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Evaluation of DNA Damage and Oxidative Stress Inductions by Excessive Medical Intake of Saline in Mice Bone Marrow Cells

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Abstract

Sodium chloride (NaCl) is widely used in various industrial, medical and food applications that increasing human exposure to it by excessive amount. However, almost all searches focused on its nephrotoxicity and adaptation of kidney cells to high salinity and only few scientific attentions were concerned on its genotoxicity. As the vast majority of drugs especially anticancer drugs diluted by saline injected by i.p. route. Hence, the oxidative DNA damage induction by excessive saline intake in mice bone marrow cells was investigated. Mice were injected i.p. with NaCl saline solution at the fixed dose level (10 ml/kg b.w. /2 days) of both the normal (0.9%) and high (3% & 5%) saline for six times and sacrificed 24 hour after the last injection. Micronucleus and comet assays and gel pulsed field electrophoresis were used to evaluate the possible genotoxicity of excessive saline on both chromosomal and DNA levels. Also, some biochemical markers of oxidative stress were assessed to shed more light on saline mechanism of action. DNA damage induction by excessive intake of both normal and high saline was evidenced by the statistical significant elevations in both micronucleated polychromatic erythrocytes level and tail moment in a dose-dependent manner. This was further evidenced by appearance of fragmentized smeared DNA on agarose gel. Moreover, excessive NaCl caused significant elevation in malondialdehyde level and decreases in reduced glutathione level and superoxide dismutase and glutathione peroxidase activities in a dose dependent manner.

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In the conclusions we can say that DNA damage induction by excessive saline intake was evidenced on both chromosomal and DNA level in mice bone marrow cells even though at the normal concentration (0.9%). Also, its reactive oxygen species generation ability was evidenced by the observed significant elevation of lipid peroxidation marker and reductions in antioxidant defense system. Therefore, it's recommended to decrease saline intake or use especially that used for drugs dilutions due to its dangerous genotoxic effects.

Keywords: excessive saline; DNA damage; oxidative stress and mice

1. Introduction

Sodium Chloride (NaCl), also known as salt, is widely used in miscellaneous applications in food, industry and medicine: food preservation e.g. smoked bacon or fish, flavor enhancement [1,2]. It is also used as a color-, fermentation-, and texture-control agent and in many brands of shampoo, toothpaste.

Moreover, its medical uses including: wounds disinfection and dilutions of many anticancer drugs e.g. 5-fluorouracil, carboplatin, etoposide and ifosfamide [3,4]. It is also used in the prevention or treatment of deficiencies of sodium and chloride ions caused by excessive diuresis or excessive salt restriction and in the prevention of muscle cramps and heat prostration resulting from excessive perspiration during exposure to high temperature [5]. Thus, human exposure to excessive amount of NaCl continuously increased and increased its risk. In the workplace, concentrated sodium chloride solutions may be irritating to the eyes and respiratory tract. Possible target organs may include the eyes, cardiovascular system, gastrointestinal system and nervous system [6].

Despite, NaCl is an essential nutrient for the normal functioning of the body e.g. nerve conduction, muscle contraction, correct osmotic balance of extracellular fluid and the absorption of other nutrients. The toxicity of excessive amount of salt has been known, exogenic intake of excessive amounts of common salt causes adverse toxic effects: increased blood pressure, muscle weakness, neurotoxicity, nephrotoxicity and cardiovascular complications [7,8]. Moreover, NaCl has been demonstrated to be a gastric tumour promoter in experimental animals and high sodium chloride intakes have been associated with incidence of stomach cancer in human populations with traditional diets of highly concentrated, salted foods.

High NaCl-induced genotoxicity has been well studied using different experimental system in *in vitro*. It has been shown to induce DNA damage in Chinese hamster ovary (CHO), murine kidney, L5178Y cells and others in culture [9-11], chromosomal aberrations in CHO cells [12,13], and mutations in a mouse lymphoma cell line [14,15].

However, the available experimental data on the *in vivo* genotoxicity of excessive NaCl are limited. In *in vivo* genotoxicity and clastogenicity were evidenced by the appearance of DNA breaks using comet assay and micronuclei induction in rat bladder epithelial cells [16], mouse renal inner medullas [7,17] in *Caenorhabditis elegans* [18], and in marine invertebrates [19]. Moreover, following high NaCl, Mre11 exits from the nucleus thus

DNA double-strand breaks accumulate in the S and G2 phases of the cell cycle, and DNA repair is inhibited [10].

Despite, the exact mechanism by which high NaCl causes DNA damage is not well known, a variety of biological responses to DNA damage caused by high NaCl have been shown: induction of cell cycle checkpoints [20-22], induction of the tumor suppressor p53 [23] and of the GADD45 growth arrest, and DNA damage-inducible proteins [20,24].

Recently, There went about studying free radical generation as a one mechanism of DNA damage induction by NaCl [25,26]. Tamura and his colleagues [7] evidenced that high NaCl intake generates reactive oxygen species mainly H_2O_2 and O_2^- in normal rat gastric epithelial cells using the electron paramagnetic resonance (EPR).

MDA is an end product of lipid peroxidation and one of the most frequently used indicators of lipid peroxidation since it is represented the level of lipid peroxidation thus it is used as a biomarker of oxidative stress in cells and tissues [28]. While, SOD and GPx called antioxidant enzymes protect the cells against reactive oxygen radicals (singlet oxygen : 1O, superoxide radical : O2 -, hydroxyl radical : -OH and hydrogen peroxide :H2O2). SOD catalyzes the dismutation of two superoxide radicals to O2 and H2O2 while GPx detoxifies H2O2 to H2O and O2, and converts lipid hydroperoxides to nontoxic alcohols [29]. Gpx requires endogenous GSH as a co-substrate. Thus, antioxidants can act as protectors that guard against oxidative damage induced by ROS [30].

