KEAP1/Nrf2 pathway in sodium fluoride-induced cardiac toxicity and the prophylactic role of vitamin C versus platelet-rich plasma

Authors: H. Labib, A. M. Badr, M. Abdelgwad, T. I. Abd el-galil

DOI: 10.5603/FM.a2021.0053

Article type: Original article

Submitted: 2021-02-19

Accepted: 2021-05-06

Published online: 2021-05-17

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The present study was conducted to investigate the role of vitamin C versus platelet-rich plasma against sodium fluoride (NaF)-induced cardiotoxicity and cell death in rats’ myocardium. Previous studies suggest that NaF decreased cellular viability and intracellular antioxidant power. The present study revealed that NaF administration caused histological alterations in the cardiac muscle and increased the accumulation of intracellular reactive oxygen species, the expression of iNOS and PCNA as well as collagen deposition in cardiac tissue. As supported by colorimetric analysis, an elevation in MDA level and a decrease in both SOD and TrX levels were seen, whereas molecular analysis revealed a decrease in Keap1 and an increase in Nrf2 and HO-1 gene expression. Pretreatment with vitamin C and PRP prior to NaF administration significantly improved the altered parameters and enhanced the cellular antioxidant capability of myocardium resulting in protection of cardiac muscle from NaF-induced cytotoxicity and apoptotic cell death. The cyto-protective activity of PRP was found to be comparable to that of the known antioxidant, vitamin C.

**Key words:** sodium fluoride, vitamin C, PRP, Keap-1, Nrf2, Trx-1
INTRODUCTION

Fluoride is an inorganic, mono-atomic anion designated the chemical formula F\(^{-}\), the salts of which are colorless/white, bitter tasting and are neutral in terms of smell. It is used in the manufacture of fluorocarbons. Although classified as a weak base, concentrated fluoride causes skin erosion [27]. Fluoride is mandatory for bones and teeth development and for minimizing bone fractures. A fluoride concentration of 0.5–1.5 mg/L in drinking water is favorable, whereas exceeding intake of fluoride is toxic [45]. Fluoride is used in tooth decay prevention and oral hygiene products in the form of sodium fluoride (NaF) or sodium monofluorophosphate. Water fluoridation depends on the fluoride [29]. The U.S. Department of Agriculture has specified the Dietary Reference Intakes of fluoride to be 10 mg/day, which is equal to 10 L of fluoridated water and 0.7 to 2.2 mg/day for the younger population [11]. A dose of 5-10g (equivalent to 32-64mg/Kg) of sodium fluoride is considered to be lethal [4, 16].

Substantial histopathological and biochemical changes are seen in the myocardial tissue of rats exposed to chronic sodium fluoride ingestion. Myocardial cell necrosis, excessive cytoplasmic vacuolation, karyolysis in myositis and degenerative changes of the myocardial fibers are some of the encountered histopathological changes. In addition, an increase in oxidative stress markers is encountered biochemically. Such histopathological and biochemical changes lead to myocardial tissue damage [9].

Platelet-rich plasma (PRP) is a product of autologous fractionation of whole blood by means of centrifugation that aims to remove blood cells and acquire a concentrate of platelet-rich plasma protein [31]. The platelets in PRP are activated by the addition of thrombin and calcium chloride, enhancing the release of growth factors from their alpha granules [6]. PRP has the ability to modify the oxidative damage seen in skeletal muscle injury by enhancement of myocytes’ mitochondrial function and increasing their antioxidant defense mechanisms [28]. PRP promotes angiogenesis in ischemic heart disease through cell signaling. Although the role of PRP to restore a functional myocardium, either by resettling exogenous or by activating native stem cells, to induce endogenous repair has been recorded in ischemic heart disease [44], its role in prevention of sodium fluoride-induced myocardial damage has not entered broad clinical practice yet.

Vitamin C (ascorbic acid) is a down regulator of oxidative stress and inflammation of the cardiac muscle [12]. Vitamin C causes enhancement of antioxidant capacity, induction of
CRYAB and Hsp70 expression, reversal of elevated cardiac enzymes and modification of the histopathological findings in myocardium [50].

Nuclear factor erythroid 2-related factor 2 (Nrf2), also known as nuclear factor erythroid-derived 2-like 2, is a transcription factor encoded by the NFE2L2 gene in humans [30]. Nrf2 is a basic leucine zipper (bZIP) protein that balances the expression of antioxidant proteins, which in turn combat the oxidative stress caused by injury and inflammation [15]. The vasculature has multiple protective means against oxidation and inflammation, many of them being regulated by the Nrf2 transcription factor. Heme oxygenase-1 (HO-1) is a Nrf2-regulated gene that protects against vascular inflammation; it is the inducible isoform of HO. Production of biliverdin, carbon monoxide, and release of ferrous iron occur as a result of an oxidative cleavage of the heme groups achieved by HO-1. It has an antioxidant, anti-inflammatory, anti-apoptotic, anti-proliferative, and immunomodulatory functions in vascular cells [3].

