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Occupational exposure in lead and zinc mines induces oxidative stress in miners lymphocytes: Role of mitochondrial/lysosomal damage

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Abstract: The purpose of this research was to determine mitochondrial and lysosomal damage and oxidative stress status in blood lymphocytes of lead-zinc miners. This research was performed in 10 mine workers who have been in contact with lead and zinc in comparison to a control group containing 10 healthy volunteers. Lymphocytes were isolated from peripheral blood using the Ficoll standard method and then mitochondrial and lysosomal damage and oxidative stress were evaluated. The level of reactive oxygen species (ROS), collapse in the mitochondrial membrane potential (MMP) collapse, and glutathione disulfide (GSSG) content, and lysosomal damage in miners were higher than the control group. Also, viability and glutathione (GSH) content were decreased. The lymphocytes of workers of a lead-zinc mine are more susceptible to oxidative stress, mitochondrial and lysosomal damage. The proper use of safety equipment can reduce the risk of toxic agents and their subsequent hazards for mine workers.

Keywords: lead; zinc; mine workers; lymphocytes; heavy metal; oxidative stress

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1 Introduction

In developing countries, heavy metals pollution is one of the serious problems and public awareness of their toxic effects is increasing (Qu et al., 2012). Occupational exposure is one of the most important sources of exposure to lead (Conterato et al., 2013; Ghanwat et al., 2016; Hsieh et al., 2017). The lead enter to the workers' body through the inhalation route, and then can accumulate in the vital organs of the body (Ghanwat et al., 2016; Hsieh et al., 2017; Kalahasthi and Barman, 2018). Inhalation, skin and ingestion are among the most important routes of exposure to zinc. According to the National Institute for Occupational Safety and Health (NIOSH), the number of workers exposed with zinc fumes is around 50,000 (Ricco et al., 2018). According to the Occupational Safety and Health Administration (OSHA), the blood lead level (BLL) in workers is less than 40 μg/dL (Basit et al., 2015).

Research has shown that heavy metals can increase the generation of reactive oxygen species (ROS) and oxidative stress through Fenton like reaction (Qu et al., 2019). Oxidative stress is one of the most important mechanisms involved in lead toxicity (Kasperczyk et al., 2014b; Qu et al., 2019; Shraideh et al., 2018). In addition, lead can induce apoptosis signaling (Qu et al., 2019). It has been shown that lead has a high affinity to the sulfhydryl groups (SH) of proteins. Sulfhydryl groups play an important role in antioxidant enzymes against the generation of free radicals (Conterato et al., 2013; Kasperczyk et al., 2014a, 2016; Shraideh et al., 2018). An in vivo research has shown that overexposure to zinc affects the function of lymphocytes. Also, zinc excess has an effect on ROS generation, induction of oxidative stress, cytochrome c release, and induction of apoptosis (Plum et al., 2010). Exposure to lead leads to defects in lymphocyte function, and also changes in the ratio of lymphocytes subpopulation (Zarei et al., 2017).

Mitochondria is the main source of ROS generation in the cell, and damage to the mitochondria causes excessive release of ROS and induction of oxidative stress (Zarei et al., 2018a). Sulfhydryl groups are located in all parts of the mitochondrial respiratory chain (Fedotcheva et al., 2012). Lead with a high affinity to sulfhydryl groups induces ROS generation and oxidative stress (Conterato et al., 2013; Kasperczyk et al., 2014a, 2016; Shraideh et al., 2018). The damage to the mitochondria can disrupt the mitochondrial membrane potential (MMP) and release the cytochrome c and eventually induce cell death. Disruption of the lysosomal membrane is due to hydrogen peroxide (H2O2) transmission of mitochondria to cytosol and activation of the fenton reaction (Zarei et al., 2018a). In this research, we aimed to study the oxidative stress marker and mitochondrial and lysosomal damage in relation to exposure to lead and zinc in peripheral blood lymphocytes taken from workers of a lead-zinc mine.