Therefore, the present study was designed to investigate excessive saline intake induced oxidative DNA damage in mice bone marrow cells. Micronucleus and comet and pulsed field gel electrophoresis assays were used as genotoxic end points. Indeed, evaluation of oxidative stress markers: MDA level, a marker of lipid peroxidation, and GSH level and SOD and Gpx activities (antioxidant defense system) was done to shed more light on NaCl genotoxicity.

2. Materials and methods

2.1 Animals

Male Swiss Webster mice aged 10-12 weeks (25-30 gm) were obtained from the animal house of National Organization for Drug Control and Research **(NODCAR)**. Animals were supplied with standard diet pellets and water that were given *ad. Libitum*. They were kept in plastic cages for 7 days to be accommodated with our laboratory conditions before being treated.

2.2 Chemicals

Sodium chloride (NaCl) was purchased in the form of an odourless white soft crystal with a distinctive taste **(Sigma chemical Co., St. Louis, MO, USA)** and dissolved in sterilized dist water to prepare the administered dose (10 ml/kg b.w.) of the different concentrations (0.9%, 3% and 5%). As i.p. route is preferred for several drugs e.g. 5-Fluorouracil, etoposide and ifosamide because of highest drug concentration after injection [3,31,32]**,** NaCl injected i.p. in this study.

2.3 Treatment schedule

Animals were divided randomly into four groups of five animals each. Negative control group (group 1) was injected i.p. with dist. H2O and the remaining three groups (2-4) were injected i.p. with the fixed NaCl dose level (10 ml/kg b.w/2 days) of the three different normal (0.9%) and high (3% and 5%) concentrations six times and scarified after 24 hour of the last injection.

2.4 Genotoxicity assays

2.4.1 Micronucleus assay

Bone marrow slides for micronucleus assay were prepared and stained according to the method described by **Schmid,** [33]: the bone marrow cells were flushed down with few drops of filtered fetal bovine serum on a clean slide and spread as smear. Air-drying, fixing and stained for 5 minutes in May-Grunwald – Giemsa stain mixture. Finally wash in distilled water and mounted with DPX. 2000 polychromatic erythrocytes per animal were scored, and the number of micronucleated polychromatic erythrocytes (MNPCEs) was determined. The results are expressed as the number of MNPCEs per 1000 PCEs. In addition, the number of polychromatic erythrocytes (PCEs) was counted in fields that contained 1000 cells normochromatic erythrocytes (NCEs) and PCEs to determine a shift in erythroblast proliferation. The values were expressed as PCEs/NCEs ratio.

2.4.2 Comet assay

The alkaline (pH >13) comet assay was performed according to the method described by [34], with minor modifications. A femur was perfused with one ml of cold mincing solution (Hanks balanced Salt Solution (HBSS) Ca^{++} and Mg^{++} free with 20 mM EDTA, 10% DMSO) using needle into a microcentrifuge tube. A 10 µl aliquot of cell suspension containing approximately 10000 cells was mixed with 70 µl of 0.5% low melting point agarose (Sigma) and spread on a fully frosted slide pre-dipped in normal melting agarose (1%). After solidification, the slides were placed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10) with freshly added 10% DMSO and 1% Triton X-100) for 24 hours at 4ºC in dark. Subsequently, the slides were incubated in fresh alkaline buffer (300 mM NaOH and 1 mM EDTA, pH>13) for 20 min. The unwinding DNA was electrophoresed for 20 min. at 300 mA and 25 V (0.90 V⁄cm) and neutralized in 0.4 M Trizma base (pH 7.5) and finally, fixed in 100% cold ethanol, air dried and stored at room temperature until they were scored. The extent of DNA migration for each sample was determined by simultaneous image capture and scoring of 100 cells at 400 x magnification using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK). The extent of DNA damage was evaluated using **tail moment**: it incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail) and calculated as: tail moment= tail length × %DNA in tail/100.

2.4.3 DNA-ladder fragmentation

According to Attia, [35], bone-marrow cells were flushed down and lysed with TE buffer for 20 min on ice before centrifugation. The soluble DNA recovered in the supernatant was incubated with 100 _g/ml proteinase K for 3 h at 56 ◦C and precipitated in 0.1 volume of 5 M NaCl and 1 volume of isopropyl alcohol overnight at −20 ◦C. The DNA pellet was then rinsed with 70% ethanol, mixed with TE buffer, and incubated with 100 _g/ml RNase A at 56 ◦C for 1 hour. Electrophoresis was carried out in a 1.5% agarose gel containing ethidium bromide. The gel was examined and photographed under UV light to visualize intra-nucleosomal DNA fragmentation (laddering), characteristic of apoptosis.

2.5 Oxidative stress assays

Bone marrow cells were flushed down by saline according to attia [35] method in 1.5 ml epiendorff, centrifuged and allowed to dry for weighting to prepare 10% homogenate for biochemical analysis.

2.5.1 Malondialdehyde (MDA) level

MDA was measured using method described by Ohkawa *et al.* [36] that depends on thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95°C for 30 min. to form thiobarbituric acid reactive product. The absorbance of the resultant pink product was measured spectrophotometrically at 534 nm. Results were expressed as nmol/g tissue used.

2.5.2 Reduced glutathione (GSH) level

Ellman's, [37] method was used to estimate hepatic GSH level and based on the reduction of Ellman's reagent [5, 5' dithio bis-(2-nitrbenzoic acid)] with glutathione (GSH) to form 1 mol of 2-nitro-5-mercaptobenzoic acid (yellow compound) per mol of GSH. The reduced chromogen is directly proportional to GSH concentration and its absorbance was determined at 412 nm. Results were expressed as µmol/g tissue used.

2.5.3 Superoxide dismutase (SOD) activity

SOD activity was measured using Nishikimi *et al*. [38] method which based on its ability to inhibit the phenazine methosulphate_mediated reduction of nitroblue tetrazolium dye. Results were expressed as µ/g tissue used.

