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The Induced Oxidative DNA Damage and Presenillin-1 Mutations by the Pharmacologically Used NaCl Saline Solutions Increase the Incidence of Alzheimer Disease in Mice

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Abstract

The addition of sodium chloride (NaCl) to food by the manufacturer or consumer and its medical use particularly as a drug dilution solvent result in elevated concentrations of sodium and chloride ions in human blood and tissues, including the brain. However, all previous studies were concerned on its induced renal toxicity and no attention on its neurotoxicity, thus the current study investigated the saline induced genotoxicity, mutagenicity and oxidative stress in mice brain tissue. Mice were administered 0.9, 3, or 5% saline solution by intraperitoneal injection (10 mL/kg body weight) every two days for a total of 6 injections and euthanized 24 h after the last injection. Multiple injections of NaCl saline solutions induced DNA damage in neurons and elicited a high incidence of presenillin-1 mutations and β-amyloid accumulation in a dose-dependent manner. These outcomes could be attributed to the observed elevations in serum cholesterol levels and increased generation of reactive oxygen species, indicated by the elevated malondialdehyde and nitric oxide levels and reduced glutathione levels and catalase activity in NaCl-treated groups. We conclude that multiple injections of NaCl solution, even at the concentration of normal saline (0.9%), caused cholesterol accumulation and oxidative DNA damage that resulting in mutations in presenillin-1 and also increased β-amyloid accumulation in the brain therapy increasing the incidence of Alzheimer disease in a dose-dependent manner in mice. Therefore, its recommended to reduce the uses of saline in drug dilutions.

Keywords: presenillin 1; oxidative DNA damage; Alzheimer disease; NaCl and mice.

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1. Introduction

Alzheimer disease is a neurodegenerative disease and represents one of the most dangerous diseases that threaten human life. It's characterized by various symptoms: patient confusion, irritability, aggression, and memory loss at its onset to depression and isolation with Alzheimer progress leading to body functions lost and death [1,2]. Despite, the main causes of Alzheimer disease are still unclear, many factors increases Alzheimer risk including age, genetics disorders, cardiovascular diseases, smoking and environmental toxins especially genotoxic oxidative stressors [3,4].

Sodium chloride (NaCl) is one of chemicals that have highly human exposure and it is urgent to study its risks on brain. The extensive uses of NaCl as food additives and preservatives and also its medical uses particularly drugs dilutions e.g. dilutions of many anticancer drugs: 5-fluorouracil, carboplatin, etoposide and ifosfamide etc, elevated the concentrations of sodium and chloride ions in human blood and tissues, including the brain thus increased its risks and toxicities [5-9].

High NaCl induced genotoxicity has been evidenced. In *in vitro* studies evidenced chromosomal aberrations inductions in CHO cells [10,11] DNA damages inductions in Chinese hamster ovary (CHO), murine kidney, L5178Y cells and others in culture [12-14] by high salinity. Mutations inductions by excessive salinity demonstrated in a mouse lymphoma cell line were shown [15,16]. But, the *in vivo* studies on high NaCl intake induced genotoxicity are limited and concerned on renal cells. High NaCl induced DNA damage and micronuclei in rat bladder epithelial cells [17], mouse renal inner medullas [5,18], mouse bone marrow cells [19], in *Caenorhabditis elegans* [20], and in marine invertebrates [21]. Thus, high NaCl affecting the genomic stability because elevating NaCl concentrations increased the number of DNA breaks in renal inner medulla [22]. Moreover, high salinity promote gastric tumor in experimental animals and increased the incidence of stomach cancer in human populations with traditional diets of concentrated salted foods [5,6].