Nrf2 and thioredoxin-1 oxidoreductase (Trx-1) play a significant role in cardiovascular diseases prevention [22]. In cardiomyocytes, Trx-1 induces electron transport chain and the citric acid cycle via PGC-1 alpha and Nrf2 [1], whereas Nrf2 promotes expression of Trx-1 [22]. The Nrf2-induced HO-1 is another feed-forward loop to the Trx-1/Nrf2/Trx-1 axis that alleviates cardiomyocyte apoptosis [39]. Kelch-like ECH-associated protein 1 (Keap1) is the main intracellular regulator of Nrf2. Under basal conditions, Nrf2 is sequestered by cytoplasmic Keap1 and targeted to proteasomal degradation [47]. During oxidative stress, the Nrf2-Keap1 interaction is dose-dependent [21]. Free and newly synthesized Nrf2 translocated to the nucleus, influence the expression of many genes coding proteins that serve as antioxidant agents, detoxifying enzymes, stress response proteins and metabolic enzymes [13].

Malondialdehyde (MDA) is a highly reactive compound that occurs as an enol. It is naturally occurring and is a marker for oxidative stress [33].

Nitric oxide synthases (NOSs) are a group of enzymes responsible for catalyzing the production of nitric oxide (NO) from L-arginine [10]. Through utilization of NADPH and O2, NO is produced by three different enzymes belonging to the family of nitric oxide synthases (NOS): nNOS/NOS1 (neuronal), iNOS/NOS2 (inducible), and eNOS/NOS3 (endothelial). The activities of nNOS and eNOS demand Ca2+ for activation and produce NO at nanomolar levels for a short time. On the contrary, iNOS are induced by multiple stressors, without a need for Ca2+ for activation, and can generate NO at micromolar levels for much longer periods. iNOS,
play a role in immune response by binding calmodulin and producing NO as an immune defense mechanism, since NO is a free radical with an unpaired electron (He et al., 2020). We herein evaluate the oxidative stress seen in cardiac muscles of NaF-induced male rats and the role of vitamin C and PRP in its prevention.

Proliferating cell nuclear antigen (PCNA), also called ‘cyclin’, is a 36KDa nuclear protein which functions as a secondary protein for DNA Polymerase delta. PCNA is involved in DNA synthesis and cellular proliferation. PCNA expression lowers during mitosis and is difficult to detect immunohistochemically, but it peaks at the G1/S of interphase for which it is considered to be a histological marker. PCNA expression is also a marker for mitotic activity [46]. The involvement of PCNA in DNA synthesis and cellular proliferation is attributed to its nature as a cell cycle regulatory protein marker. Chromosome recombination, DNA methylation, nucleic acid metabolism and RNA transcription are other cellular processes with which PCNA is associated [52]. The present study investigates the PCNA expression in cardiac muscles of NaF-induced male rats.

In this study, we evaluate the impact of NaF oral consumption on oxidative stress markers, Keap1, Nrf2 and HO-1 gene expression and histological modifications in rats’ myocardium. The prophylactic roles of oral vitamin C and parenteral PRP administration are compared when given as pretreatment prior to NaF administration.

**MATERIALS AND METHODS**

**Animals**

Thirty-six adult (2-months-old) male albino Wistar rats weighing approximately 250 grams were purchased from and housed at the animal house at the Faculty of Medicine, Cairo University. The animals were accommodated six in a cage, at a constant room temperature of 22±1 °C under a 12 h light: 12 h dark cycle. Food and water were supplied ad libitum. All procedures were in accordance with the principles of the Ethics Committee, Faculty of Medicine, Cairo University.

**Experimental design**
The rats were equally divided (n=6) into six groups where control group (I) received water ad libitum, vitamin C sham control group (II) received 100mg/kg.b.w/day of vitamin C, PRP sham control group (III) received 0.5 ml of PRP, Na fluoride group (IV) received sodium fluoride (NaF) at a dose of 25 mg/kg body weight/day (which was 1/10 of the oral LD50 values in rats) as recommended by Chinoy [8], Na fluoride +vitamin C group (V) pre administered with vitamin C daily (100mg/kg.b.w/day) 90 min before the administration of Na fluoride at a dose similar to group IV and Na fluoride +PRP group (VI) pre administered with 0.5 ml of PRP daily 90 min before the administration of Na fluoride at a dose similar to group IV and V. Na fluoride and vitamin C were administered orally via gastric intubation using an intravenous cannula, whereas PRP was injected intraperitoneally. For all groups, the duration of the experiment was four weeks.

On the 30th day, blood samples were collected from all rats by means of retro-orbital sampling under general anesthesia by use of sodium pentobarbital (40 mg/kg, i.p.). Then the rats were sacrificed by decapitation. The hearts were removed and tissue samples were taken from the heart of each rat for biochemical and histological assessment. For biochemical analyses, the tissue samples were labeled in glass bottles and frozen at -80°C to be studied later.

**Chemicals**

Vitamin C tablets were purchased from Sigma Chemical Co. (St. Louis, MO). Each tablet (100 mg) of vitamin C was crushed and dissolved in 2 mL of distilled water to acquire a 50 mg/mL suspension and was prepared just prior to administration of Na Fluoride was purchased from Sigma Chemical Co. (St. Louis, MO) in powder form and dissolved in normal saline.

