Antioxidant potential of Thymoquinone against Arsenic mediated neurotoxicity

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ABSTRACT

Introduction: Arsenic, an established poisonous metalloid has widespread occurrence in the environment which is posing a constant threat to health and survival of all living organisms. In view of arsenic induced oxidative stress and toxicity, this study focuses on the mitigatory role of thymoquinone, a major active component in the volatile oil of *Nigella sativa* (commonly known as black cumin); as its nutraceutical value can fulfil the demand of such dietary supplements in high risk population areas. **Methods:** In the current study, brain preparations of male wistar rats were used to assess different biochemical markers of oxidative stress and genotoxicity. **Results:** Significant and dose-dependent alterations in the level of enzymatic and biochemical biomarkers of oxidative stress were observed in the As-treated system. However, pre-treatment with thymoquinone brought about significant reduction in the As-induced neurotoxicity. Also, a significant decline in arsenic-induced DNA damage was recorded on pre-treatment with thymoquinone in comet assay. **Conclusion:** This study proves usefulness of antioxidant potential of thymoquione in mitigating the arsenic induced neurotoxicity.

Key words: Arsenic, DNA Damage, Neurotoxicity, Oxidative Stress, Thymoquinone.

INTRODUCTION

Arsenic (As) is widely distributed in atmosphere in various forms. It is constantly released into the environment through natural weathering of geological forms and through anthropogenic factors such as agricultural wash-off, mining, manufacturing, burning of fossil fuels and incineration. Arsenic contamination in ground water has been found in Taiwan, Mexico, Chile, Argentina, Thailand, Bangladesh and India, where levels are much higher than the maximum permissible limit i.e., 10 ppb (as per the WHO report, 2001) which raises serious concern about the well being of their inhabitants.[1]

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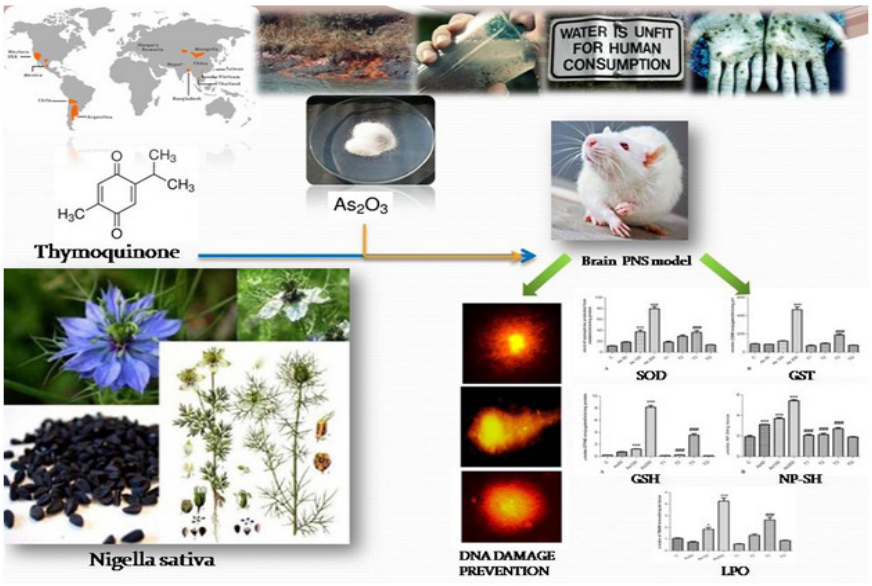
Arsenic exists in both organic as well as inorganic forms and a large fraction of it in the surface water exists as As^{5+} while ground water derived from deep anoxic wells contains As^{3+} form. The trivalent form of arsenic has been reported to be more toxic than the pentavalent form.[2] The populations at higher risk have been struggling with secondary morbidities such as increased risk for cancers,[3] cardiovascular disease[4,5] and childhood neuro-developmental deficits for decades.[6] It is a known neurotoxin that has both neurodevelopmental and cognitive effects as it can cross blood brain barrier.[7] Numerous *in vivo* studies have reported higher damages in brain regions involved in higher cognitive functions such as basal ganglia, corpus striatum, cortex and hippocampus.[8-10] Sodium arsenite, the biotransformation product of arsenic trioxide has shown marked teratogenesis including delayed maturation of Purkinje cells and their defective migration during rapid brain growth period,[11] alteration in motor behaviour[9,12] and impaired learning and memory.[13,14]