Recently, oxidative stress is considered as a main mechanism of high NaCl induced DNA damages because high NaCl intake generate reactive oxygen species (ROSs) that elevated malondialdehyde (MDA) level, a biochemical marker of oxidative stress, and disrupting the antioxidant defense systems e.g. superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) [19, 23, 24]. As previously shown, oxidative stress increases the risk of Alzheimer disease and at the same time it is the main mechanism of high NaCl induced oxidative DNA damage [3,4,25]. Thus the present study investigated the possible induction of Alzheimer disease by high salinity in mice brain cells. Comet and laddered DNA fragmentations assays detected DNA breaks and apoptotic fragmentation inductions by high salinity. While single strand conformational polymorphism (SSCP) investigated mutation in presenilin-1 (PSEN-1). Histopathological examinations estimated the histological injuries in brain. Measured Cholesterol and protein as biochemical markers of Alzheimer and biochemical determinations of oxidative stress markers.

2. Materials & Methods

2.1. Animals

Purchased the male Swiss webster mice (25-30 gm) aged 10-12 weeks from the animal house of National Organization for Drug Control and Research **(NODCAR)** and supplied them with standard diet pellets and water *ad. Libitum*, they leaved in plastic cages for one week to be acclimatized with our laboratory conditions.

2.2. Chemicals

Purchased sodium chloride (NaCl) as an odorless 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 level (10 ml/kg b.w.) of the different concentrations (0.9%, 3% and 5%)

2.3. Experimental design

The Institutional Animal Care and Use Committee (IACUC) in Faculty of Science Cairo University approved the animals care and all experiments. Divided mice randomly into four groups, five animals per each group all injected intraperitoneally (ip). Negative control group (group 1) was injected with dist. water and injected the remaining three groups (2-4) with the fixed NaCl dose level (10 ml/kg b.w/2 days) of the three different concentrations [normal (0.9%) and high (3% and 5%)] six times and scarified after 24 hour of the last injection by cervical dislocation as a physical Euthanasia method of laboratory animals death because anaesthetic agents affect central nervous system thus it can't be applied in this study.

2.4. Comet assay

Comet assay detected both single and double strand breaks by mincing small piece of brain tissue and mixed 10 µl aliquot of cell suspension containing 10000 cells with 80 µl of 0.5% low melting point agarose (Sigma) [26]. Spread on a fully frosted slide pre-dipped in normal melting agarose (1%) and placed in cold lysis buffer after solidification for 24 hours at 4ºC in dark. The unwinding DNA was electrophoresed for 20 min. at 300 mA and 25 V (0.90 V/cm) after incubation and neutralized in 0.4 M Trizma base (pH 7.5). Fixed, 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 the most common DNA damage indicators: tail length, %DNA in tail and tail moment.

2.5. Laddered DNA fragmentation assay

Apoptotic DNA fragmentation was assessed according to Sriram *et al* [27]: cells were lysed in TE lysis buffer containing 0.5% sodium dodecyl sulfate, then added 0.5 mg/mL RNAase A and incubated at 37°C for one hour. Incubated after that with 0.2 mg/mL proteinase K at 50°C overnight. Phenol extraction of DNA and precipitated by 7.5 M ammonium acetate and isopropanol. Electrophoresed fragmentized DNA in a 1% agarose gel at 70 V visualized by UV transilluminator and photography.

2.6. Single strand conformational polymorphism (SSCP) analysis

SSCP analysis investigated mutations in presenilin-1 (PS1) because its mutations are the most common cause of autosomal dominant Alzheimer's disease. Extracted genomic DNA from brain cells using **Biase *et al*. [28]** method and PS1 gene was amplified by polymerase chain reaction (PCR) using the selected primers sequences: forward: 5'AATCTACACCCCATTCACAG 3' and reverse: 5'GCCCCCAACTCTCCCACC 3' [29], and electrophoresed PCR products through 2% ethidium bromide-treated agarose gel. SSCP analysis is performed by mixing a 5 µl aliquot of purified PCR product with 5 µl of denaturing-loading dye (95% formamide, 0.1% bromophenol blue, 0.1% Xylene cyanol FF and 0.5 µl 15% Ficoll) and 5 µl of TE buffer, denaturated at 94°C for 7 min. and chilled on ice for 10 mins (Gasser *et al.*, 2006). Electrophoresed samples at 90 volt till the dye reached the bottom of the gel (about 45 mins) and stained for 10 mins in 100 ml of 1x TBE with ethidium bromide to visualize the DNA bands with the aid of shaking. Examined gel using UV transilluminator and photograph by Polaroid camera (PolaroidMP4 Land Camera).

