acid[42]. Quinalphos exposure induced similar changes in brain of *Cyprinus carpio*[43]. Also, edematous changes in brain tissue after exposure of arsenic and quinalphos when used in combination were responsible for the most severe histomorphological alterations in our study. In all, the pathological findings observed were in line with our data on alterations in antioxidant status upon administration of these toxicants. Further, the underlying antioxidant deficits were likely responsible for development of pathological lesions observed in nervous tissue. Similar to our findings, significant alterations in SOD, CAT and GPx activities and MDA levels in the brain were found to correlate well with pathological lesions in brain of common carp exposed to atrazine and chlorpyrifos[45].

CONCLUSIONS

It can be concluded from our study that the reductions in the antioxidant biomarkers as well as AChE activities and enhancement of MDA levels following repeated exposure of quinalphos and arsenic in Wistar

rats caused significant oxidative damage and further lead to development of pathological lesions in brain. More strikingly, the significantly higher alterations in the antioxidant status and histomorphology of brain, observed in the co-exposed groups as compared to the groups exposed to individual toxicants indicate that the unintended subacute concurrent exposure to arsenic and quinalphos can produce neurological health deficits among exposed populations inhabiting contaminated geographical locations.

AUTHOR'S CONTRIBUTION

All authors of manuscript participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; Parvinder Singh, Pawan Kumar Verma, Priyanka Sharma conducted the experiments, Rajinder Raina supervised and supplied the required materials for the research and histopathological studies were conducted by Shilpa Sood.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, and/or publication of this article.

ETHICAL APPROVAL

The experimental protocol was dully approved by Institutional Animal Ethics Committee (IAEC) vide proposal no 7/IAEC-17/2017.

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Original Research Article

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