Graphical Abstract

$As^{3+/5+}$ contamination in drinking water has been correlated with poorer scores in global cognition, processing speed and immediate memory.[15] Alarmingly, prolong As exposure induces changes that coincide with most of the developmental, biochemical, pathological, and clinical features of Alzheimer's disease (AD), vascular dementia and associated disorders.[16]

Reactive oxygen species mediated oxidative damage is a common denominator in As pathogenesis as it increases formation of ROS, including peroxyl radicals (ROO•), the superoxide radical, singlet oxygen, hydroxyl radical (OH•) via the Fenton reaction, hydrogen peroxide, dimethyl arsenic radical, dimethylarsenic peroxyl radical and induces lipid peroxidation which in turn generate more bioactive oxygen and nitrile molecules.[17,18]

Medicinal plants have shown significant therapeutic potential as they contain several compounds having strong antioxidant properties. Extract of seeds and oil of *Nigella sativa*, a native of South and South West Asia have been widely used since medieval ages because of their medicinal values. Thymoquinone, TQ (2-isopropyl-5-methyl-1,4-benzoquinone) is a predominant bioactive constituent in the volatile oil of *Nigella sativa* and possesses antioxidant, anti-inflammatory, anti-apoptotoic, chemopreventive and hepato-protective properties.[19-24] It plays a significant mitigatory role against various heavy metal induced toxicities.[25] The protective effect of TQ is attributed to its free radical scavenging activity,[26,27] Hence, TQ stands as an important candidate for therapeutic approach against As-induced neurotoxicity as it can cross blood brain barrier. Govil *et al.* (2012) demonstrated the suitability of Post Nuclear Supernatant (PNS) as an alternate in-vitro model for assessing ROS mediated oxidative stress induced heavy metal toxicity.[28] Similarly, Zafeer *et al.* (2012) have evinced that PNS model can be used for assessing the abrogatory role of natural compounds. In the present study, we have studied the mitigatory role of TQ pre-treatment on As-induced neurotoxicity in rat brain PNS model.[29]

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA), 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB), epinephrine, reduced glutathione (GSH) and sulfosalicylic acid were purchased from Hi-Media Labs, Mumbai, India. Thymoquinone (TQ), Arsenic trioxide (As_2O_3) and propidium iodide were purchased from Sigma Aldrich Co. (St. Louis, MO). 2,4, dinitrophenylhydrazine (DNPH), ethylene diamine tetraacetic acid (EDTA) and other routine chemicals were obtained from Merck, India Ltd.

Animals

Male wistar rats (body weight 200 ± 20 g) were used in the present study. The animal use protocol had been approved by the Institutional Animals Ethics Committee (IAEC) of Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh. Animals were housed in cages maintained under suitable conditions with temperature (25 ± 1°C), humidity (60 ± 10%), ventilation (continuous circulation of fresh air), and illumination (a 12-h dark and 12-h light cycle).

Sample Preparation

Rats were sacrificed by cervical dislocation and their brains were immediately harvested. Subsequently, these were homogenized (1:10 w/v) in 0.1 M sodium phosphate (pH 7.4) buffer with a Potter–Elvehjem homogenizer under cold conditions. The homogenate obtained was then centrifuged at 3000 rpm for 10 min at 4ºC temperature, using a REMI C-24 centrifuge (Remi Sales and Engineering Ltd., Mumbai, India), and the supernatant was collected, only after pelleting the nuclei during subcellular fractionation. The post-nuclear supernantant (PNS) was immediately used for biochemical assessments.