2.5.4 Glutathione peroxidase (GPx) activity

Paglia and Valentine [39] method was used to measure GPx activity depends on a reaction in which oxidized glutathione (GSSG), produced upon reduction of peroxides, and is recycled to its reduced state by the enzyme glutathione reductase. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (A_{340}) providing a spectrophotometric means for monitoring GPx enzyme activity. The rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample. Results were expressed as µ/g tissue.

2.6 Statistical analysis

Statistical analysis was performed using T-test was to test the significance level between different groups and negative control group. Regression analysis was also done to investigate the significant level between different NaCl concentrations and different estimated parameters. All statistics were carried out using Statistical Analysis Systems (SAS) program [40] ®.

3. Results

3.1 Micronucleus assay

As shown in table 1 excessive saline intake of both normal (0.9%) and high (3 & 5%) saline concentrations resulted in statistical significant increases in the MNPCES frequencies ($p<0.001$) and decreases in PCEs/NCEs ratio compared from control group in a dose dependent manner as shown by strong positive correlations between NaCl concentrations (0.9%, 3% and 5%) and MNPCEs frequency ($r= +0.99$) in regression analysis curve (Fig. 1).

3.2 Comet assay

Administration of NaCl different concentrations at the dose level of 10 ml/kg/2 days six times (groups 2-4) induced DNA damage as shown by the statistical significant increases ($p<0.001$) in DNA migration from nucleus (Fig. 2) assessed by tail moment (Fig. 3) when compared with negative control group (group 1). Regression analysis indicated a strong positive correlation ($r = +0.99$) between tail moment and different NaCl concentrations (Fig. 4).

Table 1: Effect of excessive saline intake on the micronucleated polychromatic erythrocytes (MNPCEs) induction and polychromatic to normochromatic (PCEs/NCEs) ratio in mice bone marrow cells.

Group	Dose level	MNPCES/1000 PCEs	PCEs/NCEs ratio
Negative control	0%	3.8 ± 0.34	1.01 ± 0.01
Normal NaCL intake	0.9%	16.20 ± 1.11***	0.76 ± 0.05***
High NaCL intake	3%	25.20 ± 1.33***	0.61 ± 0.04***
	5%	36.60 ± 2.11***	0.39 ± 0.03***

Results are expressed as mean ± S.E. *; ** and ***: significantly different at P< 0.05, P< 0.01 and P< 0.001, respectively, using student t-test.

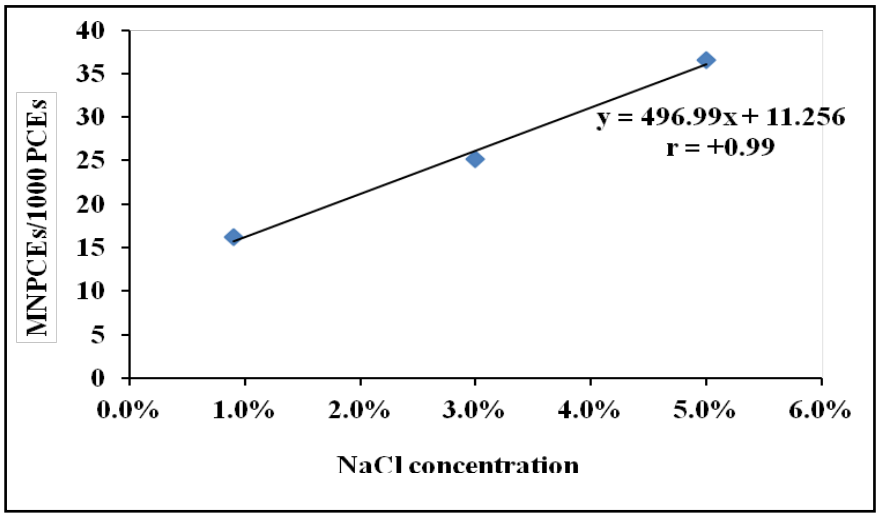


Fig.1: Regression lines and correlation coefficients for MNPCEs affected by NaCl different concentrations in bone marrow cells. Results are expressed as mean.

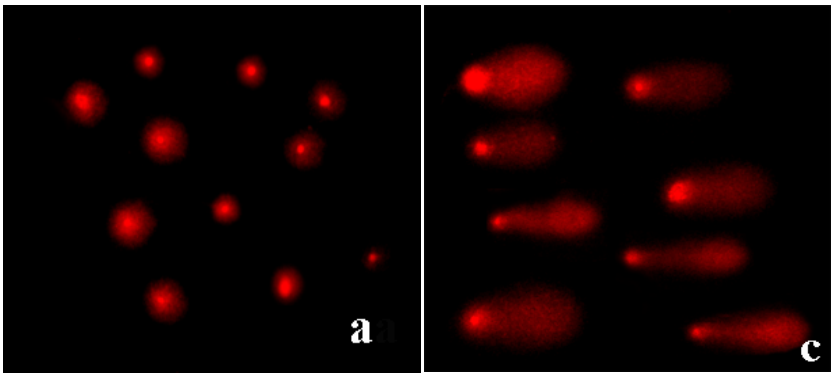


Fig 2: Representative photo for DNA damage inductions by NaCl different concentrations (C) compared with undamaged DNA (a).

