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Hajer Ben Saad
Laboratory of Pharmacology, Faculty of Medicine, 3029 Sfax, University of Sfax, Tunisia

Intissar Kammoun
Higher Institute of Biotechnology of Sfax, 3000 Sfax, Sfax University, Tunisia

Ons Boudawara
Anatomopathology Laboratory, Habib Bourguiba Hospital 3029 Sfax, University of Sfax, Tunisia

Ahmed Hakim
Laboratory of Pharmacology, Faculty of Medicine, 3029 Sfax, University of Sfax, Tunisia

Ibtissem Ben Amara
Higher Institute of Biotechnology of Sfax, 3000 Sfax, Sfax University, Tunisia

Correspondence
Hajer Ben Saad
Laboratory of Pharmacology, Faculty of Medicine, 3029 Sfax, University of Sfax, Tunisia

Preventive effect of vanillin on lipid peroxides and antioxidants in potassium bromate-induced cardiotoxicity in adult mice: Biochemical and histopathological evidences

Hajer Ben Saad, Intissar Kammoun, Ons Boudawara, Ahmed Hakim and Ibtissem Ben Amara

Abstract
Potassium bromate ($KBrO_3$) is a xenobiotic which causes several diseases. Our experimental objective was designed to evaluate the cardioprotective potential of vanillin on lipid peroxides, enzymatic and nonenzymatic antioxidants and histopathological findings in $KBrO_3$-induced cardiotoxicity in adult mice. The oral administration of $KBrO_3$ (2 g/L) to adult mice showed a significant increase in lipid peroxidation and advanced oxidation protein product levels in the heart, and a significant decrease in the superoxide dismutase, catalase, glutathione peroxidase activities and the levels of reduced glutathione in the heart. The daily administration of vanillin (100 mg/kg by intraperitonial injection) to $KBrO_3$-induced mice for a period of 15 days showed a significant decrease in the levels of lipid peroxidation and protein oxidation, and improved the antioxidant status by increasing the activities of antioxidant enzymes and nonenzymatic antioxidants. The histopathological findings of the myocardial tissue showed the protective role of vanillin in $KBrO_3$-induced mice. The results of our study showed that vanillin possesses anti-lipoperoxidative and antioxidant activites in experimentally $KBrO_3$- induced cardiac toxicity.

Keywords: *Potassium bromate, vanillin, cardiotoxicity, oxidative stress*
Abbreviations: 8-OH-dG: hydroxydeoxyguanosine; AOPP: Advanced oxidation protein product; CAT: Catalase; GPx: Glutathione peroxidase; GSH: glutathione; $KBrO_3$: potassium bromate; MDA: malondialdehyde; ROS: reactive oxygen species; ROS: reactive oxygen species; SOD: Superoxide dismutase.

Introduction
A disturbance in the pro-oxidant–anti-oxidant balance in favor of the former, leading to a potential damage, results in oxidative stress. It illustrates the steady state level of oxidative damage in a cell, tissue or organ, caused by the reactive oxygen species (ROS). These ROS react with cellular membrane lipids, nucleic acids, proteins and enzymes, resulting in cellular damage and degeneration. They are involved in the pathophysiology of many diseases, including diabetes mellitus, Alzheimer's disease, Parkinson's disease, carcinogenesis, mutagenesis and ageing [1].

Potassium bromate ($KBrO_3$) has been widely used for water disinfection, hair-coloring solutions, cosmetics, and food [2]. Toxicological studies have suggested that $KBrO_3$ is an oxidizing agent causing neurotoxicity, hepatotoxicity, thyroid toxicity, and induces the development of mesothelioma tumors in experimental animals [3, 4]. Several studies have investigated the oxidative injuries and probable mechanism of $KBrO_3$-induced carcinogenicity in experimental models [5-7]. $KBrO_3$ induces mutations, base modification, chromosomal aberrations, and alters genes expression, leading to cancer [8]. With this background, it is important that a suitable preventive/therapeutic agent for $KBrO_3$ treatment be found, which could be cheap, easily available, effective in low doses and less toxic, with a convenient mode of administration and having an antioxidant compound that could prove beneficial in chelating $KBrO_3$ from the intracellular and extracellular sites. The use of medicinal herbs and plants has proved successful with different organs against oxidative stress in various experimental models [9]. Vanilla is an extract of cured, unripe fruit of the plant Vanilla planifolia. Vanillin inhibits food spoilage in laboratory media and fruit purees [10]. It has been shown to have inhibitory effects on the initiation and promotion of carcinogenesis in different animal models [11]. This flavoring agent has abilities to scavenge superoxide and hydroxyl radicals, intermediates that have been implicated in membrane damage [12].