2 Results and discussion

2.1 Cell viability

The purpose of this study was to investigate the oxidative stress marker and mitochondrial and lysosomal damage in workers of a lead-zinc mine using blood lymphocytes. A few studies have been done on cytotoxic effects of heavy metals on human lymphocytes. Initially, the viability of lymphocytes of workers in the lead and zinc mine was evaluated. In this study, the survival of lymphocytes in

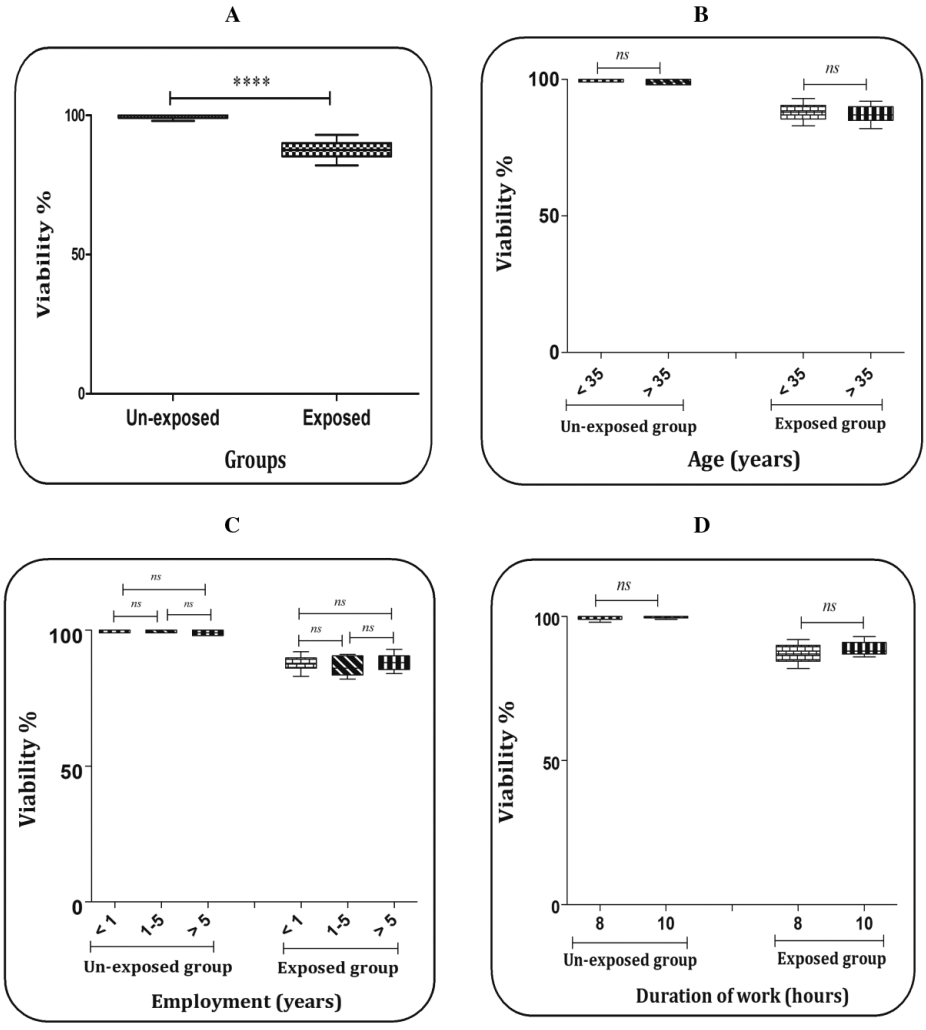

Figure 1: The cell viability assay. (a) Comparison between exposure and non-exposure groups. (b) Comparison between the exposed and unexposed groups based on age (year). (c) Comparison between the exposed and unexposed groups based on employment (year). (d) Comparison between the exposed and unexposed groups based on duration of work (hours). Data are presented as mean ± SD (n = 20). **** $P < 0.0001$.

exposed group is less than that of the un-exposed group (Figure 1a). These results are in agreement with previous studies shown the toxicity of lead and zinc in human lymphocytes using in vitro studies (Steffensen et al., 1994; Zarei et al., 2017, 2018a). In the exposed and un-exposed groups, there is a no significant difference in the cell viability based on the comparison of age, employment and duration of work (Figures 1b-d).

2.2 ROS generation

In the blood lymphocytes isolated from the exposed group, the level of ROS generation is significantly higher than that of the un-exposed group. The results show that exposure to lead and zinc increases ROS generation in isolated lymphocytes (Figure 2a). In the exposed group, there is a significant difference in the level of ROS based on the comparison of age, employment and duration of work (Figures 2b-d).