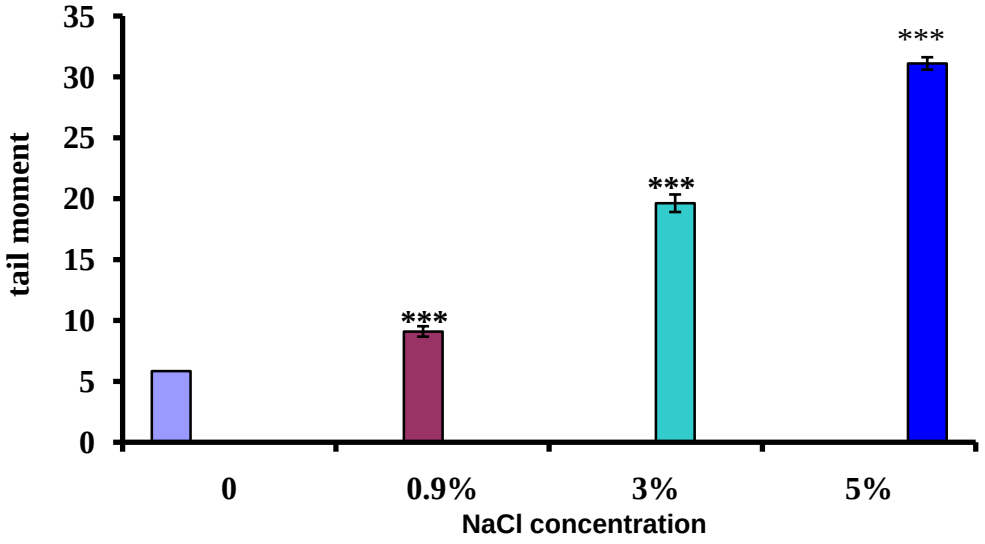


Fig. 3: Effect of excessive saline intake on DNA damage induction assessed by tail moment in mice bone marrow cells. Results are expressed as mean ± S.E. ***: statistically significant differene from control group at $p<0.001$.

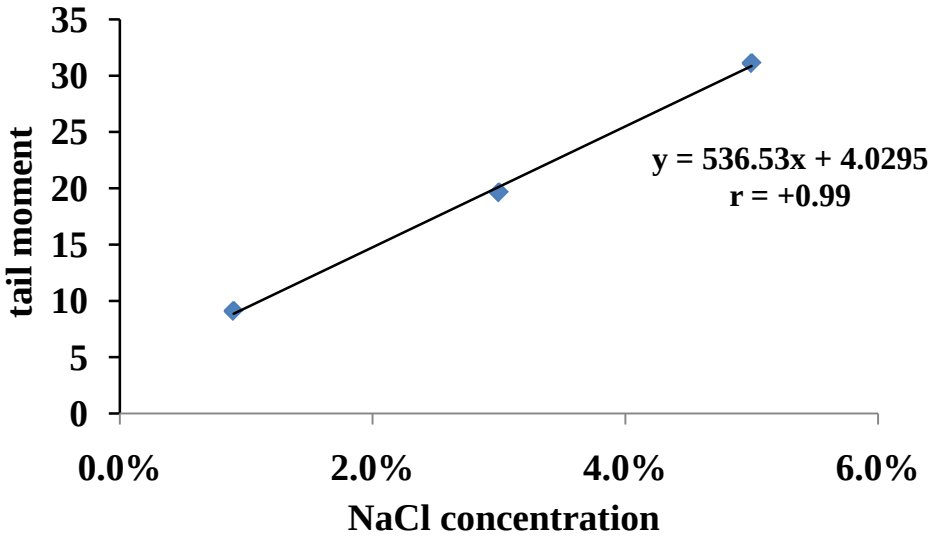


Fig. 4: Regression lines and correlation coefficients for tail moment affected by NaCl different concentrations in bone marrow cells. Results are expressed as mean

3.3 DNA- Ladder fragmentation

Qualitative analysis of DNA fragmentation was evaluated by agarose gel electrophoresis. As shown in Fig. 5 excessive saline intake resulted in a dramatic degradation of DNA in the form of smear at the three tested concentrations (0.9%, 3% and 5%). High DNA fragmentation was indicated by the very small size of the smeared fragmentized DNA compared with DNA ladder fragments size as they

reached to less than 50 base pair. In both of 3% and 5% NaCl treated groups fragmentized DNA have two size range, large and small fragment size, as indicated by the appearance of smeared DNA at both the top and bottom of agarose gel.

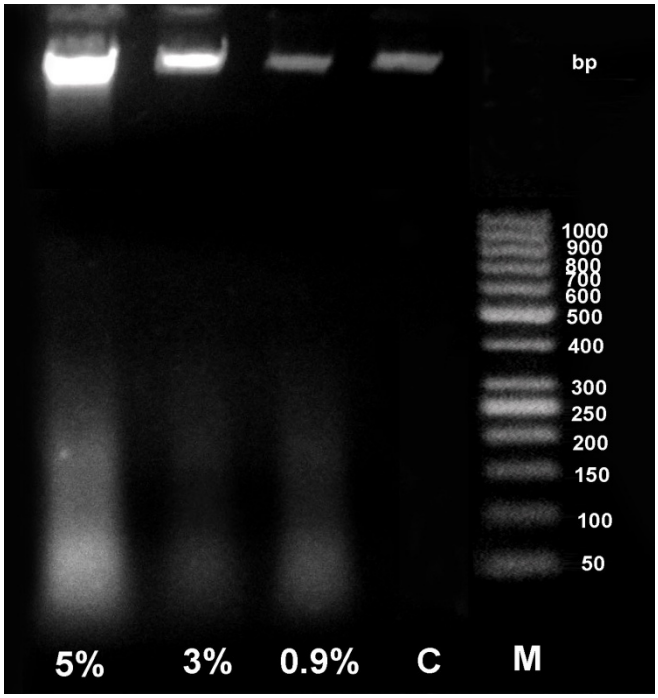


Fig 5: Agarose gel electrophoresis of DNA fragments in mouse bone-marrow cells. Lane 1: DNA marker; lane 2-4: DNA extracted from NaCl treated mice with different concentrations

3.4 Oxidative stress markers

Excessive saline intake induced oxidative stress in mice bone marrow cells as assessed by statistical significant elevations in MDA level, lipid peroxidation marker, and statistically significant decreases in the antioxidant defense systems mainly GSH level and SOD and Gpx activities (Fig. 6) compared with control group in a dose dependent manner as indicated by a strong positive (r= +0.98) and strong negative (r= -1.00, -0.99 & -0.99) for GSH, SOD and Gpx, respectively, correlations respectively between MDA level- and antioxidant defense system markers and NaCl concentration in regression analysis curve (Fig. 7).