Experimental Design

For *in vitro* evaluation of As_2O_3-induced neurotoxicity and its protection with TQ, samples were divided into eight groups namely: Control (C), As 50 (50 μM arsenic), As 100 (100 μM arsenic), As 200 (200 μM arsenic), T1 (50 μM arsenic with pre-exposure to 10 μM TQ), T2 (100 μM arsenic with pre-exposure to 10 μM TQ), T3 (200 μM arsenic with pre-exposure to 10 μM TQ) and TQ (10 μM thymoquinone only; this medial dose of thymoquinone was selected after several pilot studies).

For this assessment, PNS with TQ (10 μM) was primarily incubated at 37◦C for 1 h to replenish pre-protection before exposure to three different doses of arsenic. Thereafter, the cellular fractions were exposed to As_2O_3 for 1 h. The exposure regime was planned in such a way that the end point of all the groups took place at the same time. Subsequently, oxidative enzymes, non-enzymatic antioxidants, and oxidative stress markers were estimated.

Antioxidant Enzyme Assays

Determination of Superoxide Dismutase activity

Superoxide dismutase (SOD) activities were measured according to the method described by Misra and Fridovich.[30] The assay was based on the ability of SOD to inhibit the auto-oxidation of epinephrine at alkaline pH. The assay mixture contained 50 mM glycine buffer (pH 10.4), PNS (prepared in glycine buffer), and epinephrine. SOD activity was measured kinetically at 480 nm. The activity was measured indirectly by the oxidized product of epinephrine, i.e., adrenochrome. SOD activity was expressed as nmol of (−) epinephrine protected from oxidation/min/mg protein by the sample compared with the corresponding reading in the blank using a molar extinction coefficient of 4,020 M^{-1} cm^{-1}.

Determination of Glutathione-S-Transferase activity

The method of Habig *et al*.[31] with some modifications was used to measure Glutathione-S-Transferase (GST) activity. This reaction product was quantitated by observing the conjugation of CDNB with GSH forming a colored conjugate glutathione 2,4-dinitrobenzene. For activity measurement, the reaction mixture contained 0.1 M sodium-phosphate buffer (pH 7.4), 10 mM GSH, 10 mM CDNB, and 0.2 ml PNS. The enzyme activity was calculated as nanomoles of CDNB conjugate formed per minute per milligram of protein using a molar extinction coefficient of 9.6×103 $M^{-1}cm^{-1}$ at 340 nm.

Non-enzymatic Antioxidant Assays

Determination of Reduced Glutathione level

The level of reduced forms of GSH was determined using the method of Jollow *et al*.[32] as modified by Tabassum *et al*.[33] The thiol group (-SH) of GSH reacts with the reagent DTNB to form thio-nitro benzoic acid, TNB. Absorbance was measured immediately at 412 nm.

Determination of Non-protein Thiol level

Non-protein bound thiol (NP SH) was measured according to the method described by Sedlak and Lindsay[34] as modified by Tabassum *et al*.[33] The molar extinction coefficient of 13,100 at 412 nm was used for the determination of the NP-SH content. The values were expressed as μmol of the NP-SH/g tissue.

Oxidative Stress Markers

Determination of Lipid Peroxidation (LPO)

LPO was measured using the procedure of Uchiyama and Mihara.[35] Determination of LPO in the tissues is based on the estimation of the thiobarbituric acid reacting species (TBARS), which largely include malondialdehyde. The reaction mixture briefly consisted of 0.25 ml of sample, 10 mM BHT, 3 ml 1% (w/v) chilled ortho phosphoric acid (OPA) and 1 ml of 0.67% TBA. The mixture was incubated at 90°C for 45 min. The absorbance of supernatant was taken at 535 nm. LPO was determined as nmoles of TBA-reactive substances (TBARS) formed/h/g of tissue.

Protein Determination

Protein content in the sample was estimated using the method of Lowry *et al*.[36] using Folin's reagent and 0.02% BSA as standard.