Preparation of PRP was done by enrichment of whole blood platelets using a 2-step centrifugation technique. A 10 ml volume of whole blood was obtained from 6 randomly-picked rats into pre-chilled tubes containing Anticoagulant Citrate Dextrose Solution A (ACD-A) at a blood/ACD-A ratio of 9:1. Centrifugation of the blood samples followed, at 400 g for 10 min to obtain the 3 typical layers: RBCs at the bottom, a ‘buffy coat’ layer in the middle and acellular plasma in the supernatant. Using a sterile pipette, the upper layer and buffy coat were transferred to another neutral tube and re-centrifuged at 800 g for 10 min. Approximately, 1 ml of PRP was collected from the bottom of the tube (for a total of 6 ml of PRP solution from 6 rats). The total
platelet count in each sample of PRP was determined under a phase contrast microscope. Concentrations of the platelets in the 2 groups receiving PRPs were 692,458 ±60,287/μl and 0.5 ml of PRP was then administered intraperitoneally.

**Biochemical analysis**

Homogenation of tissue samples was achieved by washing the heart samples with distilled water to wash out the remaining blood. A 10% homogenate (1000 U) from each sample was obtained at 5-min intervals in a homogenator (IKA Labor Technic Ultra-Turrax T 25 model) with 150 mM cold KCl. The homogenates were then centrifuged at 6000 xgat +4°C for 10 minutes for supernatants to be obtained. Protein concentrations of supernatant were determined by Real Time Quantitative PCR.

**Real time quantitative PCR of studied genes**

Total RNA was extracted from heart tissue homogenate with RNA easy Min Kit (Thermoscientific, Lithuanai). The quantity and quality were assessed by Beckman dual spectrophotometer (USA). SensiFAST™ SYBR® Hi-ROX One-Step Kit, catalog no.PI-50217 V had been formulated for highly reproducible first-strand cDNA synthesis and subsequent real-time PCR in a single tube in a 48-well plate using the Step One instrument (Applied Biosystem, USA). Normalization for variation in the expression of each target gene was performed referring to the mean critical threshold (CT) values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene expression by the ΔΔCt method. Primers base sequences of the studied genes are listed in (Table 1).

**Table 1. Details about the base sequences of the primers used**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Direction and Primer sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keap-1</td>
<td>Kelch-like ECH-associated protein 1</td>
<td>Forward F:5′-TTCGCCCTACACGGCCTC-3′</td>
</tr>
</tbody>
</table>
Assessment of Trx-1 by ELISA

The amount of Trx-1 was measured using Rat (Trx) ELISA kit, Catalog #SRB-T-81639, Shanghai. The spectrophotometric absorbance was assessed at 450 nm in accordance with the manufacturer’s instructions. The results were expressed as nmol per mg protein.

Assessment of MDA and SOD by colorimetry:

The amount of MDA and activity of SOD were measured using colorimetry kit, Biodiagnostic, Catalog #: MD 2529, SD 2521 respectively in accordance with the manufacturer’s instructions. The results were expressed as nmol per g protein and U per mg protein.

Processing of specimens and stains for light microscopy

Tissue samples from left ventricle were fixed in buffered neutral formalin, processed through graded alcohols and xylene and embedded in paraffin blocks. Tissue sections of 4-6 µm were made at multiple levels. Sections were stained with hematoxylin and eosin (H&E).
For Masson’s trichrome stain, the samples were first deparaffinized and rehydrated, washed in distilled water then fixed and rinsed. Staining in Weigert's iron hematoxylin and Biebrichscarlet-acid fuchsine solution was done for 10 minutes for each stain with intervening rinsing. Differentiation in phosphomolybdic-phosphotungstic acid solution, staining with aniline blue solution, washing and dehydration were done before finally mounting with resinous mounting medium.

Immunohistochemistry for inducible nitric oxide synthase (iNOS) was performed on biopsies of deparaffinized rat cardiac tissue. Heat-induced antigen retrieval was performed using 10mM sodium citrate (pH6.0) buffer and microwaved for 8-15 minutes for exposure of target proteins. Afterwards, tissues were blocked in 3% BSA-PBS for 30 minutes at room temperature. Tissues were then probed at a dilution of 1:200 with a rabbit polyclonal antibody recognizing iNOS (ab3523, ABCAM, USA) or without primary antibody (negative control) overnight at 4°C in a humidified chamber. Tissues were washed thoroughly with PBST and endogenous peroxidase activity was stopped by the addition of a peroxidase suppressor. Detection was performed using a biotin-conjugated secondary antibody and SA-HRP, followed by colorimetric detection using DAB. Tissues were counterstained with hematoxylin and prepped for mounting.

Immunohistochemistry of proliferating cell nuclear antigen (PCNA) entailed deparaffanization and heating of paraffin-embedded heart sections in 0.01 M sodium citrate buffer (pH 6.0) for 15 minutes followed by overnight incubation with rabbit anti-PCNA antibodies (EPR382, ABCAM, USA) at 4 °C. 3,30-diaminobenzidine (DAB) was used as the chromogenic substrate and the sections were assessed by light microscopy. The characteristics and morphology of myocardial fibers for all previously mentioned stains were observed under a 400x magnification using a Leica DM 750 light field microscope and a Leica ICC50 HD camera.

**Statistical analysis**

Data were coded and entered using the GraphPad Prism version 7. Data was summarized using mean and standard deviation. Comparisons between groups were done using one way analysis of variance (ANOVA) with Tukey’s multiple comparisons test when comparing more one variable in more than 2 groups.