Studies showed that excessive exposure to heavy metals such as lead and zinc increases the level of ROS and oxidative stress (Akram et al., 2018; Kasperczyk et al., 2017; Plum et al., 2010; Zarei et al., 2017, 2018a). Another study showed that the level of free radicals in workers exposed to lead was higher than that of the control group (Fracasso et al., 2002). A significant increase in ROS generation was observed in the blood lymphocytes isolated from the lead and zinc mine workers. Mitochondria (complexes 1 and III) are considered as one of the important sources in the generation of ROS. ROS play various roles in the different concentrations (Dan Dunn et al., 2015; Li et al., 2013; Zorov et al., 2014). It has been shown that heavy metals with an effect on mitochondria increase the generation of ROS

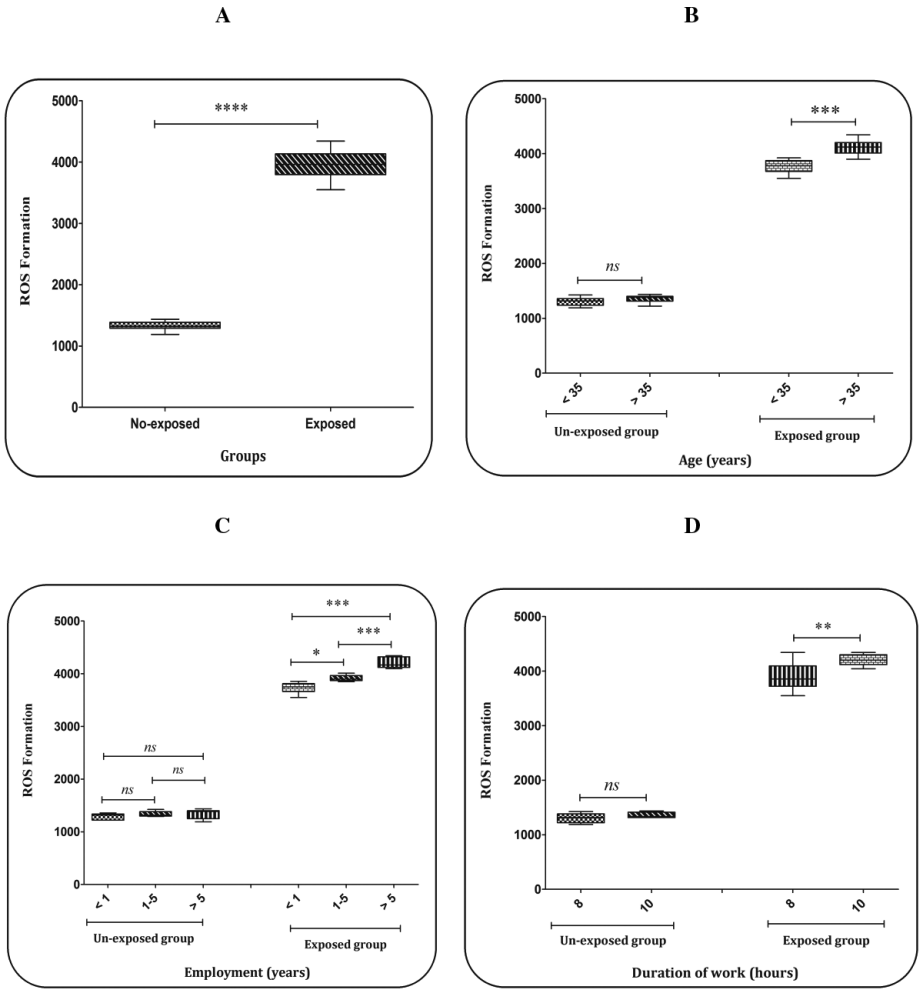

Figure 2: The mitochondrial ROS assay. (a) Comparison between exposure and non-exposure groups. (b) Comparison between the exposed and unexposed groups based on age (year). (c) Comparison between the exposed and unexposed groups based on employment (year). (d) Comparison between the exposed and unexposed groups based on duration of work (hours). Data are presented as mean ± SD (n = 20). * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001. The unit of measurement of ROS formation is based on fluorescent intensity.