4. Discussion

Although NaCl is being used increasingly in various applications, e.g. food preservation, flavor enhancement, disinfection, fermentation and dilutions of several drugs thereby increasing humans exposure, there is insufficient knowledge concerning the NaCl genotoxicity in bone marrow cells. Therefore, the present study was designed to investigate DNA damage and oxidative stress induction by NaCl in mice bone marrow cells.

In the present study animals were treated with NaCl by the i.p. route. Since the administration by i.p. route permits a marked exposure of bone marrow cells to the agents tested and drugs concentration was highest after i.p. injection versus other routes in all tissues e.g. 5-Fluorouracil, etoposide and ifosamide [3,32]. As 3% and 5% NaCl are widely used to provide electrolytes and are a source of water for hydration, these concentrations and normal saline 0.9% concentrations were used in this study to investigate genotoxic effects of excessive saline intake.

Since micronuclei arise either from a broken chromosome part or intact whole chromosomes lagging behind at the anaphase stage of the cell division or from oxidative DNA damage as previously reported [16,41], so the observed statistical significant elevations in MNPCEs (Table 1) evidenced chromosomal damage induction by excessive intake of both normal and high saline concentrations. Moreover, NaCl cytotoxicity was evidenced by the observed significant decreases in PCEs/NCEs ratio in a dose dependent manner.

The recent discovery that high NaCl causes DNA double-strand breaks added it to the list of genotoxic stresses known to damage DNA [7]. In our study comet assay and pulsed field gel electrophoresis evidenced the DNA damage induction by high NaCl intake as indicated by the statistical elevations in DNA migration assessed by tail moment (Fig. 3) and the appearance of fragmentized smeared DNA on agarose gel (Fig.5) in accordance with previous studies in various systems other than bone marrow cells [16-18].

In this study, the appearance of fragmentized smeared DNA reached to less than 50 bp compared with DNA marker further evidenced the dramatic DNA damage by the high NaCl intake showed by by high migration rate of breakage DNA fragments on agarose gel. In a harmony with the previous study of Dmitrieva *et al*. [7] showed that high NaCl damaged DNA as manifested by DNA fragmentation and double-breaks induction in mouse inner medullar cells.

Although the exact mechanism for NaCl–induced DNA damage is not well known, either physical distortion in chromatin structure or the generation of free radicals (secondary to cellular disruption) have been postulated as being responsible for inducing damage to the DNA phosphodiester backbone [9]. This study was focused on estimating oxidative stress as a one of genotoxic mechanisms of NaCl DNA damage induction.

Despite, sodium and chloride often function together within body as positively charged sodium and negatively charged chloride are the predominant electrolytes in the fluid surrounding cells. Salt-induced high osmotic pressure environment inhibited a mitochondrial electron transfer system to involve superoxide anion production using therapy increasing superoxide anion radicals [27].

Oxidative stress has been recognized as one of the main causes of DNA damage as reactive oxygen species (ROS) overproduction can cause severe damage to cellular macromolecules, especially the DNA [42]. ROS generation by salt intake was evidenced in this study by significant elevations in MDA

level in accordance with the finding of previous studies [25-27] demonstrated that high NaCl produces ROS mainely H_2O_2 and superoxide anion. MDA has been shown to react with DNA under physiological conditions to form a variety of adducts, and combines with several functional groups on DNA molecules [43,44].

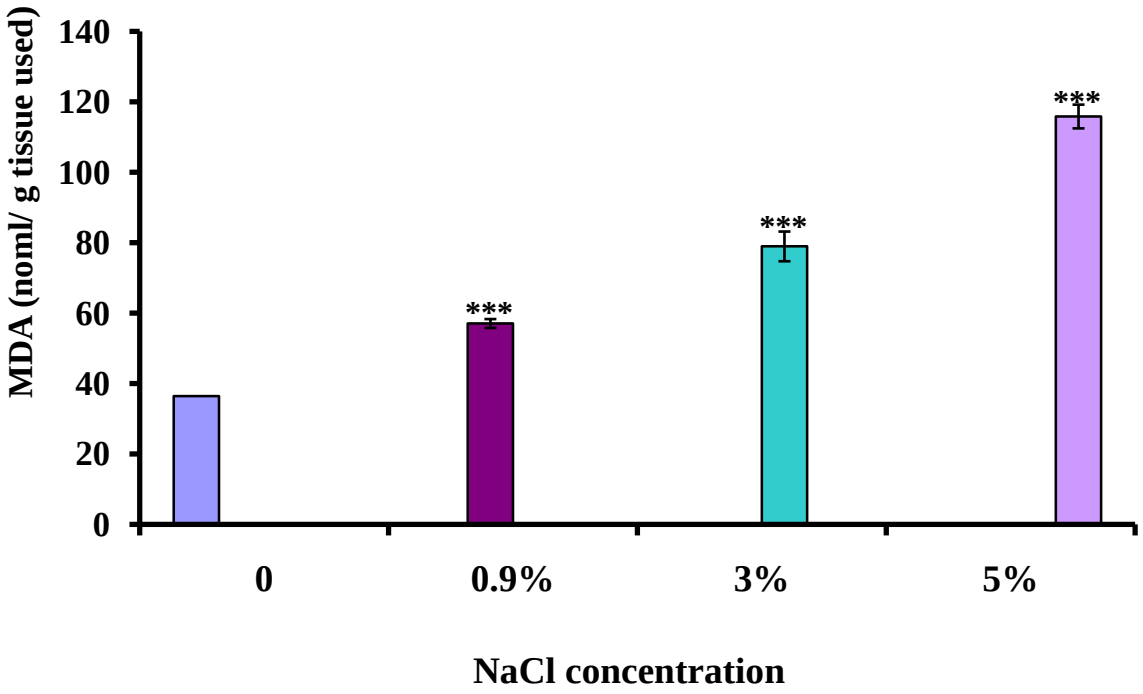
In consequence, excessive saline intake decreased significantly the antioxidant defense system members mainly GSH level and SOD and Gpx activities that further confirmed the previously reported oxidative stress induction by high NaCl [25-27]. As GSH role in DNA synthesis [45] and its involvement in the repair of DNA damage has been reported [46], the observed decreased cellular GSH levels and capacity for GSH synthesis increases the sensitivity of bone marrow cells to oxidative damage.