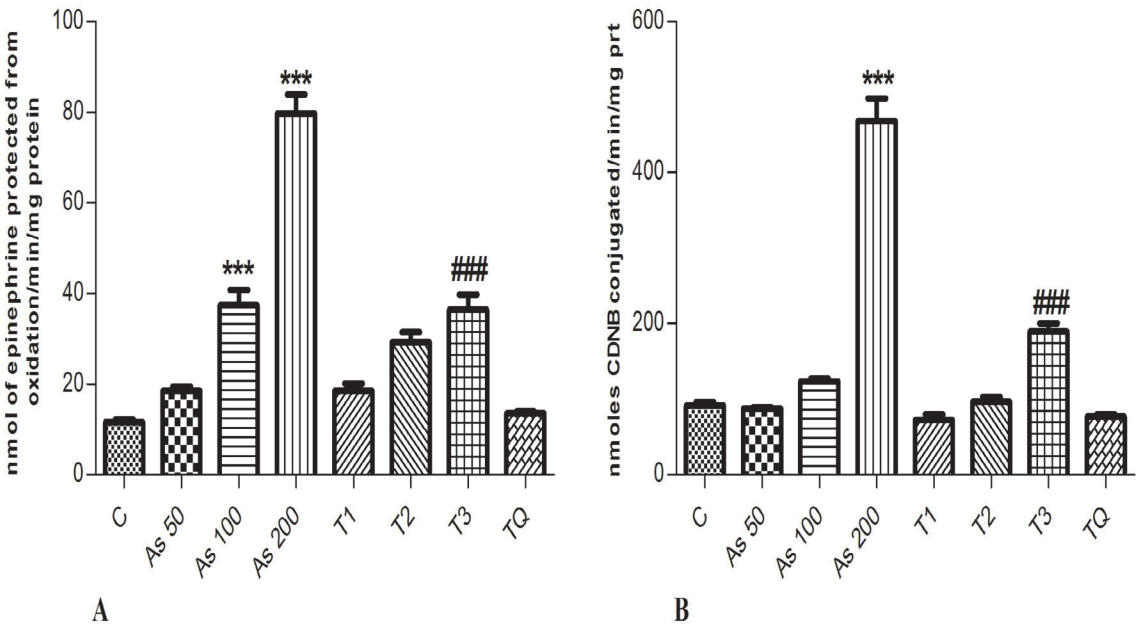


Figure 1: (A) Effect of As_2O_3 (50 μM, 100 μM and 200 μM) and TQ (10 μM) on SOD activity in the brain of rat. Each value represents mean ± SE (n=6); (B) Effect of As_2O_3 (50 μM, 100 μM and 200 μM) and TQ (10 μM) on GST activity in the brain of rat. Each value represents mean ± SE (n=6)

Significant differences were indicated by ***p<0.001 when compared with control and ###p<0.001 was used to show significance when compared to the As_2O_3 exposure group.

Assessment of DNA damage by Comet Assay

DNA damage in brain cells, contrary to PNS system was assessed using neutral comet assay as described by Singh *et al*.[37] with slight modifications. The entire protocol was performed under dim-light to avoid additional DNA damage. Freshly collected brain was first washed with chilled phosphate buffered saline-calcium magnesium free (PBS-CMF) and then minced to prepare cell suspension. The cells were suspended in 1% low melting point agarose (LMPA) overlaid on slides precoated with a fine layer of 1% normal-melting agarose. A third layer of 1% LMPA was poured and slides were allowed to dry. Three sets of treatment groups were taken i.e., control (C), 100 μM As (As 100) and treatment group consisting of 10 μM TQ and 100 μM As (T). The entire treatment time lasted for an hour. Afterwards, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 0.2 mM NaOH, 1% Triton X-100, and 10% DMSO; pH 10) for 2 h at 4ºC to lyse cells and facilitate DNA unfolding. This was followed by electrophoresis in TBE buffer (Tris, EDTA, boric acid; pH 8) at 24 V and 300 mA current for 45 min.

Slides were dried and stained with 1X propidium iodide (Sigma Aldrich, CAS No. 25535-16-4) solution. Photographs were obtained at 400X using Nikon Eclipse Ci-L fluorescence microscope. Comets were selected randomly and analyzed with Cometscore TM software (version 1.5, TriTek Corporation, Sumerduck). Degree of DNA damage was represented as percent DNA in tail.

