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The Effect of Sodium Metabisulphite on Locomotor Activity in the Experimental Model of Parkinson's Disease: The Role of Cyclooxygenase

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Abstract

Parkinson's disease (PD) is characterized by a progressive and selective loss of dopaminergic neurons in substantia nigra (SN). PD is the second most common neurodegenerative disease after Alzheimer disease. Although the exact cause of disease is not known, oxidative stress, neuroinflammation, mitochondrial dysfunction and microglial activation are observed in PD.

The aim of this study was to investigate the mechanism underlying possible toxic effects of sulphite on the experimental model of PD. Male Wistar rats were assigned into one of four groups Control (Control), Sulphite-treated (Sulphite), 6-hydroxydopamine (6-OHDA)-injected (6-OHDA) and sulphite-treated and 6-OHDA-injected (6-OHDA+Sulphite). Sodium metabisulphite was administered at a dose of 100 mg/kg/day for 45 days by gavage. Experimental PD was created stereotactically via the unilateral infusion of 6-OHDA into the medial forebrain bundle (MFB). 6-OHDA-injected rats exhibited reduced locomotor activity compared to control. A significant increase in catalepsy was found in the 6-OHDA-injected group as compared to the the control group. Plasma levels of S-sulfonate increased in Sulphite and 6-OHDA+Sulphite groups as compared to their respective controls. Cyclooxygenase (COX) enzyme activity, prostaglandin E2 (PGE2) and nuclear factor kappa B (NF-κB) levels increased in the 6-OHDA group as compared to control. The tyrosine hydroxylase (TH)-positive immunostainingdecreased significantly in the 6-OHDA-injected group where the sulphite and control groups had almost the same immunoreaction for the dopaminergic neurons. In conclusion, sulphite is not a potentially aggravating factor for the activity of COX and the levels of PGE2 or NF-κB in a 6-OHDA-induced experimental model of Parkinson's disease.

Keywords: Parkinson's disease; Sulphite; Cyclooxygenase; Nuclear factor kappa B; PGE2; 6-OHDA

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer disease (AD) which affects about 1.8% of old people over 65 years of age [1]. Parkinson's motor findings are characterized by slowing movement, tremor, rigidity and deterioration of locomotor coordination. Progression of disease causes walking, swallowing and speech difficulties and impairement of cognitive functions. PD is characterized by the progressive loss of dopamine (DA) and dopaminergic neurons in the substantia nigra (SN) or striatum [2]. The Etiology of PD is not completely clear yet, however several hypotheses to explain neuronal death has been put forward, such as; mitochondrial dysfunction, exitotoxicity and inflammation [3,4]. Besides these hypothesis, there are several studies about oxidative stress [5,6]. Since reactive oxygen species are accumulated more in dopaminergic neurons, it makes these neurons more vulnerable to neural degeneration [7]. Hydrogen peroxide (H_2O_2) and superoxide (O^{2-}) radicals form as a result of enzymatic metabolism of DA. In addition, when the auto-oxidation product of DA combine with iron (Fe), it leads to the formation of DA-Quinones that causes the formation of hydroxyl radicals (OH) [7,8]. Futhermore, the synthesis of DA by Tyrosine hydroxylase (TH) and catabolism by monoamine oxidase (MAO) can result to the formation of H_2O_2 [5,9]. Reactive oxygen species (ROS) have been detected in the SN of patients with PD and in the experimental PD model of animals [9]. Mitochondria which is the main source of ROS contributes to the pathogenesis of PD [10-12]. Increased oxidative stress causes oxidation of proteins, nucleic acids and lipids and leads to cellular damage [13]. The increasement of ROS activates the transcription factors such as nuclear factor kappa-B (NF-κB), which causes pro-inflammatory gene expression, like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [14]. The nuclear translocation of NF-κB (p65) has been shown to increase in the dopaminergic neurons of mice with experimental PD [15]. In addition, COX-2 activity and Prostaglandin E2 (PGE2) levels have been shown to increase and a strong correlation between this increase and neurodegeneration has been demonstrate [16,17].