**RESULTS**
**Histological results**

Hematoxylin and Eosin-stained sections of the control and sham control groups showed normal histological architecture of heart cardiac muscle. The cardiac fibers exhibited cross-striations, branching and one or two central vesicular, open faced nuclei with surrounding perinuclear sarcoplasm. The terminal ends of adjacent cardiac muscle fibers revealed characteristic and dense-staining, end-to-end junctional complexes called intercalated disks (Figure 1).

In the heart tissues of the Na fluoride group (IV), vascular dilatation and congestion were detected along with Zenker’s degeneration of the myocardium. Loss of cross striations, fragmentation and separation of the fibers were also seen (Figure 2). In the heart tissues of groups V and VI, an apparent restoration of the normal architecture was featured in addition to mild hyperemia at the myocardial interstitial intervals and in the capillaries (Figure 3). In Masson’s trichrome-stained sections, the myocardial fibers were found to show a minimal positive reaction in groups I, II, III, V and VI, whereas a strong positive reaction was observed in group IV (Figures 4 and 5).

The immunohistochemical expressions of iNOS and PCNA in the cardiac tissue are shown in Figures 6 and 7 respectively. A weak expression was presented in the cardiac muscle of the control groups. However, iNOS and PCNA were significantly positively expressed in cardiac muscle, which presented as dark brown granules, in the NaF group. The apparent reduction in PCNA immunopositivity in group VI was not as marked as that of group V. The relative densities of Masson’s trichrome, iNOS and PCNA in the myocardium of the NaF group was also significantly higher ($P < 0.05$) than the control group and lower ($P < 0.05$) in the vitamin C and PRP groups than the NaF group (Tables 2 and 3).
Figure 1. Hematoxylin&Eosin-stained sections of a. control, b. vitamin C sham control and c. PRP sham control adult rat cardiac muscle exhibiting distinct cross-striations (arrows). Cardiac muscle fibers exhibit branching (circle) and one or two central vesicular nuclei (N) with perinuclear sarcoplasm (arrow heads). The terminal ends of adjacent cardiac muscle fibers reveal characteristic intercalated disks (tailed arrow). Narrow intercellular spaces with blood capillaries are seen (zigzag arrow) H&E x400.
**Figure 2.** Hematoxylin& Eosin-stained sections of Na fluoride-treated adult rat cardiac muscle, showing Zenker’s degeneration with widely separated myocardial fibers (double arrow), loss of cross-striations and fragmentation (F). Some myocardial fibers exhibit a wavy orientation (W). Areas of normal histological architecture in section (d) alternate with areas of loss of architecture in section (b). Dilated and congested arteriole (A) and venule (V) are seen. Pyknotic nuclei (P) and ghost cells (G) are noticeable along with areas of inflammatory cellular infiltration (arrows) H&E x400.
**Figure 3.** Hematoxylin&Eosin-stained sections of a and b. Na Fluoride + vitamin C-treated, c. Na- fluoride + PRP-treated adult rat cardiac muscle exhibiting normalization of histological architecture. Distinct cross-striations (arrows) are seen. Cardiac muscle fibers show branching (circle) and central vesicular nuclei (N) with persistence of some nuclear pyknosis (P). The terminal ends of adjacent cardiac muscle fibers show intercalated disks (tailed arrow). Intercellular spaces with blood capillaries are also seen (zigzag arrow) H&E x400.
Figure 4. Masson’s trichrome-stained sections of a. control, b. vitamin C sham control and c. PRP sham control adult rat cardiac muscle exhibiting positive reaction (arrows). Masson’s trichrome x 400. Blue color indicates positivity.
Figure 5. Masson’s trichrome-stained sections of a. Na fluoride-treated adult rat cardiac muscle exhibiting a strong positive reaction (arrows), b. Na fluoride + vitamin C-treated and c. Na-fluoride + PRP-treated adult rat cardiac muscle exhibiting positive reaction (arrows). Masson’s trichrome x 400. Blue color indicates positivity.
Figure 6. iNOS immunohistochemistry of adult rat cardiac muscle in a. control, b. vitamin C sham control and c. PRP sham control exhibiting minimal iNOS-immunopositivity. d. apparent increase in iNOS-immunopositivity in Na-fluoride-treated rat myocardium. Minimal iNOS expression in e. Na Fluoride + vitamin C-treated, and f. Na-fluoride + PRP-treated rats. (iNOS immunohistochemistry, hematoxylin counterstain, ×400). Brown color indicates positivity.
Figure 7. PCNA immunohistochemistry of adult rat cardiac muscle in a. control, b. vitamin C sham control and c. PRP sham control exhibiting minimal PCNA-immunopositivity. d. apparent increase in PCNA-immunopositivity in Na-fluoride-treated rat myocardium. Minimal PCNA expression in e. Na Fluoride + vitamin C-treated and moderate expression in f. Na- fluoride + PRP-treated rats (PCNA immunohistochemistry, ×400). Brown color indicates positivity.

Biochemical and Molecular Results

Oxidative markers

MDA level in nmol/gm tissue
A statistically significant difference was seen in MDA level between the different study groups (p value <0.0001). MDA level in the NaF-treated group (14±0.46) was significantly increased compared to the control group (1.3±0.13) (p value <0.0001). In the vitamin C and PRP-treated groups, significant reductions in MDA level (4.1±0.52 and 8.1±0.28 with p values <0.0001 and =0.0003 respectively) were observed when compared to the NaF-treated group. In addition, there was a statistically significant decrease in MDA level in the vitamin C group compared to PRP group (p value = 0.0017) (Figure 8.A).