Statistical Analysis of Data

Results were expressed as mean ± standard deviation (SD). Entire data was analyzed using analysis of variance (ANOVA) followed by Tukey's test. Values of $p<0.05$ were considered as significant. All the statistical analyses were performed using graph pad prism 5 software (Graph Pad Software Inc., San Diego, CA).

RESULTS

Effect on Activities of Enzymatic Antioxidants

SOD Activity

SOD activity showed a dose-dependent increase in the arsenic exposure groups. As_2O_3 treatment resulted in significant ($p<0.001$) enhancement in As 100 and As 200 as compared to control (C). 10 μM TQ pretreatment did not show any significant difference in T1 and T2 than their

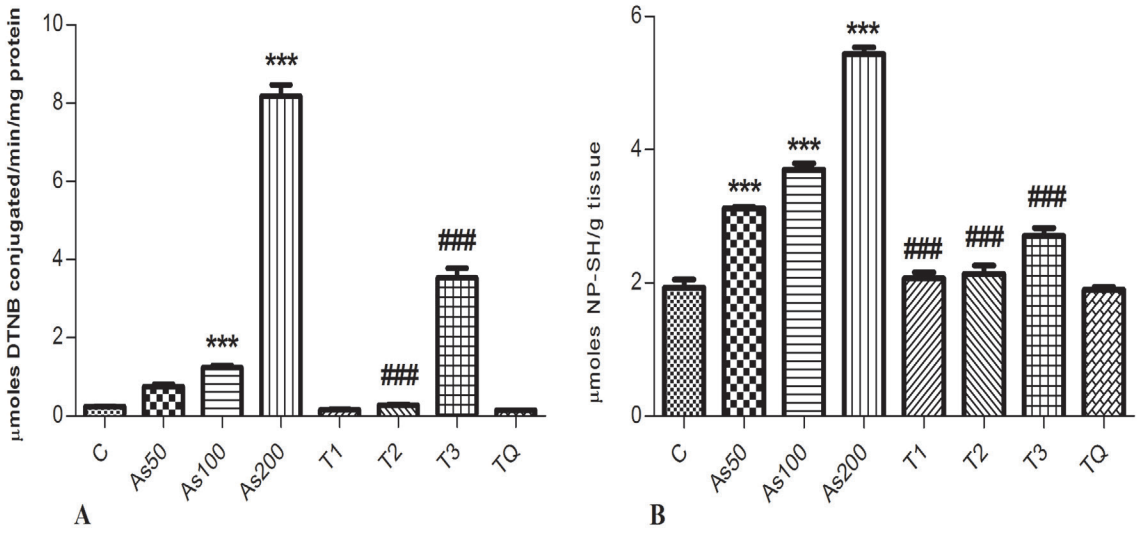


Figure 2: (A) Effect of As_2O_3 (50 µM, 100 µM and 200 µM) and TQ (10 µM) on GSH activity in the brain of rat; (B) Effect of As_2O_3 (50 µM, 100 µM and 200 µM) and and TQ (10 µM) on NP-SH in the brain of rat. Each value represents mean ± SE (n=6)

Significant differences were indicated by ***p<0.001 when compared with the control group and ###p<0.001 was used to show significance when compared to the As_2O_3 exposure group.

respective exposure groups. While, a significant decrease (p<0.001) was observed in the T3 group as compared to the consequent As 200 exposure group thus affording protection against SOD activity (Figure 1a).

Glutathione-S-Transferase activity

A dose dependent increase in the GST activity was observed in the exposure groups while the activity decreased in the subsequent treatment groups. A significant increase (p<0.001) in the GST activity was observed in the As 200 exposure group but no significant difference was seen in As 50 and As 100 as compared to control. In the treatment groups, GST activity was reduced in comparison with their corresponding As-treated groups with a significant decrease (p<0.001) in activity in the T3 group (Figure 1b).