On the other hand, in food and beverages, sulfur dioxide and various sulphite salts such as sodium metabisulphite and potassium sulphite are used common for a variety of preservative properties such as antimicrobial, antioxidant and leads to delayed undesirable change in color. Sulphite salt react with water leading to production of bisulphite, sulphite and sulphitedioxide [18,19]. Previous studies indicated that ingested sulphite can be absorbed to the circulation and distributed essentially to all body tissues including central nervous system (CNS) [20-22]. Whatever the source, the sulphite, with well-known toxic properties, should be metabolized effectively in the body [23,24]. Sulphite is oxidized by the mitochondrial enzyme sulphite oxidase (SOX) and converted into inorganic sulphate (SO_4^{2-}), which is a harmless compound. SOX activity in tissues varies; for example, while the liver, kidney and the heart have high SOX activity, the brain, spleen

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and the testis have low activity of SOX [25]. The SOX enzyme is at high levels under normal conditions; however, the enzyme concentration or activity may be decreased in aging or in certain disease states [22]. For the first time in 1967, due to the increased endogenous sulphite in individuals with genetically low SOX enzyme levels, severe neurological dysfunction, mental retardation and early death have been demonstrated [26]. If there is a deficiency of SOX or exposure to excessive sulphite, sulphite is catalysed by peroxidases. The one electron oxidation of bisulphite produces the sulphur trioxide radical which causes the formation of superoxide, hydrogen peroxidixe and peroxyl radicals. Sulphite radicals induce lipid peroxidation, mitochondrial damage and DNA injury [22].

Sulphite exposure was found to increase the protein expressions of COX-2 mRNA and COX-2 of the respiratory system cells [27,28]. This finding indicates that this pathway may also have a role in the apoptosis [29]. Sulphite has been shown to increase the levels of PGE2 in various tissues [30,31]. Furthermore, the pro-inflammatory effect of sulphite has been shown and in this effect, NF-κB signal transduction pathway has been indicated as the possible pathway [32].

6-OHDA, which is the specific neurotoxin for dopaminergic neurons, is widely used in experimental PD models [33]. In experimental PD, which is formed with 6-OHDA, it has been shown to cause an increase in COX-2 activity and an increase in the levels of NF-κB [34-37]. However, PGE2 EP1 receptor antagonist has been detected to protect the 6-OHDA-induced neurodegeneration of dopaminergic neurons of rats.

This study aimed to investigate the effect of sulphite diet on experimental PD model induced by 6-OHDA. In the current literature, there is no *in vivo* study investigating the effect of excessive sulphite exposure to contemporary eating habits on the pathogenesis of PD. In order to close this gap in the literature, we determined the subject of our project as the effect of chronic sulphite on the experimental PD model that will be formed with 6-OHDA neurotoxin. Within the scope of this study, to confirm the sulphite diet, plasma levels of S-sulphonate in the experimental animals were measured. Effect of sulphite on locomotor activity was investigated on 6-OHDA-induced experimental PD rats. TH-positive neurons were detected with immunohistochemistry. COX-2 enzyme activity NF-κB and PGE levels were measured. In the literature, the effect of sulphite on locomotor activity and the mechanism of this effect is unknown.

Materials and Methods

Animals

Male wistar rats (3 month old, weighing 250-300 g) were obtained from Akdeniz University Animal Care Unit. The animals were housed in stainless steel cages (5-6 per cage) in an air-conditioned room (22 ± 2°C with a 12:12 hour light:dark cycle). All experimental protocols conducted on rats were performed in accordance with the standards established by the Institutional Animal Care and Use Committee at Akdeniz University Medical School.