2.7. Histopathological examination

Fixed brain tissue at once in 10% formalin and exposed for later processing until reached to sectioning into thin slices (4–5 µm thickness) and stained with hematoxylin–eosin. Evaluation was done unaware of the treatments using light microscopy (U-III Multi-point Sensor System; Nikon, Tokyo, Japan).

2.8. Biochemical analysis

Brain tissue of different groups was further processed for biochemical estimation of cholesterol and total protein levels using Richmond [30] and Gornal *et al*. [31] methods respectively. Results were expressed as mg/g tissue for cholesterol and g/g tissue used for total protein.

2.9. Oxidative stress assays

Oxidative stress inductions by high salinity were investigated in this study by biochemical evaluations of both malondialdehyde (MDA) and nitric oxide (NO) levels using **Ohkawa *et al*. [32]** and **Montgometry and Dymock [33]** methods, respectively. Disturbance in antioxidant defense systems was estimated by measuring reduced glutathione (GSH) level and catalase (CAT) activity using **Ellman [34]** and **Aebi [35]** methods, respectively.

2.10. Statistical analysis

In this study the Statistical Package for the Social Sciences (SPSS) version 20 package software was used for statistical analysis. Student t-test was done to test the significance level between the different groups and negative control group. One way analysis of variance (ANOVA) was done to test the effect of NaCl different concentrations on the tested parameters.

3. Results

3.1. DNA damage inductions

Figure 1 showed representative photo for the observed DNA damages in different groups. Multiple injections with NaCl (0.9%, 3% and 5%) resulted in statistical significant elevations in tail length and tail moment (Table 1) in a concentration dependent manner as indicated by one way ANOVA analysis for both tail length ($F= 20.25$ $p<0.001$) and tail moment ($F= 11.94$ $p<0.01$). On contrary, the %damaged DNA in tail elevated statistically in group treated with 5% saline.

3.2. Laddered DNA fragmentation

Apoptotic DNA fragmentation by NaCl multiple administrations (0.9%, 3% and 5%) was indicated by the smeared pattern of running genomic DNA on agarose gel in a concentration dependent manner (Figure 2).

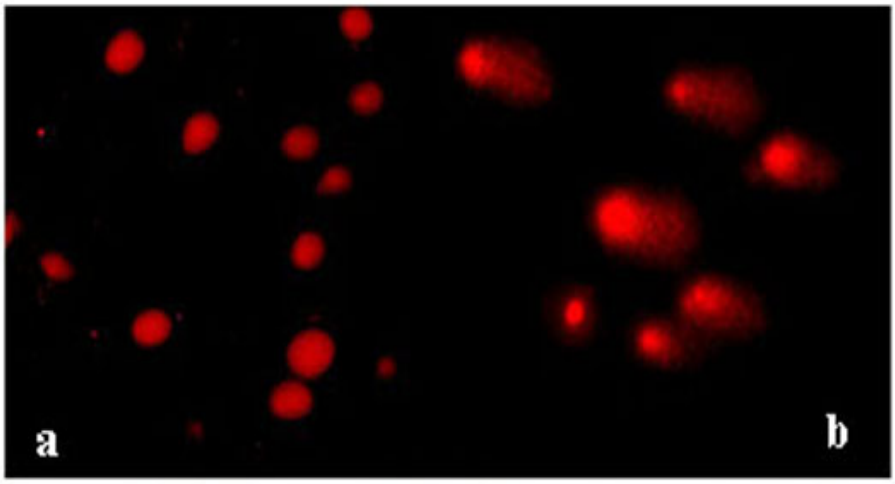

Figure 1: Representative image of undamaged DNA in control mice treated with distilled water (a) and damaged DNA in mice administered NaCl (b).