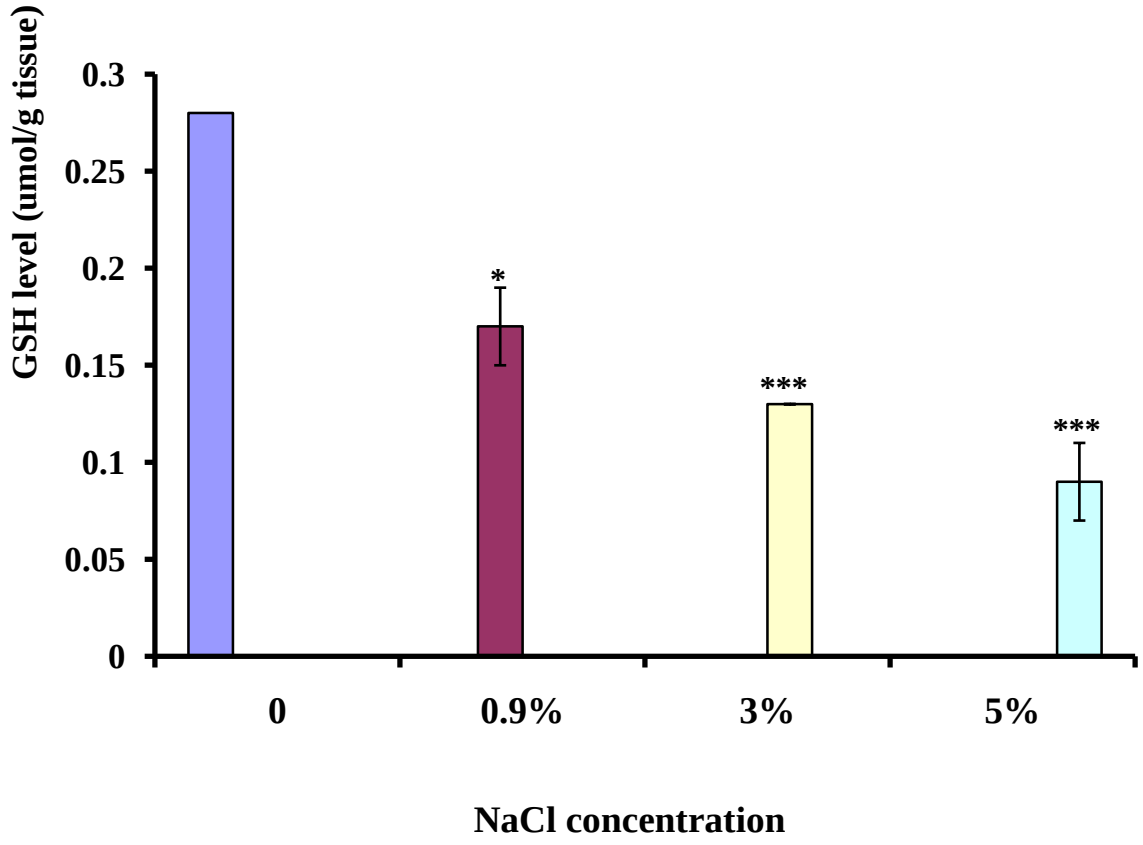
SOD and Gpx enzymes are among the endogenous antioxidant enzymes that play a pivotal role in the elimination of the superoxide radical and hydrogen peroxide since SOD converts $O_2^{\cdot -}$ hydrogen peroxide that then detoxified by Gpx into H2O [30]. Thus, in this study the observed significant decreases in SOD and Gpx activities confirmed the recently reported superoxide anion ($O_2^{\cdot -}$) generation by high NaCl [25,27]. Moreover, as Gpx requires endogenous GSH as a co-substrate, decreased Gpx activity may also attributed to the observed decreases in GSH level after high salt intake.

These obtained results are supported by the study of Zhang *et al.* [26] who demonstrated that an amplification of oxidative stress induced in DSS rats fed a high salt diet synergistically by an increase in the ROS-generating system and a decrease in the ROS-eliminating system, as shown in the increase in superoxide production and the urinary excretion of H2O2.

Additionally, The functional groups of SOD (NH_2 and ε-amino groups of lysine and the SH group of cysteine) are highly prone to oxidative damage [47]. Conversion of SH groups into disulphides and other oxidized species (e.g. oxyacids) is one of the earliest events observed during radical-mediated oxidation of proteins [48].

Regression analysis confirmed the dose dependent relationship between oxidative DNA damage induction by saline intake and its concentration in agreement with previous studies [7,26]. Thus, these results evidenced that saline must be take in a limited usage.


MDA (noml/ g tissue used)
140
120
100
80
60
40
20
0
***
***
***
0
0.9%
3%
5%
NaCl concentration


GSH level (umol/g tissue)
0.3
0.25
0.2
0.15
0.1
0.05
0
*
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0
0.9%
3%
5%
NaCl concentration