Effect on Activities of Non-Enzymatic Antioxidants

Reduced Glutathione level

As_2O_3 treatment to PNS significantly enhanced (p<0.001) the GSH level as compared to control at As 100 and As 200 while no significant change was seen at the lowest dose (AS 50). In As_2O_3 combined with TQ pretreatment (T2 and T3 respectively), the level of GSH got significantly depleted (p<0.001) as compared to their corresponding As_2O_3 alone groups. The GSH level in TQ treatment showed no significant difference when compared with control (Figure 2a).

Non-Protein Thiol level

As_2O_3 treatment significantly increased (p<0.001) the NP-SH content in all three exposure groups as compared to control (C). As_2O_3 exposed alongwith TQ pre-treatment groups (T1, T2 and T3) showed a significant decrease (p<0.001) in the NP SH level as compared to their corresponding groups. However, TQ alone group did not exhibit any change in the NP-SH level when compared with control (Figure 2b).

Membrane damage by Lipid peroxidation

The rate of LPO enhanced with the increasing doses of As_2O_3 and a significant increase of p<0.005 and p<0.001was seen in As 100 and As 200 exposure groups. Consequently, a significant decrease (p<0.001) was obtained in T3 group as compared to its corresponding exposure group (Figure 3).

DNA Damage

DNA strand breaks in brain cells (rather than PMS system) were determined using comet assay and the results are shown in Figure 4 and 5. No comets were observed in the control group as shown in Figure 4a. Exposure to arsenic (100 µM) showed typical comet with a long tail indicating heavy DNA damage than control (p<0.001) as shown in Figure 4b and Figure 5. Pre-administration of TQ showed less pronounced damage to DNA (p<0.001) as compared to its corresponding exposure group (Figure 4c).

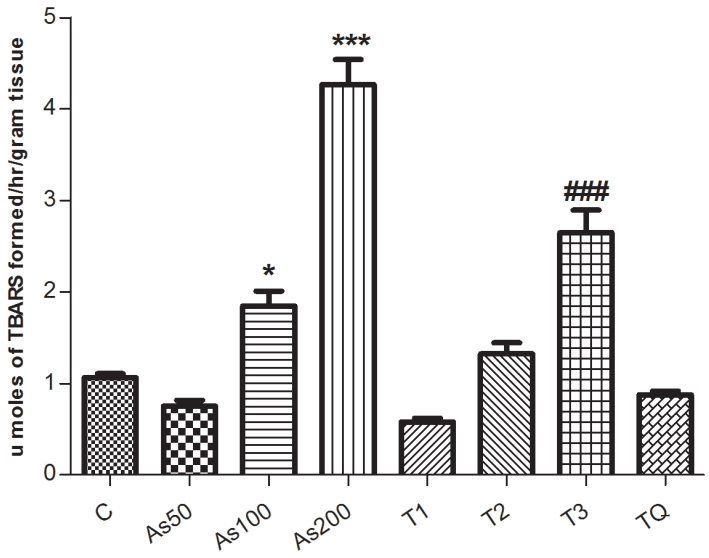


Figure 3: Effect of As_2O_3 (50 µM, 100 µM and 200 µM) and and TQ (10 µM) on LPO in the brain of rat. Each value represents mean ± SE (n = 6)

Significant differences were indicated by *p<0.05 and ***p<0.001 when compared with the control group and ###p<0.001 was used to show significance when compared to the As_2O_3 exposure group.