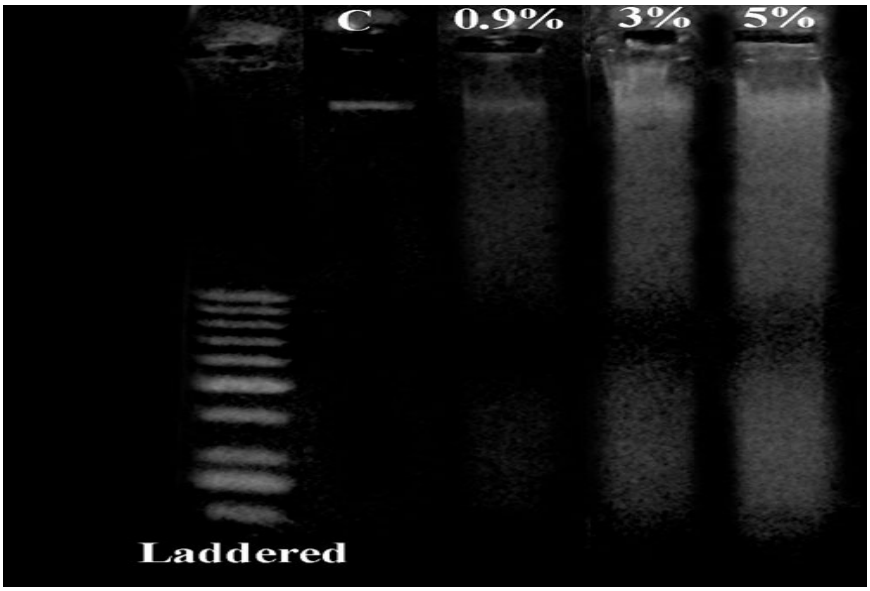

Figure 2: Gel electrophoresis showing the smear pattern of genomic DNA of NaCl-treated groups compared with the normal pattern observed in the negative control group (C).

Table 1: The effect of administration of a range of NaCl concentrations on tail length, %DNA in tail, and tail moment in brain cells of treated mice.

Group	Tail length (µm)	%tail DNA	Tail moment
Negative control	7.22 ± 0.23	19.74 ± 3.96	1.55 ± 0.45
NaCl (0.9%)	17.09 ± 2.87***	18.87 ± 3.08	3.48 ± 1.14**
NaCl (3%)	24.23 ± 2.84***	23.15 ± 2.49	6.39 ± 1.23***
NaCl (5%)	41.55 ± 10.04***	27.71 ± 5.59*	12.36 ± 4.79**
One way ANOVA	F= 20.25 P<0.001	F= 6.24 P<0.05	F= 11.94 P<0.01

Results are expressed as means ± SD. *, **, and *** indicate statistically significant difference compared to the negative control group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3.3. SSCP analysis

Results of SSCP analysis are shown in Figure 3. PS1 gene was mutated in mice treated with NaCl different concentrations (0.9%, 3% and 5%) in a concentration dependent manner as two (40%), three (60%) and four (80%) mice were mutated out of five in normal (0.9%) and high (3% and 5%) saline treated groups, respectively.

3.4. Histopathological examination

No any pathological changes was detected in the negative control groups that characterized by healthy brain tissue (Figure 4). But, multiple injections with NaCl resulted in pathological injuries into brain tissues even normal saline (0.9%) caused focal leucocytic cells infiltrations and beta amyloid deposition in brain tissues (Figure 4). Brain damage was increased by increasing saline concentration as leucocytic infiltrations and beta amyloid deposition increased in groups treated with saline (3% and 5%) in a concentration dependent manner. Neurons degenerated and vacuoles detected in brain treated by NaCl (5%) (Figure 4).

3.5. Biochemical analysis

Biochemical analysis of brain cholesterol and total proteins showed statistical elevations in both brain cholesterol and total protein levels compared with the negative control group (Figure 5) in a concentration dependent manner as evidenced by one way ANOVA analysis $p<0.001$ and $F= 38.33$ for cholesterol and $F= 22.21$ for total protein.