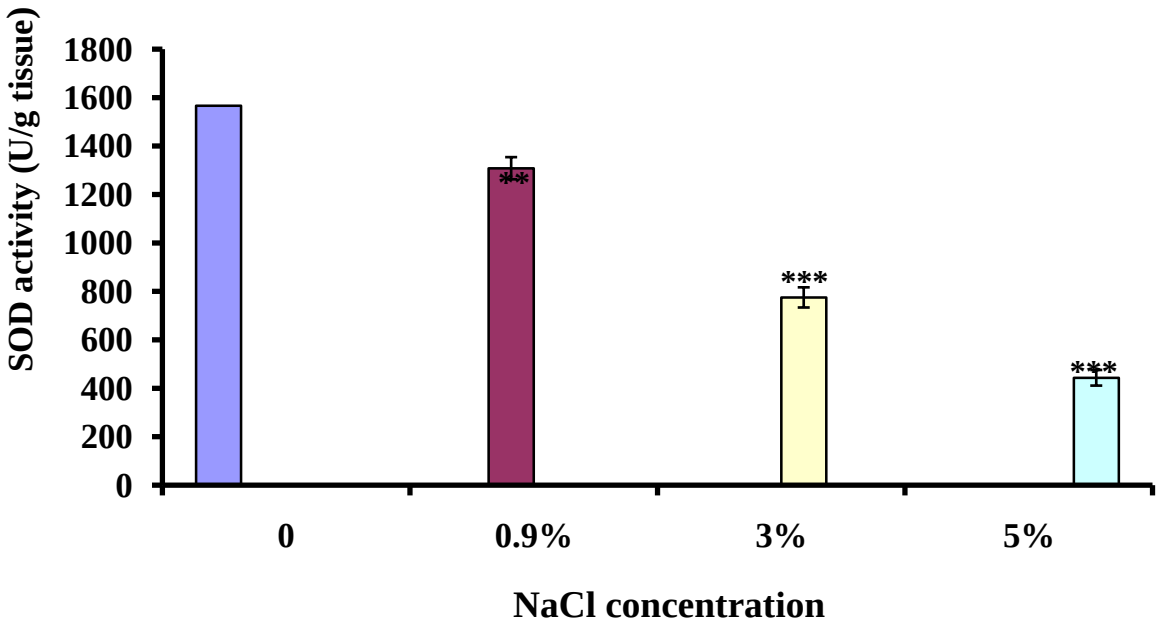
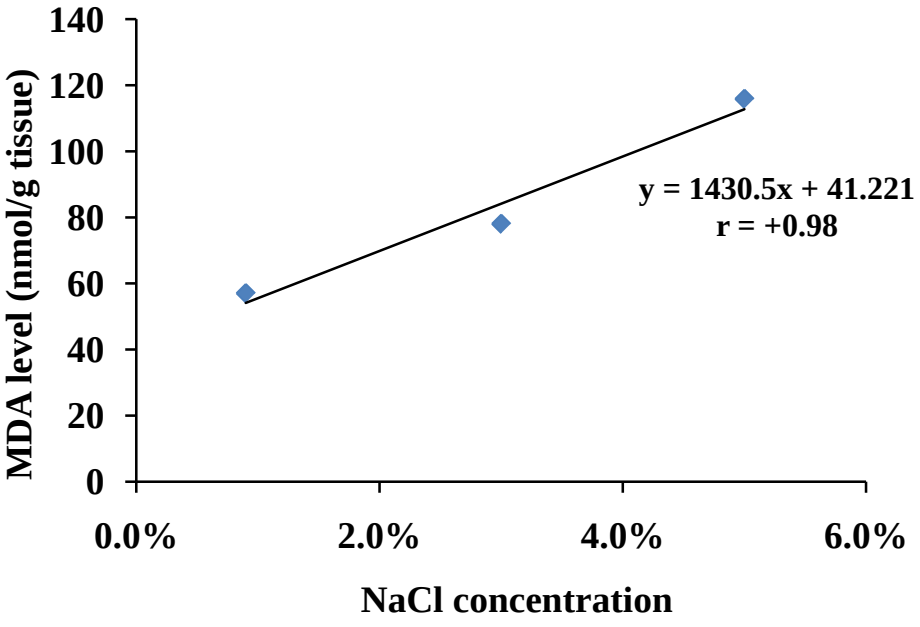
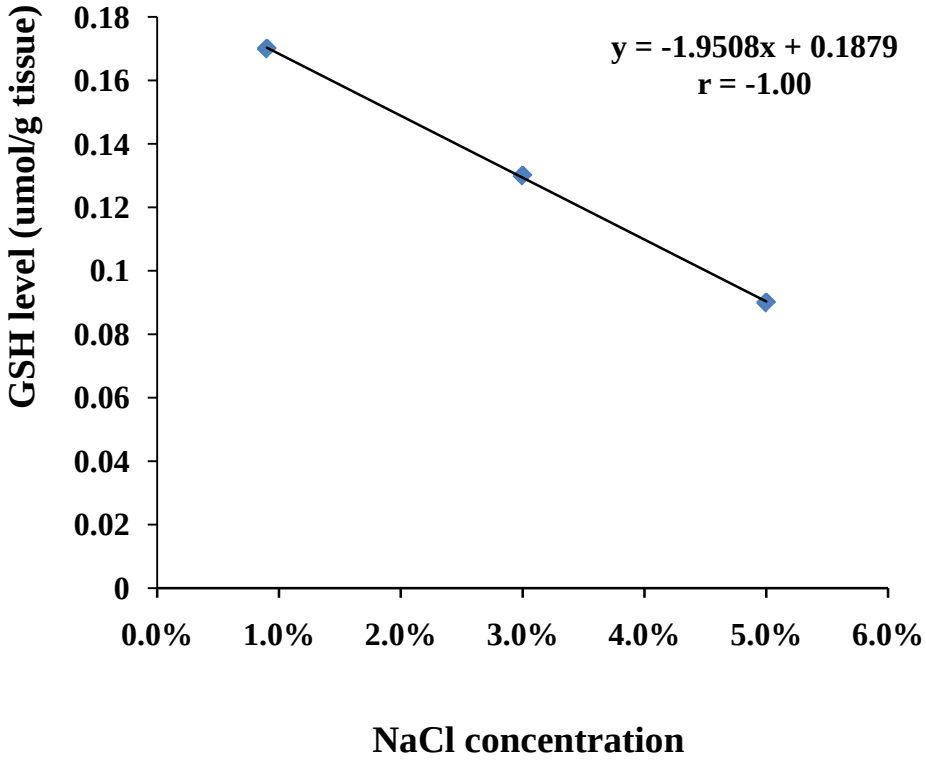
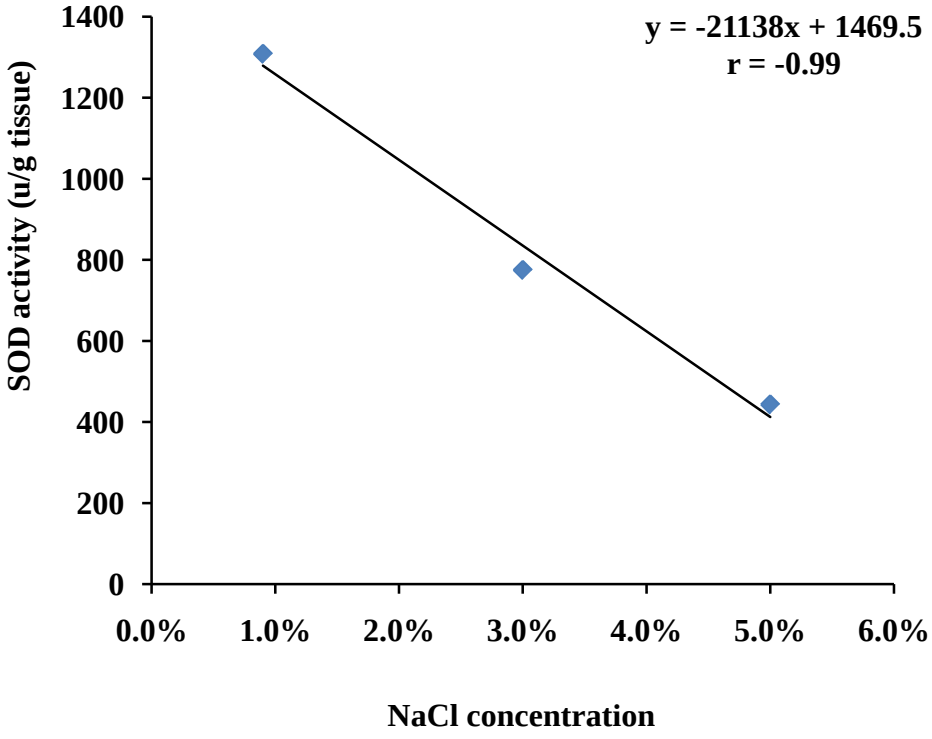