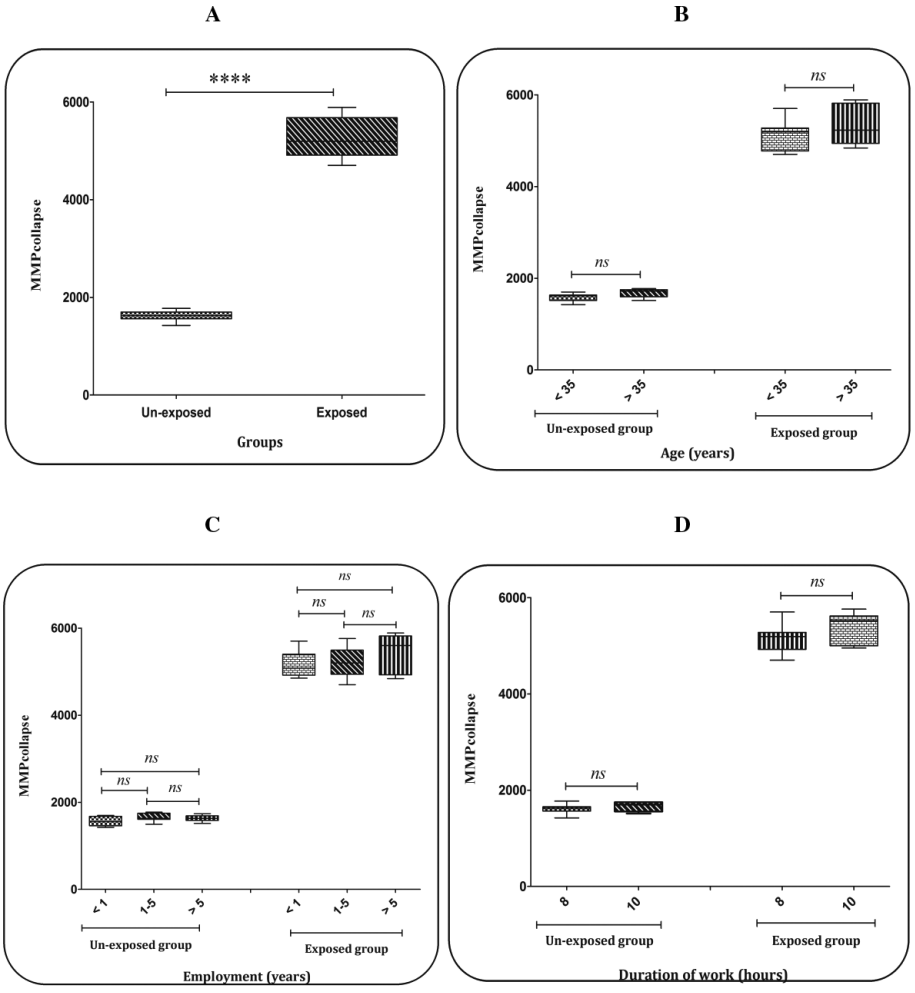

Figure 3: MMP assay. (a) Comparison between exposure and non-exposure groups. (b) Comparison between the exposed and unexposed groups based on age (year). (c) Comparison between the exposed and unexposed groups based on employment (year). (d) Comparison between the exposed and unexposed groups based on duration of work (hours). Data are presented as mean ± SD (n = 20). **** P < 0.0001. The unit of measurement of MMP collapse is based on fluorescent intensity.

(Belyaeva et al., 2012; Ercal et al., 2001; Liu et al., 2017; Ranjbar et al., 2014). Therefore, damage to mitochondria with consequences such as an increase in the generation of ROS, induces oxidative stress and cell death.

2.3 MMP assay

As shown in Figure 3, there was a significant MMP collapse in the blood lymphocytes isolated from lead and zinc mine workers compared to control group (Figure 3a). An increase in fluorescence intensity (Rh 123) indicates a collapse in the MMP. In the exposed and un-exposed groups, there is a no significant difference in the MMP collapse based on the comparison of age, employment and duration of work (Figures 3b-d).

MMP collapse was evaluated as one of the important parameters of mitochondrial damage. A significant collapse in MMP was observed in the blood lymphocytes isolated from the lead and zinc mine workers compared to control healthy group in our research. Changes in MMP are one of the important indicators for the evaluation of mitochondrial function. Also, the collapse of the MMP is associated with cell death induction. Therefore, mitochondria in in vitro toxicity research are usually used as an important target (Kamogashira et al., 2017; Sakamuru et al., 2016). These results are in agreement with previous studies (Chen et al., 2003; Zarei et al., 2017, 2018a).