Many pharmacological activities were reported of vanillin as an antioxidant [13], a hepatoprotective [14] and a nephroprotective [15], which urged our research on vanillin against $KBrO_3$-induced toxicity. There is, however, paucity of information on the possible effect of vanillin on oxidative membrane damage induced by $KBrO_3$. Therefore, the present paper describes the ameliorative effects of vanillin on $KBrO_3$-induced oxidative stress in mice heart.

The aim of this study was to examine the protective effect of vanillin against damage inflicted by $KBrO_3$ poisoning to the heart of adult mice. The antioxidant effect of vanillin is likely to counteract or minimize the undesirable effects induced by $KBrO_3$.

Materials and methods

Experimental animals

All the experiments were carried out with male Swiss mice weighing 40–45 g. The experiment was carried out according to the general guidelines on the use of living animals in scientific investigations approved by the Ethical Committee of the Sciences Faculty of Sfax.

Experimental design

The animals were grouped as twelve mice in each group: control; $KBrO_3$ (2 g/L by their drinking water); $KBrO_3$ + vanillin (100 mg/kg body weight by intraperitoneal injection) and vanillin. After 15 days of treatment, all the mice were sacrificed by cervical decapitation. Heart tissue was excised immediately and rinsed in ice-chilled normal saline. A known weight of the heart tissue was homogenized in 5.0 ml of 0.1M Tris–HCl buffer (pH 7.4) solution. The homogenate was centrifuged and the supernatant was used for the estimation of various biochemical parameters.

Biochemical assays

The heart malondialdehyde (MDA) concentrations, index of lipid peroxidation, were determined spectrophotometrically according to Draper and Hadley [16]. In brief, an aliquot of heart extract supernatant was mixed with 1 mL of trichloroacetic acid and centrifuged for 10 min. An amount of 1 mL of TBA reagent was added to 500 µL of supernatant and heated at 90°C for 15 min. The mixture was then cooled and measured for absorbance at 532 nm. The MDA levels were expressed as nmol of MDA/mg protein.

Advanced oxidation protein product (AOPP) levels were determined according to the method of Kayali *et al*. [17]. The concentration of AOPP for each sample was calculated using the extinction coefficient of 261 cm^{-1} mM^{-1} and the results were expressed as µmoles/mg protein.

Superoxide dismutase (SOD) activity in the heart was assayed by the method of Beauchamp and Fridovich [18]. Superoxide radicals react with nitroblue tetrazolium in the presence of reduced nicotinamide adenine dinucleotide and produce formazon blue. SOD removes the superoxide radicals and inhibits the formation of formazon blue. The intensity of the colour is inversely proportional to the activity of the enzyme and read at 560 nm. The activity of SOD was expressed as units/mg protein.

Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler [19]. GPx catalyzes the oxidation of reduced glutathione by cumene hydroperoxide. The oxidized reduced glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH, in the presence of reduced glutathione reductase and nicotinamide adenine dinucleotide phosphate reduced form (NADPH). The decrease in absorbance at 340 nm was measured. The enzyme activity was expressed as nmol of GSH oxidized/min/mg protein.

Catalase (CAT) activity was assayed by the method of Aebi [20]. Enzymatic reaction was initiated by adding an aliquot of 20 µL of the homogenized heart and the substrate (H_2O_2) to a concentration of 0.5 M in a medium containing 100 mM phosphate buffer (pH 7.4). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of µmol H_2O_2 consumed/min/mg of protein.

Heart reduced glutathione (GSH) contents were determined by Ellman's method [21], modified by Jollow *et al*. [22] based on the development of a yellow color when 5,5-dithiobis-2 nitro benzoic acid was added to compounds containing sulfhydryl groups. In brief, 3 mL of sulfosalicylic acid was added to 500 µL of heart homogenate in phosphate buffer for deproteinization and the mixture was centrifuged at 2500g for 15 min. Ellman's reagent was then added to 500 µL of supernatant. The absorbance was measured at 412 nm after 10 min. Total reduced glutathione content was expressed as µg/mg of protein.

Histopathological examination

The heart tissue obtained from all experimental groups were washed immediately with saline and then fixed in 10% buffered neutral formalin solution. After fixation, the heart tissue was processed embedding in paraffin. Then, the heart tissue was sectioned and stained with hematoxylin and eosin (H&E) and examined under high power microscope (320×) and photomicrographs were taken.