Fig. 6: Effect of excessive saline intake on MDA level, GSH level, SOD activity and Gpx activity in mice bone marrow cells. Results are expressed as mean ± S.E. *, **, ***: statistically significant differene from control group at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.




y = -1.9508x + 0.1879
r = -1.00
GSH level (umol/g tissue)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.0%
1.0%
2.0%
3.0%
4.0%
5.0%
6.0%
NaCl concentration


y = -21138x + 1469.5
r = -0.99
SOD activity (u/g tissue)
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1200
1000
800
600
400
200
0
0.0%
1.0%
2.0%
3.0%
4.0%
5.0%
6.0%
NaCl concentration

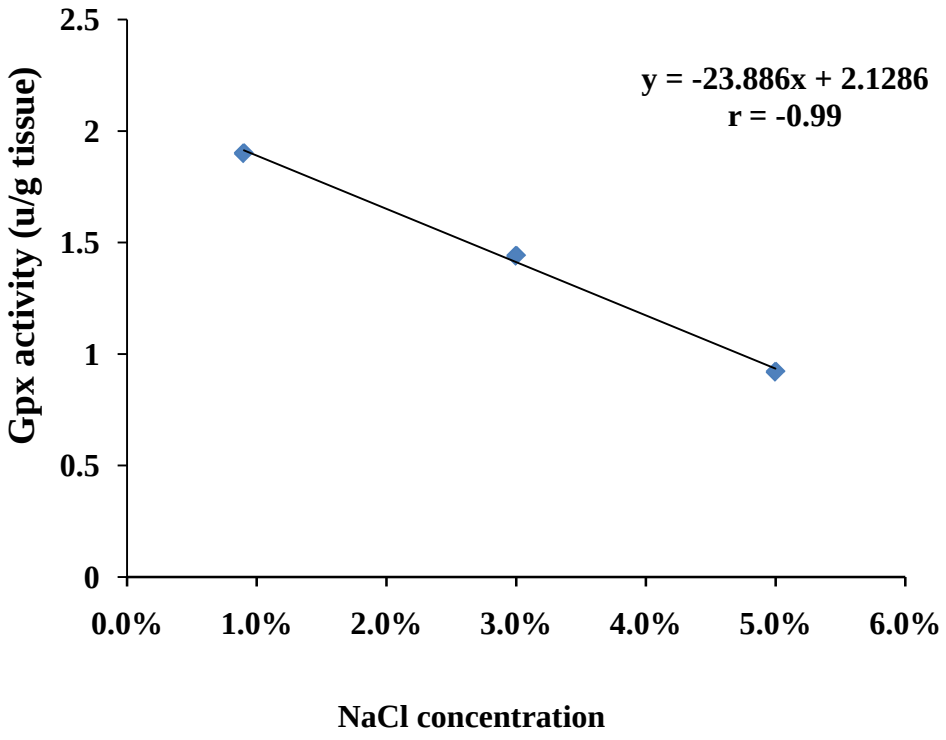

Fig. 7: Regression lines and correlation coefficients for MDA level, GSH level, SOD activity and Gpx activity affected by NaCl different concentrations in bone marrow cells. Results are expressed as mean

5. Conclusion

In conclusion**:** DNA damage induction by excessive saline intake was evidenced by the observed significant increases in micronuclei and tail moment in mice bone marrow cells that may be attributed to ROS generation as evidenced by significant elevations in lipid peroxidation marker (MDA) and antioxidant defense systems mainly GSH, SOD and Gpx in a dose dependent manner. Thus, its recommended to decrease saline usage especially in drugs dilution to avoid its risks including genotoxicity.

Acknowledgement

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