2.4 GSH and GSSG content assay

The results showed that GSH content in workers in lead and zinc mine was significantly lower than un-exposed group (Figure 4a). Also, GSSG content in workers in lead and zinc mine was significantly higher than control group

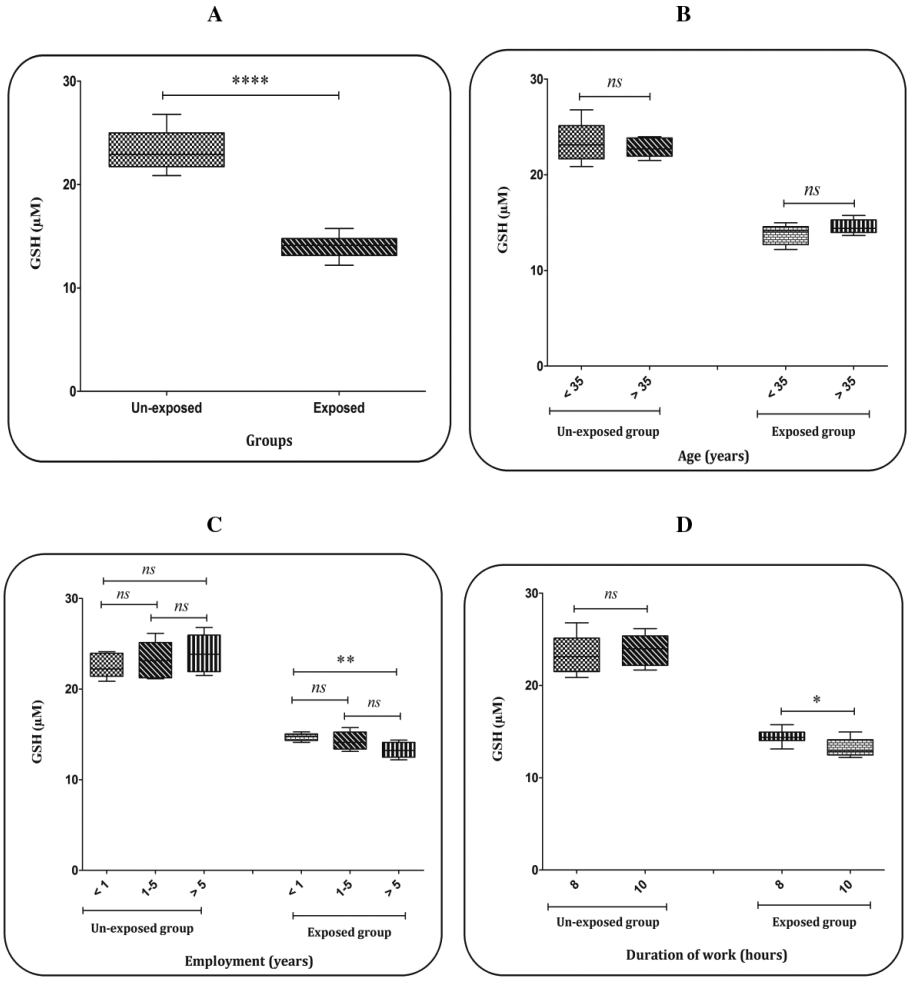

Figure 4: GSH content assay. (a) Comparison between exposure and non-exposure groups. (b) Comparison between the exposed and unexposed groups based on age (year). (c) Comparison between the exposed and unexposed groups based on employment (year). (d) Comparison between the exposed and unexposed groups based on duration of work (hours). Data are presented as mean ± SD (n = 20). * P < 0.05, ** P < 0.01 and **** P < 0.0001.

(Figure 5a). In the exposed group, the results showed a significant difference between GSH (Figures 4c,d), and GSSG (Figures 5c,d) levels based on employment and duration of work.

Heavy metals such as lead and zinc have the ability to reduce GSH level which plays an important role in the antioxidant system (Conterato et al., 2013; Kasperczyk et al., 2014a, 2017; Shraideh et al., 2018; Zarei et al., 2017, 2018a). Our results indicated a decline in GSH content and increased GSSG content in blood lymphocyte taken from worker groups compared to those of control group. It has been suggested that one of the mechanisms by which the level of GSH content is reduced is due to the high tendency of lead to complex sulfhydryl groups. This group plays an important role in antioxidant defense (Kasperczyk et al., 2016; Shraideh et al., 2018).