Experimental Design

Rats were randomly divided into four experimental groups as follows: control (Control); sulphite- treated (Sulphite); 6-OHDA-injected (6-OHDA), and sulphite-treated + 6-OHDA injected (6-OHDA+Sulphite). Sulphite in the form of sodium metabisulphite ($Na_2S_2O_5$) (100 mg/kg/day nominal dose) was given to the treatment groups for 45 days by gavage [38]. In order to eliminate the effects of daily gavage; other groups received similar volume of water alone. Food and water were provided ad libitum through the experiments [39].

Surgery

At the 38th day of the experimental period lesion surgery was conducted under chloral hydrate (Merck, Darmstadt, Germany) (400 mg/kg, ip.) anesthesia in a stereotaxic frame. 6-hydroxydopamine hydrochloride (6-OHDA-HCl) (Sigma, Steinheim, Germany) was dissolved in 1% ascorbate (Sigma, Steinheim, Germany) saline. For injection, a burr hole was drilled above the injection site and the tip of the syringe lowered into the target. The right medial forebrain bundle (MFB) was lesioned by injection of freshly prepared, 12 µg (freebase) of 6-OHDA-HCl (3 µl of 4 µg/µl solution at 1 µl/min with 3 min for difusion) [40,41] at stereotaxic coordinates AP-2.2, ML ± 1.5 (from bregma) and DV -8.0 below dura [40-42]. After injection the cannula was left in place for three minutes before slowly retracting it. Finally, the skin incision was sutured. All groups of animals continued to receive their usual treatments for additional days until sacrification.

Behavioral tests

Locomotor activity test: Seven days after the injection of 6-OHDA, locomotor activity was measured with an open-field activity monitoring system (MAY 9908 model Activity Monitoring System: Commat Ltd, Turkey) [40,41]. This system had eight Plexiglas cages (42 cm x 42 cm x 30 cm) equipped with infrared photocells. Fifteen photocell emitter and detector pairs were located 4.5 cm above the floor at intervals of 2.5 cm on the both counter sides of each activity cage, and another 15 photocell pairs were located 11.5 cm above the floor. Interruptions of photocell beams were detected by an IBM-compatible computer system, and the location of the animal was calculated by the soft ware at 0.1 s sensitivity. If the calculated locations were completely changed, this was expressed as ambulatory activity. Other behavioral responses that caused interruptions of beams but not changes in location were presented as horizontal activity. Vertical activity, such as rearing, was detected by the photocells located 11.5 cm above the floor. The system also gives the total locomotor activity and the total distance moved. For testing all parameters the rats were placed in the center of the open field and recorded for 5 min. Before placing another animal, the open field was cleaned with 10% ethanol to attenuate odors [24,40,41]

Catalepsy test: To determine the degree of catalepsy, a typical symptom of Parkinsonism, grid test was performed. Each rat was gently hung by the forepaws on a grid (size 56.5 × 23.5 cm; mesh 1 × 1 cm; wire diameter 2 mm). The time taken to actively displace at least one of the paws (descent latency) was noted and taken as a quantitative assessment of degree of catalepsy. Each rat was tested three times on the grid and an average of the three values was taken as descent latency time [42].

Tissue and blood collection

At the end of 6th week, heparinized blood was collected from the abdominal aorta of rats under urethane (Sigma, Steinheim, Germany) anesthesia (1.5 g/kg, ip) and used for determination of plasma S-sulfonate levels. The animals were then killed by exsanguination. For immunohistochemical analyses 6 rats per group were perfused transcardially with 100 ml heparinized phosphate buffered saline (PBS; pH 7.4) followed by 100 ml, 10% formaline (Merck, Darmstadt, Germany). Their brains were carefully removed from the skull and then rapidly and carefully suspended in 10% formaline solution at overnight. For biochemical analyses rats were perfused transcardially with 100 ml heparinized PBS (pH 7.4) and brain tissues were removed immediately.

The obtained SN tissues were flash-frozen in liquid nitrogen and stored at -80°C for later biochemical analysis.