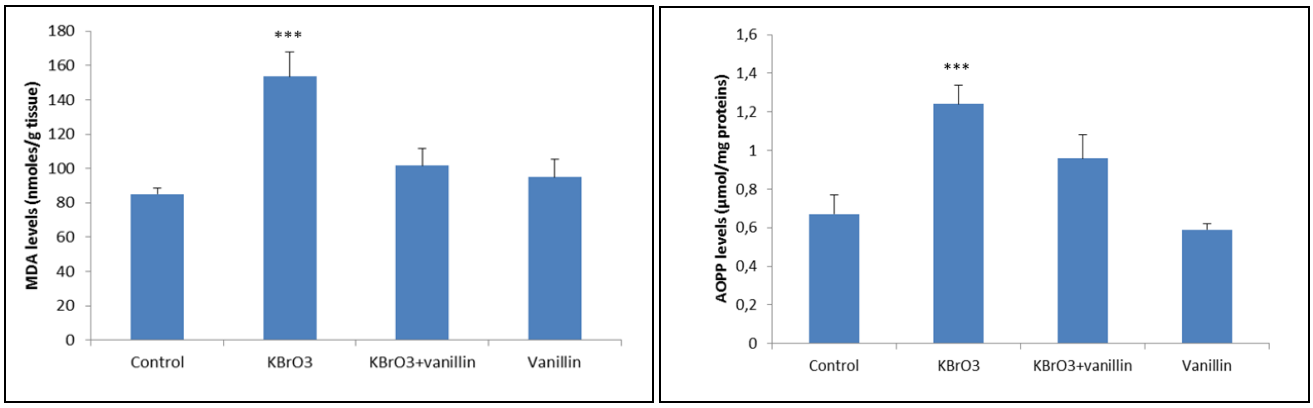
Statistical analysis

The data were analysed using the statistical package program Stat View 5 Software for Windows (SAS Institute, Berkley, CA, USA). Statistical analysis was performed using one way analysis of variance followed by Fisher's protected least significant difference test as a post hoc test for comparison between groups. All values were expressed as the means ± standard deviation (SD). Differences were considered significant at $P<0.05$ level.

Result

Oxidative damages

The inhibitory effect of vanillin on $KBrO_3$ induced peroxidative damage is shown in Fig. 1. The levels of MDA and AOPP were significantly increased ($p< 0.001$) in heart tissue of $KBrO_3$ treated animals when compared to controls. Supplementation of vanillin alleviated the MDA and AOPP levels in comparison with $KBrO_3$-treated group. No significant differences in the values were observed in mice treated with vanillin only compared to control mouse values.



(A) (B)

Fig 1: Malonaldialdehyde (MDA) and advanced oxidation protein products (AOPP) levels in the heart of adult mice controls or treated during 15 days with $KBrO_3$, $KBrO_3$+ vanillin and vanillin alone.
Values are expressed as means ± S.D for 10 animals in each group. Comparisons are made between treated vs control group: ***<0.001.

Antioxidant activities

Table 1 illustrate the levels of enzymatic antioxidants namely catalase, SOD and GPx in the heart of control and experimental mice. A significant decrease in the activities of heart enzymatic antioxidants (SOD, CAT and GPx) in $KBrO_3$ mice is seen. Treatment with vanillin alone did not affect any of the tested parameters. However, in their combinations with $KBrO_3$, all antioxidant enzymes were normalized to their control values. The vanillin abolished the oxidative damage and toxicity induced by $KBrO_3$ accompanied by an amelioration of antioxidant enzyme activities.

Table 1 illustrates the effect of vanillin on the levels of GSH in the heart in normal and $KBrO_3$-induced mice. Mice induced with $KBrO_3$, showed a significant (P< 0.001) decrease in the levels of GSH on comparison with normal control mice (table 1). The administration of vanillin (100 mg/kg) to $KBrO_3$-induced mice significantly (P<0.01) increased the levels of GSH when compared with $KBrO_3$-alone induced mice.