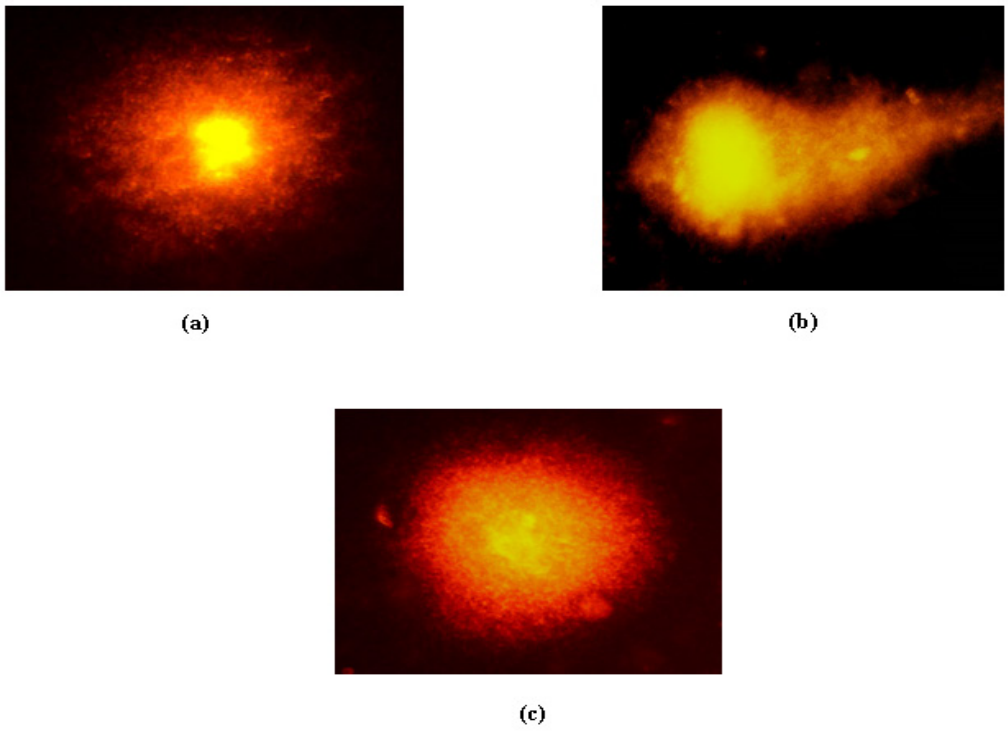


Figure 4: Effect of arsenic exposure on DNA in rat brain cells, 400X (a) Control, (b) As100, (c) Treatment (As100 + TQ).

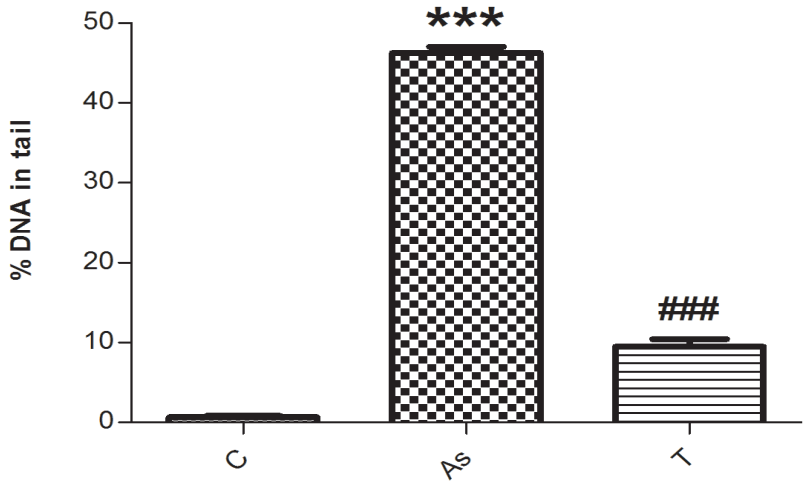


Figure 5: Graph showing % DNA damage in tail in control, As100 and Treatment (As 100 + TQ)

Significant differences were indicated by ***p<0.001 when compared with the control group and ###p<0.001 was used to show significance when compared to the As_2O_3 exposure group.

DISCUSSION

Our results indicated a considerable change in various enzymatic and non-enzymatic parameters of oxidative stress and an increase in DNA strand breaks in cells exposed to a medial concentration of As (100 μM) were observed. Dimethylarsenic peroxyl radical (DMPR) as suggested by Rin *et. al*[38] has been assumed to play a major role in DNA damage and production of superoxide anions.[39,40] Thus, DNA damage is believed to trigger the production of more superoxide anions thereby compelling the cells to induce greater SOD activity. Furthermore, TQ prophylactic pre-treatment in our system causes mitigation of DNA damage as evident by comet assay and depletion of SOD activity in pre-treatment groups. (Figure 1a; 4 & 5).