2.5 Lysosomal damage

As shown in Figure 5, there was a significant lysosomal damage in the blood lymphocytes isolated from the lead and zinc mine workers compared to un-exposed group (Figure 6a). The results show that lysosomes are similar to mitochondria targeted at lead and zinc. In the exposed and un-exposed groups, there is a no significant difference in the lysosomal damage based on the comparison of age, employment and duration of work (Figures 6b-d). Disruption of the lysosomal membrane is due to hydrogen peroxide (H_{2O2}) transmission from mitochondria to cytosol and activation of the fenton reaction (Zarei et al., 2018a). Our results indicated a damage in the lysosomal membrane in miners group compared to the control group.

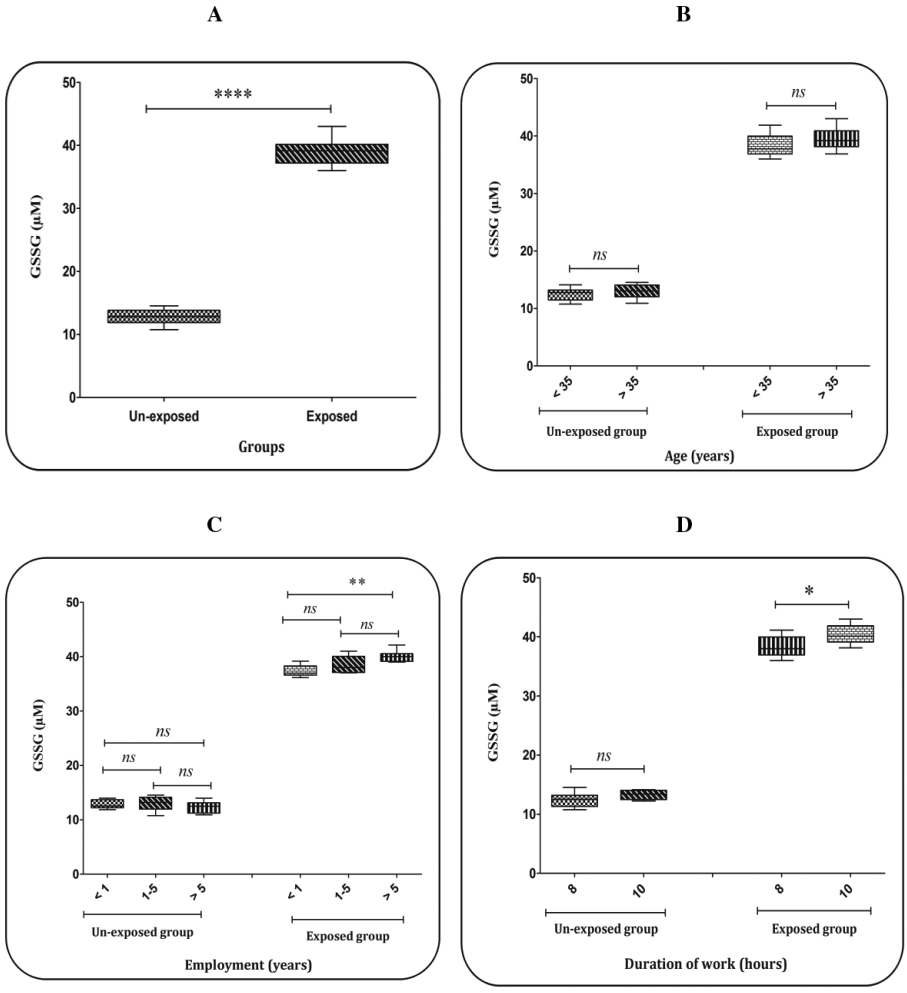

Figure 5: GSSG content assay. (a) Comparison between exposure and non-exposure groups. (b) Comparison between the exposed and unexposed groups based on age (year). (c) Comparison between the exposed and unexposed groups based on employment (year). (d) Comparison between the exposed and unexposed groups based on duration of work (hours). Data are presented as mean ± SD (n = 20). * P < 0.05, ** P < 0.01 and **** P < 0.0001.

3 Conclusion

In conclusion, we showed that mitochondria and lysosomes are targeted at lymphocytes isolated from workers in lead and zinc mine. Also, the level of ROS, oxidative stress, collapse of MMP and GSSG content has increased in the lymphocytes isolated from lead and zinc miners.

Experimental

Chemical

2,7-dicloroflurescein diacetate (DCHF-DA), rhodamine 123 (Rh 123), MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) reagent and acridine orange were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest commercial grade available.