**SOD level in U/gm tissue**

A statistically significant difference was seen in SOD level between the different study groups (p value = 0.03). SOD level in NaF-treated group (2.9±0.05) was significantly decreased compared to the control group (4.0±0.37) (p value =0.033). In the vitamin C and PRP-treated groups, a non-significant increase in SOD level (3.8±0.16 and 3.3±0.27 with p values = 0.067 and = 0.37 respectively) were observed when compared with the NaF-treated group. In addition, there was no statistically significant difference in SOD level in the vitamin C group when compared with PRP group (p value = 0.37) (Figure 8.B).

**TrX-1 level in ng/gm tissue**

There was no statistically significant difference in TrX-1 level among the different study groups (p value = 0.23). Its level in the control group was 21±0.7 compared to 15±0.56 in NaF-treated group. TrX-1 level in the vitamin C and PRP-treated groups was 23±3.3 and 30±11 respectively (Figure 8.C).

**Gene expression**

**Keap1**

A statistically significant difference was seen in Keap1 gene expression among the different study groups (p value =0.01). Keap1 gene expression in NaF-treated group (4.5±0.99) was significantly increased compared to control group (1±0.007) (p value= 0.0008). In the vitamin C and PRP-treated groups, significant reductions in Keap1 gene expression (1.9±0.16 and 2.2±0.28 with pvalue <0.025 and 0.03 respectively) were observed when compared to the
NaF-treated group. In addition, there was no a statistically significant difference in Keap1 gene expression in the vitamin C group compared to PRP group (p value = 0.93) (Figure 8.D).

**Nrf2**

A statistically significant difference was seen in Nrf2 gene expression among the different study groups (p value =0.032). Nrf2 gene expression in NaF-treated group (0.35±0.03) was significantly decreased compared to the control group (1±0.014) (p value= 0.026). In the vitamin C and PRP-treated groups, there was no a statistically significant difference between the PRP (7.7±0.11) and vitamin C (0.8±0.11) pretreated groups compared with NaF-treated group (p value = 0.1 and 0.08 respectively). In addition, there was non-significant difference in Keap1 gene expression in the vitamin C group compared to PRP group (p value = 0.99) (Figure 8.E).

**HO-1**

A statistically significant difference was seen in HO-1 gene expression among the different study groups (p value <0.0001). HO-1 gene expression in NaF-treated group (0.26±0.03) was significantly decreased compared to the control group (1±0.02) (p value <0.0001). In the vitamin C and PRP-treated groups, significant difference in HO-1 gene expression (0.68±0.02 and0.61±0.03 with p value =0.001 and = 0.0005 respectively) were observed when compared to the control group. A statistically significant increase in HO-1 gene expression in PRP and vitamin C groups was seen when compared to NaF treated group (p value = 0.0005 and 0.001 respectively) (Figure 8.F).
Figure 8. Different levels of biochemical and molecular markers. Data were expressed as Mean ± SD, p value <0.05 was significant.

(*) significant difference versus control group
(#{}) significant difference versus Naf-treated group
($) significant difference versus PRP-treated group

Statistical results

Statistical results for Masson’s trichrome optical density and iNOS and PCNA immunohistochemical expression are shown in Table 2 and Table 3. Masson’s trichrome’s optical density of the NaF-intoxicated group (16.371± 1.171) significantly increased compared to the control (1.183±0.123) and the sham control (1.191±0.91) groups. The groups that were pretreated with vitamin C and PRP prior to sodium fluoride intoxication showed a significant
reduction in optical density (1.505± 0.423) and (6.682±1.401) respectively when compared to the NaF group. Pretreatment with vitamin C (8.924± 0.486) and PRP (12.326±0.98) significantly lowered the immunoexpression of iNOS when compared to the NaF group (19.176±3.327), while the expression of the latter was significantly increased in comparison to the control (1.851±0.064) and sham control (1.924±1.09) groups. Intoxication with NaF caused a significant increase in PCNA immunoexpression (874±1.09) when compared to the control (70±0.44) and sham control (76±0.53) groups. Vitamin C (132± 0.411) and PRP (352±0.82) PCNA expression were decreased when compared to the NaF group. A statistically significant difference between the vitamin C and PRP-pretreated groups and the control groups was seen in all measured parameters.

**Table 2.** Multiple comparisons of $p$ values for Masson’s trichrome, iNOS and PCNA expression between the different experimental groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Masson’s trichrome</strong></td>
<td></td>
</tr>
<tr>
<td>Control vs Sham control</td>
<td>1.000</td>
</tr>
<tr>
<td>NaF-treated</td>
<td>0.000</td>
</tr>
<tr>
<td>Vitamin c</td>
<td>0.002</td>
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<tr>
<td>PRP-treated</td>
<td>0.031</td>
</tr>
<tr>
<td>NaF vs Sham control</td>
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</tr>
<tr>
<td>Vitamin c</td>
<td>0.000</td>
</tr>
<tr>
<td>PRP-treated</td>
<td>0.022</td>
</tr>
<tr>
<td>PRP vs Sham control</td>
<td>0.029</td>
</tr>
<tr>
<td>Vitamin c</td>
<td>1.000</td>
</tr>
<tr>
<td>Vitamin c vs sham control</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>iNOS</strong></td>
<td></td>
</tr>
<tr>
<td>Control vs Sham control</td>
<td>1.000</td>
</tr>
<tr>
<td>NaF-treated</td>
<td>0.000</td>
</tr>
<tr>
<td>Vitamin c</td>
<td>0.000</td>
</tr>
<tr>
<td>PRP-treated</td>
<td>0.019</td>
</tr>
<tr>
<td>NaF vs Sham control</td>
<td>0.000</td>
</tr>
<tr>
<td>Vitamin c</td>
<td>0.000</td>
</tr>
<tr>
<td>PRP-treated</td>
<td>0.000</td>
</tr>
</tbody>
</table>
\[ P < 0.05 \text{ is considered significant.} \]