3.6. Oxidative stress markers

Results are summarized in table 2. Both of normal (0.9%) and high (3% and 5%) saline concentrations caused statistical significant elevations in MDA ($p<0.001$) and NO levels by about 51, 194 and 383% and 64, 143 and

263% respectively for the three NaCl concentrations. In contrast, both of GSH level and CAT activity were statistically significantly decreased by about 38, 51 for GSH and 63% and 43, 68 and 76% for CAT in groups treated with both normal and high saline compared with the negative control. One way ANOVA revealed the statistical dependence ($p<0.001$) of both MDA ($F= 207.64$) and NO ($F= 71.71$) elevations and CAT ($F= 18.72$) decreases on NaCl concentrations.

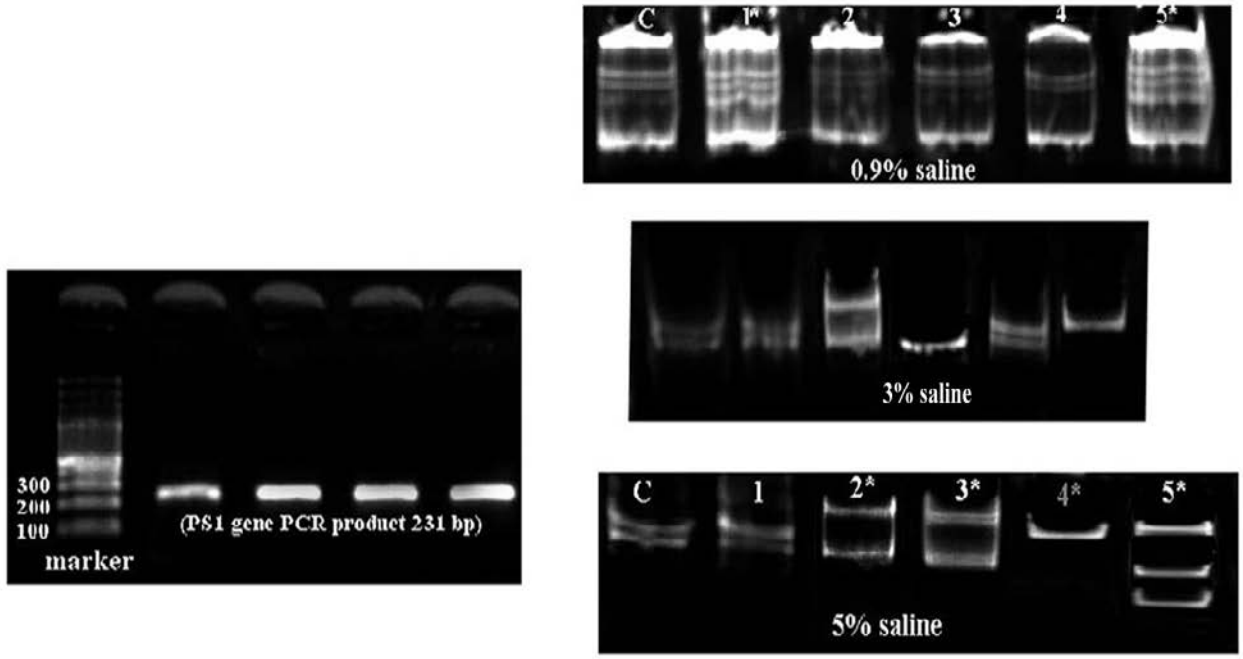

Figure 3: PCR- SSCP analysis for PS1 gene showing PCR product for PS1 gene and SSCP pattern of it in NaCl treated groups. * indicating mutation in PS1 gene compared with normal control (C) pattern.

4. Discussion

Continues and wide human exposure to NaCl by using it in many food, industrial and medical products, particularly in diluting drugs, increase its risks by elevating sodium and chloride ions concentrations in circulations thus in tissues. But almost there are no studies on the possible Alzheimer's disease induction by excessive saline intake. Thus, this study aimed to study effect of NaCl multiple administrations on the incidence of Alzheimer's disease induction. Both of 3% and 5% NaCl have high medical applications and i.p. route provides the highest drugs concentration versus other routes in all tissues e.g. 5-Fluorouracil, etoposide and ifosamide [9,36]. Thus, i.p route was used in this study and NaCl three concentrations (0.9%, 3% and 5%) could be considered as realistic concentrations.