Also, an increase in the activity of GST was observed Figure 1b) which indicates its catalysing effect for conjugation of a variety of electrophilic substrates to the thiol group of GSH to produce less toxic forms.[28] The elevated level of GST facilitates detoxification of toxic intermediates by forming complexes with protective ligands. The significant increase ($p<0.001$) in the GST activity at 200 μM concentration of As indicates a higher demand of detoxification machinery to cope up the As-induced oxidative stress. However, on pre-treatment with 10 μM TQ, there is a significant decrease ($p<0.001$) in the GST activity thereby, indicating the diminished demand for such detoxification system. This observation, further substantiate its mitigatory role against As-induced oxidative stress.

Thiol groups play an important role in the intracellular protection against toxic compounds and also in the detoxification and excretion of xenobiotics.[41] An elevation in the GSH content in the As-exposed group suggests that it scavenges free radical generated during oxidative stress (Figure 2a). Therefore, greater release of GSH occurs as the level of arsenic exposure increases. But co-treatment with TQ was significantly effective in the prevention of oxidative damage induced by As, which resulted in significant lowering of GSH concentration in brain in the treatment groups, as TQ induces greater coupling of electrophilic intermediates with GSH.[42] The plausible mechanisms might be related to the ability of TQ to suppress oxidant formation and maintain the GSH ratio. Similarly, NP-SH levels also increased significantly in brain when exposed to As. Pre-treatment with TQ is supposed to modulate the NP-SH levels, thereby protecting against As-induced oxidative damage.[29] Similar findings were obtained by Ismail *et al* (2013) in primary cultured cerebellar granule neurons where TQ prevented β-amyloid induced neurotoxicity.[43]

LPO refers to the oxidative degradation of lipids, in which ROS steal electrons from the lipids in cell membrane resulting in cell damage. In our study, statistically significant increase in LPO was observed on exposure to different doses of As when compared to control. Pre-protection with thymoquinone leads to a decrease in LPO level when compared with subsequent treatment groups.

Further more, comet assay was done to assess DNA damage which showed elongated tails in the As (100 μM) exposed group while the length of tails got reduced

significantly on pre-treatment with TQ (Figure 4 & 5). TQ also possesses various pharmacologically beneficial effects against many cytotoxic insults. These effects have been reported both *in vitro* and *in vivo* with degeneration caused by L-dopa in SH-SY5Y human neuroblastoma cells, serum/glucose deprivation induced cell death and chronic toluene exposure.[44-46]

CONCLUSION

The results of this study establish the mitigatory role of TQ against arsenic trioxide induced neurotoxicity and recommend the use of thymoquinone as a nutraceutical for the population living in arsenic hotspots or in adjoining areas where there is higher risk of arsenic exposure through contaminated water resources. Furthermore, *in vivo* studies are required to establish the therapeutic efficacy of thymoquinone against arsenic induced neurotoxicity.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

As:	Arsenic, TQ: Thymoquinone
DMPR:	Dimethylarsenic peroxyl radical
GSH:	Reduced glutathione
GST:	Glutathione-S-transferase
NP-SH:	Non-Protein thiols
LPO:	Lipid peroxidation
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase

Highlights of the paper

- Thymoquinone (2-Isopropyl-5-methylbenzo-1, 4-quinone) is an active constituent present in the oil of *Nigella sativa* seeds (locally known as *black seed plant* or *Kalonji*).
- Thymoquinone has a strong free radical scavenging activity and shows anti-inflammatory and chemopreventive properties.
- The current study investigates the protective effect of thymoquinone against Arsenic induced oxidative stress and genotoxicity using PNS of rat brain as an *in vitro* model.
- Pretreatment with 10 μM thymoquinone mitigates oxidative stress and genotoxicity induced by Arsenic.

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