**Table 3.** Mean ±SD for Masson’s trichrome, iNOS and PCNA expression between the different experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Masson’s trichrome</th>
<th>iNOS</th>
<th>PCNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mean ±SD</td>
<td>1.183±0.123</td>
<td>1.851±0.064</td>
</tr>
<tr>
<td>Sham control</td>
<td>Mean ±SD</td>
<td>1.191±0.91</td>
<td>1.924±1.09</td>
</tr>
<tr>
<td>NaF-treated</td>
<td>Mean ±SD</td>
<td>16.371±1.171+</td>
<td>19.176±3.327+</td>
</tr>
<tr>
<td>Vitamin C-treated</td>
<td>Mean ±SD</td>
<td>1.505±0.423+*</td>
<td>8.924±0.486+*</td>
</tr>
<tr>
<td>PRP-treated</td>
<td>Mean ±SD</td>
<td>6.682±1.401+*</td>
<td>12.326±0.98+*</td>
</tr>
</tbody>
</table>

+ P < 0.05, significant difference compared to that of the control group.

* P < 0.05, significant difference compared to that of the NaF-treated group.

**DISCUSSION**
Sodium fluoride toxicity continues to be a major health problem worldwide. In the current study, myocardial damage was induced in rats by oral ingestion of NaF. The purpose of our study was to evaluate the potential cardio protective role of vitamin C and PRP in NaF-induced cardiac toxicity in rat model. This was performed with reference to biochemical, molecular, immunohistochemical and histopathological changes.

Zhao et al., [52] suggested that DNA replication is an indicator of cellular proliferation. The authors also stated that DNA replication is lowered, because of the synthesis of PCNA, in inactive cells but varies according to the phase of the cell cycle, making PCNA expression a marker of cellular proliferation. In the present study, PCNA expression in the cardiac muscle was assayed by immunohistochemistry. In agreement with our results, Zhao et al., [52] observed a weak PCNA expression in the control group, and a significant positive PCNA expression in the fluoride-administered group of adult male rats’ testes. They concluded that spermatogenesis dysfunction and damage of tissue ultrastructure was caused by fluoride-induced apoptosis to which the positive PCNA expression was related, since according to the authors, apoptosis is associated with proliferation. Although previous studies have pointed out a reduction in cellular proliferation in response to fluoride exposure, the authors mentioned that cell proliferation could be maintained through the overexpression of PCNA as a response to fluoride-induced cellular apoptosis.

Shenoy et al., [42] assessed the effect of NaF administration at different concentrations on myoblast proliferation. In contrast our study where NaF was administered at a dose of 25mg/kg, the author concluded that at a low concentration of 1.5 ppm (1 ppm being equivalent to 1 mg/kg), NaF administration resulted in myoblast proliferation and myotubular hypertrophy via an IGF-1/AKT pathway activation, whereas at a higher concentration of 5 ppm, NaF caused myotubular atrophy via an ubiquitin-proteosome pathway. Enhancement of skeletal muscle catabolism, production of ROS and inflammatory cytokines were also reported by the authors when higher concentrations of NaF were administered. As regards apoptosis, the authors recorded that via PI3K/Akt signaling, NaF was capable of induction of apoptosis and reduction in cell viability when administered in a dose higher than 40 ppm.

Luo et al., [26] investigated the underlying molecular mechanism of NaF-induced cytotoxicity and cell-cycle alterations in renal cells. In contrast to the present study, the authors reported that at more than 12mg/kg body weight, a NaF-induced G2/M phase cell-cycle arrest
was seen, leading to a significant increase of cell percentage present in the G2/M phase. According to the authors, the cycle arrest occurred by activating the ATM-Chk2-p53/Cdc25C signaling pathway which inhibited cellular proliferation and was associated with down regulation of PCNA mRNA expression.

In the present study, an increase in collagen fibers deposition was seen in the NaF group, as assayed by Masson’s trichrome staining and image analysis. Chen et al., [7] investigated the role of tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor-inducible molecule 14 (Fn14) axis in myocardial fibrosis. The authors observed that stimulation of rat cardiac fibroblasts (CFs) with TWEAK increased CFs numbers and collagen synthesis via activation of the nuclear factor-kappaB (NF-κB) pathway and a subsequent production of metalloproteinase-9 (MMP-9). The authors emphasized the importance of the TWEAK/Fn14 axis in control of myocardial fibrosis.