Results of the comet assay evidenced the strand breaks inductions by both normal and high saline concentrations as indicated by the significant elevations in tail length, %DNA in tail and tail moment in a concentration dependent manner in a harmony with earlier studies [5,19]. Appearance of the smeared pattern of genomic DNA on agarose gel further evidenced DNA damage inductions and fragmentation by the three tested NaCl concentrations.

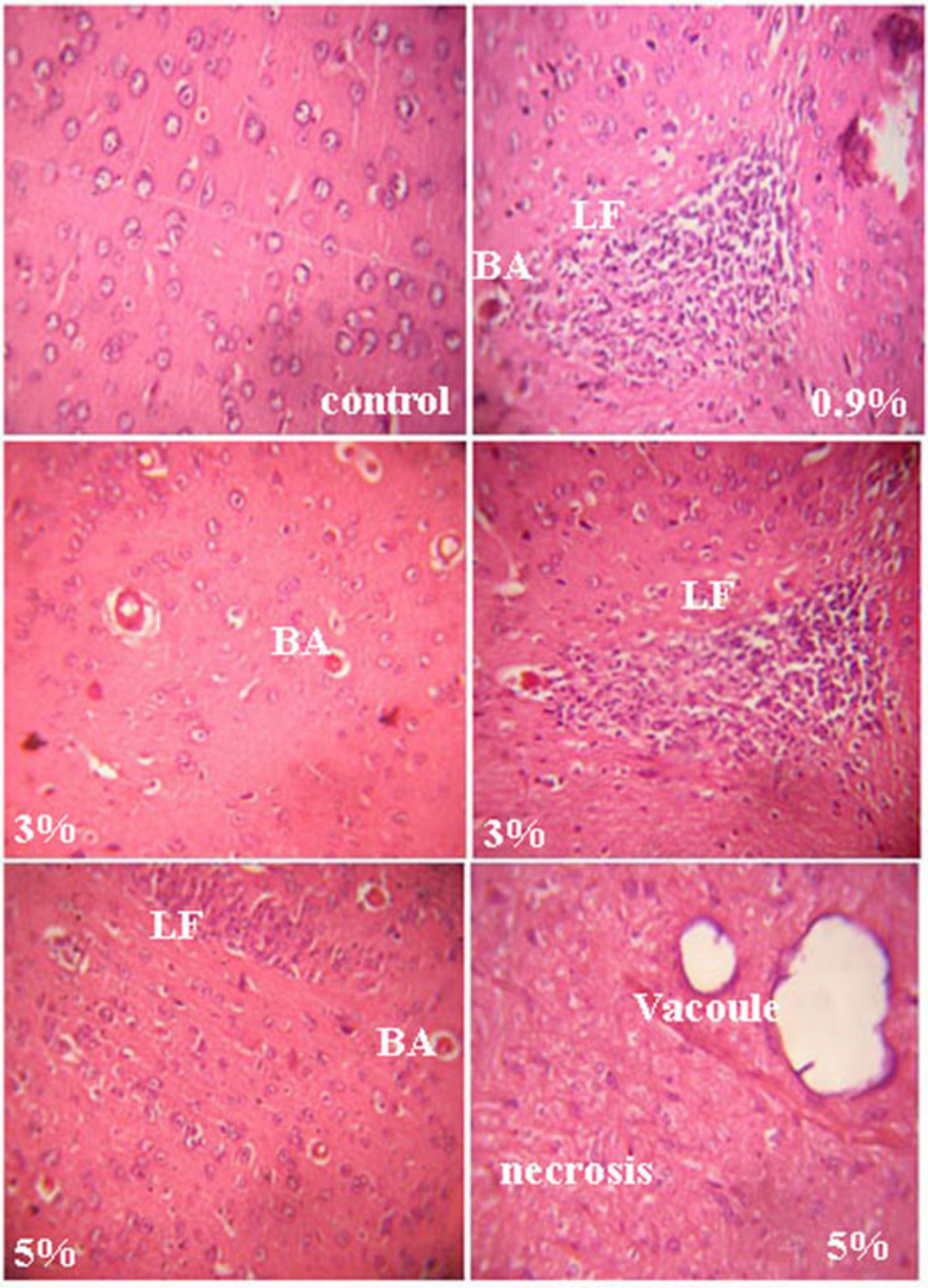

Figure 4: Histopathological examination of brain tissue showing injuries in NaCl-treated groups, compared with the normal healthy brain of the negative control group (C), obtained by hematoxylin and eosin staining at 400×. Administration of NaCl at all tested concentrations (0.9, 3, and 5%) caused leukocyte infiltration (LF) and increased β-amyloid accumulation (βA) in a dose-dependent manner. Neural degeneration, necrosis, and vacuolation were observed.