Study population

The exposed group consisted of 32 lead-zinc mine workers (32 male and 0 female). 9 were smokers and 23 non-smokers; 0 were drinkers and 32 were non-drinkers; 3 donors had chronic disease or medicine consumption. The control or non-exposed group consisted of 28 volunteers, office workers, who had never been occupationally exposed to known toxic substances (28 male and 0 female), 6 among them were smokers; 0 were drinkers and 28 were non-drinkers; 2 donors had chronic disease or medicine consumption. In this study,

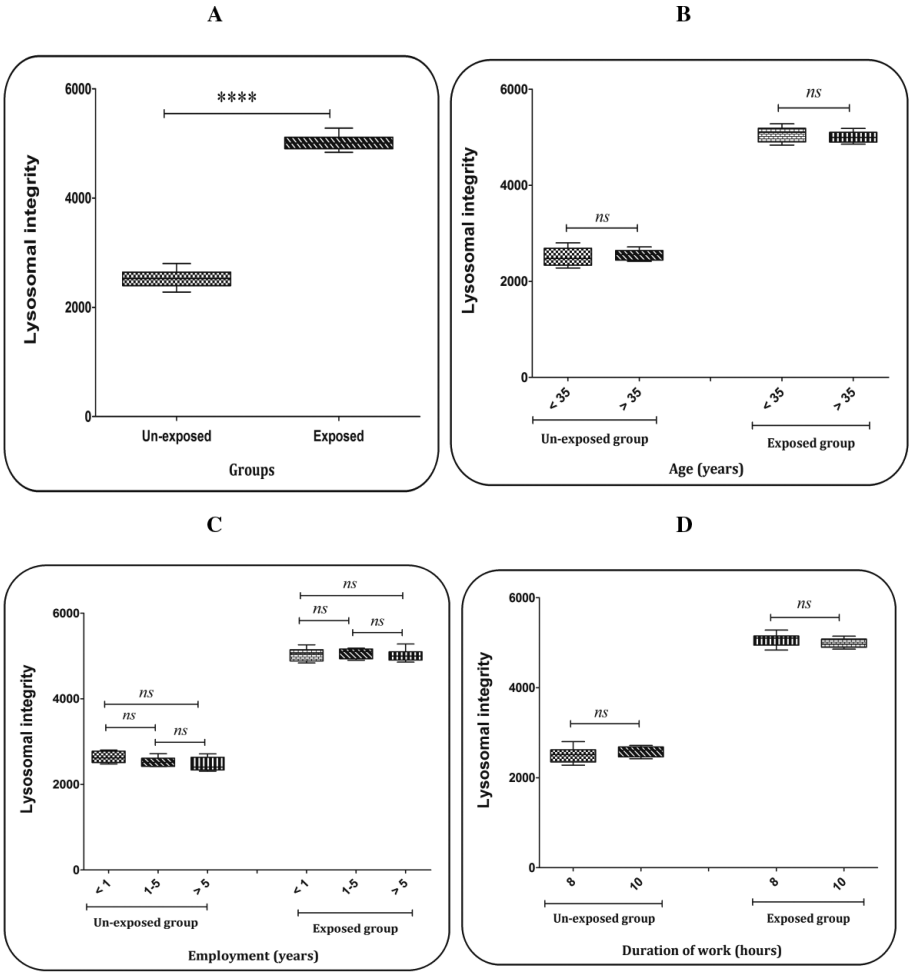

Figure 6: Lysosomal damage assay. (a) Comparison between exposure and non-exposure groups. (b) Comparison between the exposed and unexposed groups based on age (year). (c) Comparison between the exposed and unexposed groups based on employment (year). (d) Comparison between the exposed and unexposed groups based on duration of work (hours). Data are presented as mean ± SD (n = 20). **** P < 0.0001. The unit of measurement of lysosomal damage is based on fluorescent intensity.

exclusion criteria include smoking habits and acute and chronic disease (such as diabetes)/medicine consumption. Demography and other characteristic of donors from both groups are shown in Table 1. Finally, according to the exclusion criteria, 20 donors in each group were selected to study. The present study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences as ID IR.SBMU.PHARMACY.REC.1397.202. After became aware of our study donors are asked to fill out the approval form.