In contrast to the NaF-induced collagen deposition seen in the current study, Gupta et al., [17] detected that fluoride disturbs collagen synthesis causing the cells responsible for its synthesis to produce larger amounts of under-hydroxylated, inadequately cross-linked and rapidly catabolized collagen and/or non-collagenous proteins as a compensation. The net result is a decrease in the collagen content of tissues.

In the present study a statistically significant increase in NaF-induced iNOs expression was observed. In line with the observations of this study, Oyagbemi et al., [35] reported an increase in oxidative stress markers in cardiac tissue, in response to NaF administration. The latter authors recorded abnormalities in the histological architecture of the heart tissue and an increase in nuclear factor kappa beta (NF-κB) expression as assayed by immunohistochemistry. The authors indicated that NaF-induced alterations were achieved through generation of reactive oxygen species and activation of cardiac NF-κB expression, oxidative stress being an activator of NF-κB. The authors mentioned that activation of NF-κB results in its release from cytosolic inhibitors and its translocation to the nucleus and consequently to the expression of NF-κB-target genes causing tissue inflammation and damage.

The mechanism of NaF-induced oxidative stress was explained by Gupta et al., [18]. The authors reported that fluoride causes a respiratory burst and oxygen free radicals production. The authors stated that hydroxyl radicals and superoxide radicals prevail at low and high concentrations of fluoride. The authors attributed the fluoride-induced oxidative stress to its
ability to cause mitochondrial swelling and disruption and alteration of enzymes of cellular respiration. The net result is a decrease in adenosine triphosphate concentration, which in turn induces production of hydrogen peroxide and reactive oxygen species.

The current investigation revealed an increase in NaF-induced iNOS expression. He et al., [20] examined the possible effect of fluoride exposure on the expression of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) on cultured chondrocytes. The authors stated that production of NO can be under control of iNOS and that both low and high levels of F-significantly increased the activity of iNOS and consequently the level of NO leading to oxidative tissue damage.

Ngoc et al., [34] investigated the manner of NaF-induced cell death and the mechanisms involved, where they found that it occurs mainly by apoptosis rather than necrosis. The authors reported that administration of more than 1 mMNaF leads to apoptosis through hydroxyl radical-dependent and caspase- and JNK mediated pathways. According to the authors, GADD45α plays a crucial role in the induction of apoptosis, in which its transcription and function are controlled either by JNK1 or JNK2. NaF administration stimulated the induction of GADD45α, whereas a JNK specific inhibitor inhibited such effect. The authors also reported that ROS are normally produced at low concentrations in a constant manner in living organisms, serving the function of immune cells. However, over-expression or decreased removal of intracellular ROS induces oxidative damage to cells and tissues. The authors mentioned that through an elevation of oxidative stress-mediated lipid peroxidation and subsequent mitochondrial stress, that fluoride induces apoptosis. They suggested that ROS are mediators of NaF-mediated apoptosis, where mitochondrial stress is at least in part involved. The authors concluded that the mitochondrial- and caspase-mediated signaling accompanied by intracellular ROS accumulation are involved in NaF-induced apoptosis. The authors also concluded that JNK-GADD45α- and p53-mediated signaling is critical for NaF-mediated apoptosis, where ROS act as the most crucial upstream mediator.

In agreement with the histological results of the current work, Oyagbemi et al., [35] reported an infiltration of the myocardial interstitium by inflammatory cells in response to NaF treatment. Yildirim et al., [49] investigated the effect of sodium fluoride on cardiac histopathology. The investigation revealed hyperemia of interstitial vessels, hyaline degeneration andZenker’s necrosis in muscle fibers and mononuclear cell. A study done by Basha and Sujitha,
recorded myocarditis with cloudy swelling, necrosis, hemorrhage, inflammation, and atherosclerosis in cardiac tissue with chronic fluorosis. Another experimental fluorosis study done by Al Shahat and Naggar [2] revealed capillary congestion, mononuclear cell infiltration and hemorrhage in the myofibrillar interval, severe muscle degeneration, and cytoplasmic vacuolization. In our study, the NaF group revealed Zenker’s degeneration with wide separation of myocardial fibers, loss of cross-striations and fragmentation. Dilatation and congestion of blood vessels, nuclear pyknosis and inflammatory cellular infiltration were also noticeable.

The heart is vulnerable to oxidative stress due to the abundance of mitochondria; the site of basal ROS generation and due to its inherent low antioxidant defenses [51]. Vitamin C is an electron donor, and this is the basis of all its known functions. It is a soluble antioxidant that prevents oxidative damage by eliminating free radicals [36]. In the present study, vitamin C significantly ameliorated the NaF-induced alterations seen in rats’ myocardium.

The results of Ghosh et al., [14] suggest that vitamin C is capable of intracellular antioxidant enzymes restoration and maintenance of endogenous antioxidant molecules’ levels, leading the authors to include it as a positive control to verify the experimental set up against sodium fluoride-induced oxidative stress and cell death.

Sirtuin 1 (Sirt1), which is a NAD-dependent class III histone deacetylase, plays a fundamental role in multiple cellular processes via deacetylation. It controls mitochondrial health by limiting the generation of mitochondrial-derived ROS (mROS). Sirt1 deacetylases manganese superoxide dismutase (SOD2); a key enzyme involved in regulating mROS production, consequently increasing SOD2 activity [48]. Low levels of Sirt1 are associated with increased mROS levels [38]. Liu et al., [25] mentioned that Sirt1 expression has antioxidant regulatory effects in cardiac tissue where it regulates antioxidant systems.