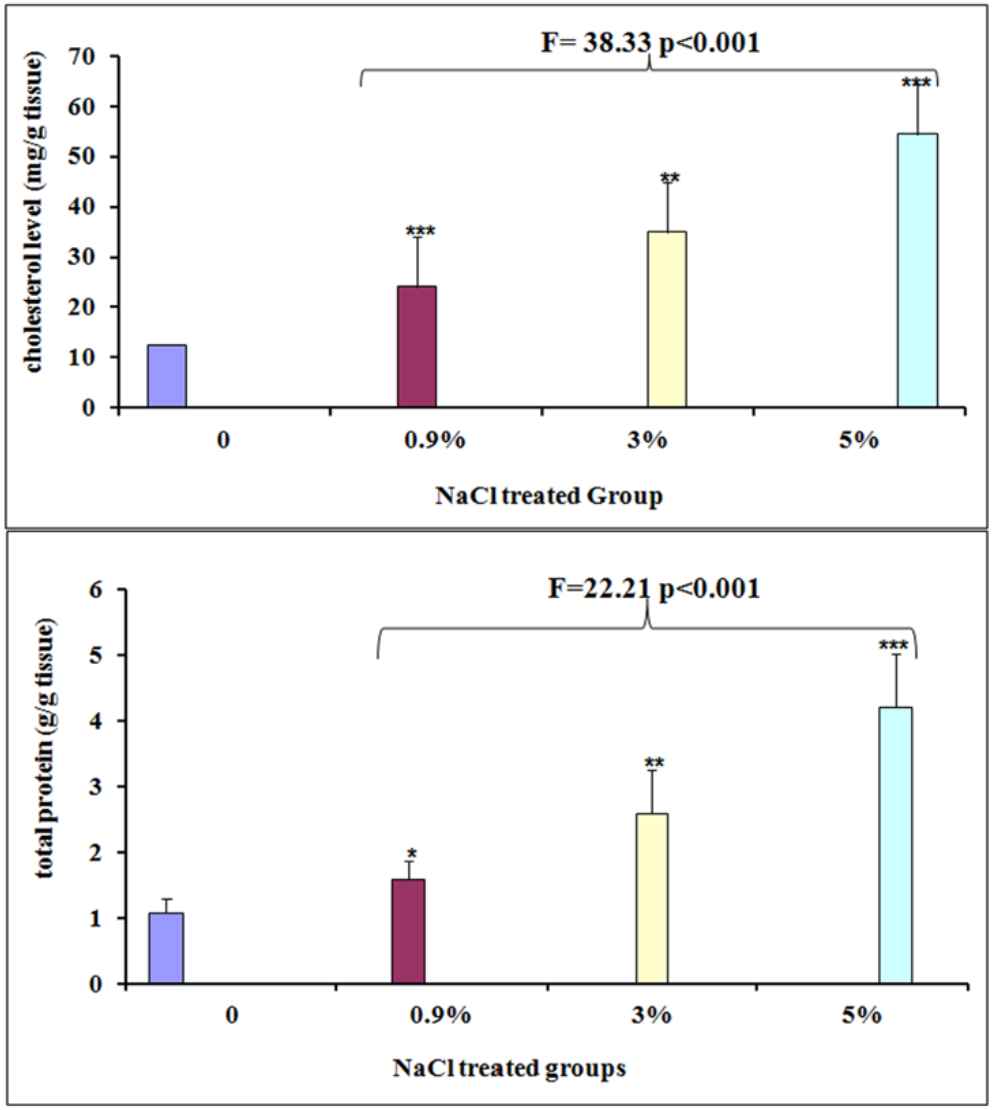

Figure 5: Levels of cholesterol and total protein in NaCl-treated groups. Results are expressed as means ± SD. *, **, and *** indicate statistically significant differences at $P < 0.05$, 0.01, and 0.001, respectively, compared with the negative control group, evaluated using Student's *t*-test. One-way ANOVA was used.

These results revealed that NaCl induced DNA damage increases the risk of Alzheimer disease as recently shown oxidative DNA damage is considered as a main mechanism of neurodegenerative diseases including Alzheimer's disease. Our study further confirmed this mechanism by the observed significant elevations in MDA and NO levels that evidenced the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation [19, 24, 37]. These ROS and RNS can attack DNA inducing strand breaks and disrupting antioxidant defense system and this could explain the observed DNA strand breaks and the reported decreases in GSH level and CAT activity in different NaCl treated groups in a concentration dependent manner.

As Oxidative DNA damage is an early event in Alzheimer disease causing modification of DNA bases by DNA direct interaction with ROS resulting in mutations and altered protein synthesis [38]. Our results of SSCP analysis

confirmed by the observed high incidence of PS1 gene mutations and resulting in significant increases in β-amyloid production in different NaCl treated groups in a harmony with the previous study that indicated that all mutations in PS1 gene increase the β-amyloid productions [39]. The β-amyloid production is also increased by the observed elevated cholesterol level in NaCl treated groups as β-amyloid generation and clearance are regulated by the cholesterol level and elevated cholesterol levels increase β-amyloid in cellular and most animal models of Alzheimer disease [40].