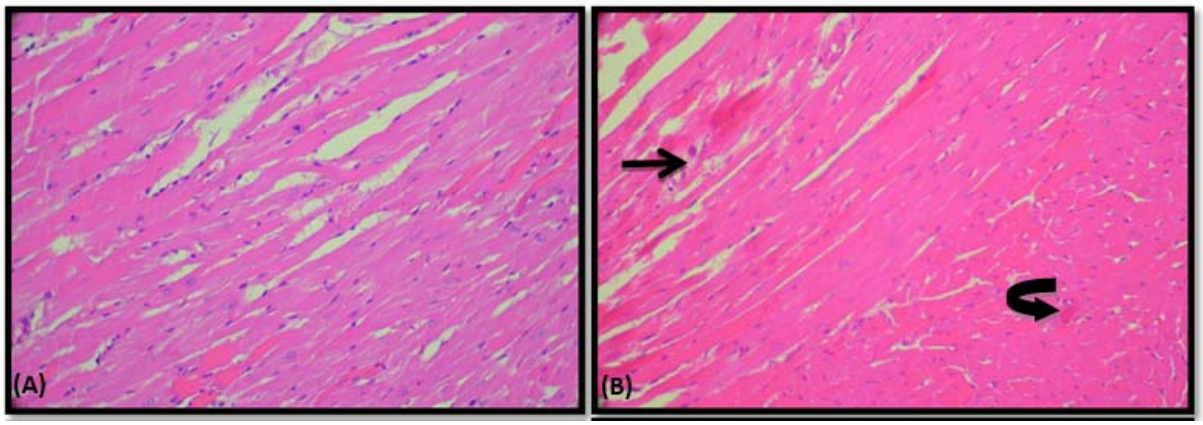
Table 1: Enzymatic antioxidant activities (glutathione peroxidase, catalase and superoxide dismutase) and the glutathione levels in the heart of adult mice controls or treated during 15 days with $KBrO_3$, $KBrO_3$+ vanillin and vanillin alone.

Parameters and treatments	GP_X	GSH	Catalase	SOD
Control	8,34±0,92	401,5±39,11	7,92±0,41	93,29±11,16
$KBrO_3$	3,71±0,53 ***	214,37±41,82***	4,21±0,33 ***	52,44±10,31 ***
$KBrO_3$+vanillin	6,18±0,44	306,49±38,62 **	4,87±0,18 **	64,33±8,11 **
Vanillin	8,27±0,29	391,36±21,19	7,19±0,72	87,42±7,43

SOD: unit/mg protein.
GPx: nmoles of GSH oxidized/min/mg protein.
CAT: nmol H_2O_2/min/mg protein.
GSH: µg/g tissue
Values are expressed as means ± S.D for 10 animals in each group. Comparisons are made between treated vs control group: **P< 0.01; ***<0.001.

Histopathological study

Heart tissue of the control mice showed normal myocardial fibers and muscle bundles with normal architecture (Fig. 2A). Heart tissue of $KBrO_3$-treated mice showed separation of myocardial fibers with vascular congestion, edema, leucocyte inflammatory, and myocardial necrosis (Fig. 2B). Myocardial section of vanillin treated mice showed slightly separated myocardial fibers with small focus of inflammatory mononuclear collections with the absence of necrotic damage (Fig. 2C).Vanillin alone-treated showed normal myocardial fibers with no pathological changes (Fig. 2D).



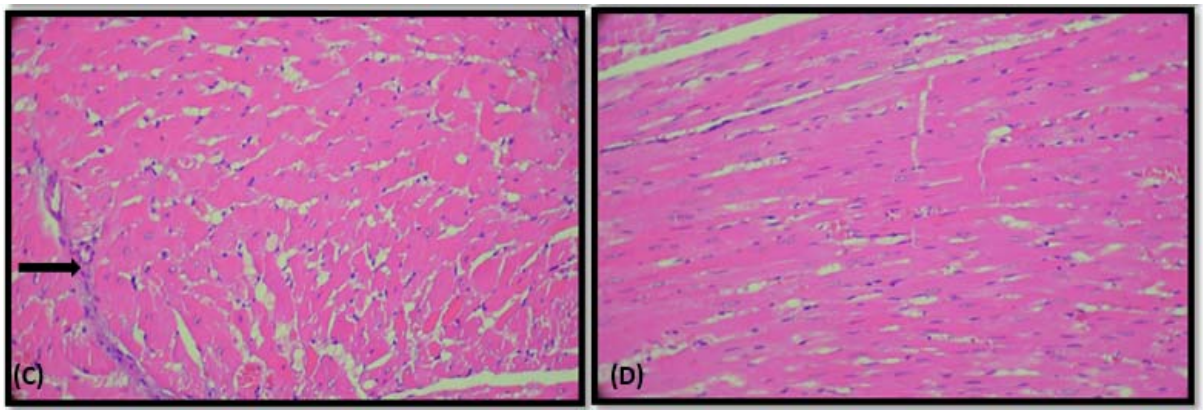


Fig 2: Heart histological sections of adult mice: controls and experimental group, controls (A), $KBrO_3$ (B), $KBrO_3$+ vanillin (C) and vanillin (D) showing the histopathological changes. Optic microscopy: HE (400X).