Peng et al., [37] investigated the beneficial effects of vitamin C against NaF-induced cytotoxicity and the underlying molecular mechanisms. The authors found that NaF caused cytotoxicity, stimulated mitochondrial reactive oxygen species (mROS) production, and prompted apoptosis in F9 embryonic carcinoma cells. NaF administration was also accompanied with decreased Sirt1 protein expression. They found that NaF-induced mitochondrial oxidative injuries were significantly attenuated by overexpression of Sirt1 or incubation with Mito-TEMPO (a SOD2 mimetic). The authors reported that pretreatment with vitamin C enhanced the expression of Sirt1 and decreased NaF-induced mitochondrial oxidative stress and apoptosis. The
authors confirmed that vitamin C-induced reduction in mROS and apoptosis was blocked by a knockdown of Sirt1 by means of inhibiting Sirt1-SOD2 signaling. They also reported that sodium-dependent vitamin C transporter 2 (SVCT-2) siRNA was capable of hindering the capacity of vitamin C to stimulate Sirt1/SOD2 signaling.

Zaki et al., [51] revealed that the modulatory effect of mesenchymal stem cells (MSCs) pretreated with platelet-rich plasma (PRP) on doxorubicin (DOX)-induced cardiotoxicity was superior to the modulatory effect of MSCs alone. The authors mentioned that oxidative stress underlies the DOX-induced cardiotoxicity and accordingly found the oxidant marker MDA to be elevated, while the antioxidant marker SOD decreased in DOX-treated groups. A significant improvement of both markers in the MSC- and PRP/MSC-treated groups was seen with a significant MDA difference in favor of PRP/MSC-treated group. A 100% higher Bcl2/Bax ratio was seen after MSCs were pretreated with PRP when apoptosis was assessed. In line with the present results, PRP showed a similar preventive role like vitamin C.

In the cardiovascular system, important antioxidative molecules are catalase, superoxide dismutases (SOD), glutathione, glutathione S-transferases, glutathione peroxidases, hemeoxygenases, thioredoxinreductases, and thioredoxins. The expression of those antioxidative molecules can be regulated by the transcription factor Nuclear factor erythroid 2-related factor 2 [22].

The potential antioxidant properties of PRP and vitamin C to antagonize cardiac toxicity of NaF were studied in the current experiment. Their antioxidant potential was investigated through their effect on the Keap1/Nrf2/HO-1 as a redox signaling pathway. In the current study, the cardiac toxicity with NaF caused the depletion of SOD, TrX1 and increase of MDA level and increase of Keap1 levels, and it decreased Nrf2 and HO-1 mRNA expressions levels, which are markers of oxidative stress. Many studies demonstrated that NaF administration reduces the activity of mitochondrial antioxidant enzymes leading to cardiotoxicity, finally resulting in myocardial necrosis [9]. Activation of Nrf2 is done to antagonize reactive oxidants, while decreased Nrf2 activity has the opposite action [43]. The treatment of tumor cells with vitamin C reduced the expression of Nrf2 at the mRNA and protein levels [32]. These are studies agreed with our study, PRP and vitamin C reduced Keap1 expression and increased the nuclear accumulation of Nrf2, which is associated with higher expression levels of HO-1, SOD, and mitochondrial antioxidant enzymes. These results are agreed with earlier studies suggesting PRP
and vitamin C as an anti-oxidative stress agent [19, 23]. Lawal et al., [24] investigated the antioxidant efficacy of heated garlic juice (HGJ) in liver against ascorbic acid (AA) in rats exposed to cadmium. Their results showed that ascorbic acid was a more effective antioxidant than heated garlic juice in avoiding cadmium-induced oxidative damage in liver and its action intervened through Nrf2-keap1 pathway. Saif-Elnasr et al., [41] studied the treatment of hepatotoxicity induced by γ-radiation in albino rats by PRP and/or LMC. Their study revealed that treatment with PRP and/or LMC decrease hepatic MDA levels and increase Nrf2 levels. Another report proposed that vitamin C reduced the levels of the oxidants malondialdehyde in acute and regular exercise [40]. The present study proved that PRP and vitamin C can be used a cardioprotective agent in NaF toxicity, which is possibly associated with the modulation of the Keap1/Nrf2 pathway, stimulating the nuclear accumulation of Nrf2 and the upregulation of phase II antioxidant enzyme expression such as HO-1, SOD, and TrX-1.

CONCLUSIONS

The presented results suggest that pretreatment with vitamin C and PRP possess a potential cardio-protective effect against sodium fluoride intoxication in adult male albino rats. This study is the first to show that the cardio-protective effect of PRP, against sodium fluoride intoxication in the homogenates of rat heart tissues is comparable to that of vitamin C and might be considered for clinical trials. The results can be viewed as a starting point for further applications of this natural compound in the pharmaceutical industry after performing clinical researches. Our data illustrates a new molecular mechanism underlying the ability of vitamin C and PRP to be explored for future treatment of NaF-induced cytotoxicity.

Compliance with ethical standards and Conflict of interest:
The authors declare that they have no conflict of interest.

Ethical approval
All protocols for animal experiments were approved by the institutional animal Ethical Committee, Cairo University, Egypt.

There was no financial support for the research, authorship and/or publication of this article received by the authors.
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