Table 2: The effect of administration of a range of NaCl concentrations on markers of oxidative stress in brain cells of treated mice.

Group	MDA level (nmol/g tissue)	NO level (µmol/g tissue)	GSH level (µmol/g tissue)	CAT (U/g tissue)
Negative control	54.20 ± 4.55	9.29 ± 2.26	1.66 ± 0.06	12.02 ± 3.85
NaCl (0.9%)	82.00 ± 6.96***	15.23 ± 2.79**	1.03 ± 0.34**	6.87 ± 0.60*
NaCl (3%)	159.60 ± 18.39***	22.61 ± 1.85***	0.81 ± 0.05***	3.82 ± 1.73**
NaCl (5%)	261.80 ± 14.17***	33.74 ± 2.63***	0.62 ± 0.23***	2.91 ± 0.31**
One Way ANOVA	F=207.64 P<0.001	F= 71.71 P<0.001	F=3.73 p>0.05	F= 18.72 P<0.001

Results are expressed as means ± SD. *, **, and *** indicate statistically significant difference compared to the negative control group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, using Student's *t*-test.

Biochemical data of total protein in our study confirmed β-amyloid accumulation in the brain tissue by the concentration dependent increases in total protein and by also by the histopathological examination as shown by the appearance of eosinophilic β-amyloid protein granules in NaCl treated mice. Additionally, the leucocytic cells infiltrations in brain cells evidenced inflammatory response to multiple NaCl intakes and vacuolation confirmed the degeneration of brain after NaCl injections.

5. Conclusion

Multiple injections with NaCl different concentrations caused oxidative DNA damages via ROS generations which react with DNA bases and lead to PS1 gene mutations. PS1 mutations and cholesterol level elevations by NaCl resulting in beta amyloid accumulation in neurons leading to neurons degenerations and increases the incidence of Alzheimer disease in a concentration dependent manner.

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