Arrows indicate → : Vascular congestio; → leucocyte inflammatory cells; ↺ Necrosis

Discussion

Toxicity of many metals, including $KBrO_3$, is associated with the increased production of ROS leading to an oxidative stress in cells [23]. The most abundant ROS generated in living cells are superoxide anions and their derivatives, particularly highly reactive and damaging hydroxyl radical which induces the peroxidation of cell membrane lipids [24]. MDA is one of the metabolic products of lipid peroxides generated by the reaction of lipid oxidation induced by oxygen free radicals in tissues. Lipid peroxidation, a type of oxidative degeneration of polyunsaturated fatty acids, has been linked with altered membrane structure and enzyme inactivation [25]. In our study, the increased MDA content in the $KBrO_3$-treated group in heart indicates severe oxidative stress. Many reports showed that excessive $KBrO_3$ treatment led to severe oxidative stress and increased lipid peroxidation [26]. It could be suggested that elevated lipid peroxidation may result from the decreased activities of antioxidant enzymes. Glutathione is one of the essential compounds for the regulation of a variety of cell functions. It has a direct antioxidant function by reacting with superoxide radicals, peroxy radicals and singlet oxygen followed by the formation of oxidized glutathione and other disulfides. GPx is a GSH-dependent antioxidant enzyme [25]. On the other hand, SOD catalyzes the dismutation of the superoxide anion (O_2) into H_2O_2, which is then detoxified to H_2O by catalase [24]. Catalase is a common enzyme found in nearly all living organisms. Its functions include catalyzing the decomposition of hydrogen peroxide to water and oxygen [27]. The present results support the hypothesis that the mechanism of $KBrO_3$ toxicity is related to the depletion of the antioxidant defense system. A significant decline in antioxidant enzyme activities and increase in free radicals in experimental models as well as in subjects is typical during the regimens of commonly used chemotherapy, and this is particularly related to $KBrO_3$ treatment [28].

In the present study, the observed decline in the level of GSH in $KBrO_3$ treated mice as compared with control group indicated that the depletion of GSH resulted in enhanced lipid peroxidation, and that excessive lipid peroxidation caused increased GSH consumption. An activity of GSH-dependent antioxidant enzyme, GPx activity was significantly reduced in $KBrO_3$ group as compared with control group. The reduction observed in the activity of GPx might be due to the decreased availability of its substrate, reduced glutathione [25]. The decrease in SOD activity could cause the initiation and propagation of lipid peroxidation in the $KBrO_3$ treated mice [29]. The decreased SOD activity is insufficient to scavenge the superoxide anion produced during the normal metabolic process [30]. The reduction in the activities of the SOD and catalase may be due to the increased generation of ROS such as superoxide and hydrogen peroxide, which in turn leads to the inhibition of the activities of these enzymes [25]. It is well known that ROS are constantly generated *in vivo* for physiological purposes. However, ROS production beyond the ability of an antioxidant system can cause oxidative damage to lipids, nucleic acids and proteins, resulting in oxidative stress. Catalase scavenges H_2O_2 that has been generated by free radicals or by SOD in the removal of superoxide anions. When mice were treated with vanillin prior to $KBrO_3$ administration, the reduction of SOD, CAT and GPx activity was inhibited. Vanillin alone did not cause significant changes in the GSH content and the activities of GPx, CAT and SOD. The protective effects of vanillin are due to the presence of polyphenols. In fact, Moskaug *et al*. [31] reported that polyphenols modulate the expression of an important enzyme in both cellular antioxidant defenses and the detoxification of xenobiotics. Akihiro *et al*. [32] clearly confirm our results and have verified the beneficial effect of this molecule.

On the other hand, the histopathological examination of the heart confirms the protective effect of vanillin against $KBrO_3$ induced oxidative damage of this organ system. The histological changes seen in the heart of mice treated with $KBrO_3$ were characterized by the separation of myocardial fibers with inflammatory mononuclear collections, and myocardial necrosis. Supplementation of vanillin in the $KBrO_3$-treated mice improved the histological alterations in $KBrO_3$- intoxicated group and reduced heart injury, which could be attributed to the antioxidant proprieties of vanillin. From the overall results, it was concluded that vanillin exhibited antioxidant and anti-peroxidative properties, which could have a beneficial effect against oxidative heart damage induced by $KBrO_3$. The chemical properties of vanillin in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers and singlet oxygen quenchers predict their antioxidant activity [32].

In conclusion, the effects of vanillin as observed in this study could help to prevent and decrease the degree of myocardial injury in $KBrO_3$- treated mice as well as to improve myocardial function. In view of this, vanillin could potentially be used as a drug to ameliorate cardiomyopathy induced by xenobiotics.

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