The blood lymphocytes isolation

Blood samples were taken from 10 lead-zinc mine workers and 10 control workers and samples were kept in heparin syringes. Briefly, ficoll plaque (3 mL) was used for the isolation of lymphocytes and then centrifugation was performed (2500 rpm for 20 min). The plasma layer is slowly detached and the white layer that is suspended in the lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH = 7.4) of the erythrocyte is human lymphocytes, and then incubated for 5 min at 37°C. In the next step, 10 mL PBS was added to the tube. Then, cells were centrifuged at 1500 rpm and the supernatant was eliminated. Finally, cells with RPMI-1640 contain 10% fetal bovine serum (FBS) at 2000 g for 7 min were washed twice and 1×10^6 cell diluted in 1 mL of RPMI-1640 contain 10% FBS and 1% antibiotic (penestrep) for use in the different test (Assadian et al., 2019).

Cell viability assay

Cell viability was assayed using MTT assay. Blood lymphocytes (1×10^4 cells/well) were placed in 96-well plates with RPMI 1640, supplemented with 10% FBS and antibiotics (50 U/mL of penicillin and 50 µg/mL

Table 1: Demography and other characteristic of donors from both groups.

		Exposed group	Control group
Number (n)		32	28
Smoking Habits		9	6
Medicine consumption/ diseases (acute and chronic)		3	2
Alcohol consumption		0	0
Age	< 35	9	12
	> 35	11	8
Gender	Male	32	28
	Female	0	0
Employment	< 1	6	5
	1-5	5	7
	> 5	9	8
Duration of work	8 h	13	15
	10 h	7	5
	12 h	0	0
	16 h	0	0

streptomycin). In the next step, MTT (5 mg/mL in RPMI 1640) was added to each well and incubated for 4 h after which the plate was centrifuged at 1800 x g for 5 min at 4°C. After careful removal of the medium, DMSO (100 µL) was added to each well, and plates were shaken. Then, absorbance was recorded at 570 nm using microplate reader (Tecan, Rainbow Thermo, Austria) (Zhang et al., 2008).

Reactive oxygen species (ROS) assay

After isolation, human lymphocytes were incubated with 500 µL of dichloro-dihydro-fluorescein Diacetate (DCFH-DA; at the concentration of 10 µM) solution for 20 min in 37°C. The human lymphocytes were washed with PBS. In the final step, fluorescence intensity was measured using a fluorescence spectrophotometer (Shimadzu RF5000U, Japan) at 495 and 530 nm excitation and emission wavelength. The results were expressed as fluorescent intensity (DCF) per 10^6 cells (Assadian et al., 2018).

The mitochondrial membrane potential (MMP) assay

At first and after removing the supernatant by centrifuging at 1000 × g for 5 min, 500 µL of 5 rhodamine 123 (Rh123; at the concentration of 5 µM) was added to the human lymphocytes suspension and incubated for 10 min. In the final step, fluorescence intensity was measured using a fluorescence spectrophotometer (Shimadzu RF5000U, Japan) at 470 and 540 nm excitation and emission wavelength. The results were expressed as fluorescent intensity (Rh 123) per 10^6 cells (Zhao et al., 2010).

Glutathione (GSH) and oxidized glutathione (GSSG) assay

After isolation, TCA 10% (0.5 mL) was added to the human lymphocytes and then centrifuged at 11 000 RPM for 2 min. In the next step, supernatant (0.5 mL) was diluted by the addition of EDTA buffer (4.5 mL). In addition, 1000 microliter of diluted supernatant was added to phosphate-EDTA buffer (2.8 mL) and the ortho-phthalaldehyde (OPA; 100 µL) solution. Finally, after incubation at room temperature for 15 min, fluorescence intensity was assayed at the λ Ex = 350 nm and λ Em = 420 using a fluorescence spectrophotometer (Shimadzu RF5000U, Japan) (Assadian et al., 2019; Hilf and Hissin, 1976).

Lysosomal damage assay

To perform this test, 1000 µL of acridine orange at the concentration of 5 µM was added to the human lymphocytes (100 µL) and incubated for 10 min in 37°C. After washing the lymphocytes, the fluorescence intensity (acridine orange) was assayed at the λ Ex = 470 nm and λ Em = 540 using a fluorescence spectrophotometer (Shimadzu RF5000U, Japan).

Statistical analysis results

Data were analyzed using SPSS software version 20 and GraphPad Prism 5 (Graphpad Software, La Jolla, CA). The Kolmogorov-Smirnov test was used to examine the distribution of data. Due to the normal distribution of data, one-way ANOVA test, followed by the post hoc Tukey test and independent sample T-Test were used to analyze the data. Values less than 0.05 were considered significant levels.

Conflict of interest: The authors declare that there is no conflict of interest.

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