Cyclooxygenase (COX) activity assay

To measure COX activity in SN tissues, COX Activity Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) was used. This kit measures the peroxidase activity of COX. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine at 590 nm. The kit can assay samples containing COX activity between 13 and 63 nmol/min/ml. Results are expressed as U/mg protein [41,43].

Determination of prostaglandin E2 (PGE2) tissue content

Prostaglandin E2 concentration in the SN tissues was measured using Prostaglandin E2 Enzyme Immunoassay (EIA) Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The relative concentration of PGE2 was normalized against total protein content of the tissue samples. Results are expressed as pg/µg protein. [41,43].

Nuclear factor Kappa-B (NF-κB) immunoassay

To measure NF-κB in SN, a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) NF-κBp65 kit (Invitrogen Corporation, CA, USA) was used. A monoclonal antibody specific for NF-κBp65 (regardless of phosphorylation state) has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing NF-κBp65, control specimens, and unknowns, are pipetted into these wells. During the first incubation, the NF-κBp65 antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody, specific for NF-κBp65, is added to the wells. During the second incubation, this antibody serves as a detector by binding to the immobilized NF-κBp65 protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase labeled anti-rabbit IgG (Anti-Rabbit IgG-HRP) is added. A substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of NF-κBp65 present in the original specimen. Results are expressed as ng/mg protein [41,43].

Protein measurements

Protein concentrations were measured at 595 nm by a modified Bradford assay using Coomassie Plus reagent with bovine serum albumin as a standard (Pierce Chemical Company, Rockford, IL) [44].

Immunohistochemistry

We used tyrosine hydroxylase (TH) immunoreactivity to mark dopaminergic neurons in SN of PD model. TH immunohistochemistry, paraffin sections were deparaffinized, rehydrated and blocked for endogenous peroxidase activity with methanol containing 3% H_2O_2 for 15 min and for nonspecific binding with universal blocking reagent (BioGenex, San Ramon, CA, USA) for 10 min at room temperature. mouse anti-TH (sc # 25269) (1/200) were applied for 2 h at room temperature in a humidified chamber. For negative controls the primary antibodies were replaced by appropriate isotype antibodies at the same concentration. After several washing steps in PBS, sections were incubated with biotinylated goat anti-rabbit IgG (1/400 dilution Vector Lab # BA100) or biotinylated horse anti-mouse IgG secondary antibody (1/400 dilution Vector Lab. # BA9200 Burlingame, CA, USA) for 1 h followed by LSAB streptavidin-peroxidase complex (Dako, Carpinteria, CA, USA) incubation for 20 min and were rinsed with PBS. Antibody-antigen complexes were visualized by incubation with diaminobenzidine (DAB) chromogen (BioGenex). Sections were counterstained with Mayer's hematoxylin (Dako), dehydrated, mounted and examined by a Zeiss-Axioplan (Oberkochen, Germany) microscope.

Statistical analysis

Differences in data were analyzed via oneway analysis of variance (ANOVA) followed by Tukey's Post Hoc Test for suit with normal dispersion and Kruskal Wallis followed up Dunn's test for not suit with normal dispersion. P values less than 0.05 were considered significant.

Results

Behavioural tests

Motor activity: Changes in locomotor activity performances are shown in Figure 1. 6-OHDA-injected rats exhibited significantly reduced ambulatory, vertical, horizontal and total locomotor activity as compared to controls. In addition, total distance moved by rats in 6-OHDA-injected group was decreased significantly as compared to control. On the other hand, there were no differences among locomotor activity performance of 6-OHDA+Sulphite group as compared to the 6-OHDA group.

Catalepsy test: The degree of catalepsy is shown in Figure 2. According to the tests, a significant increase in catalepsy was found in 6-OHDA-induced group as compared to control. Moreover, the cataleptic state was not significant in 6-OHDA+Sulphite group when as compared to the 6-OHDA group.

Total cyclooxygenase (COX) activity in the substantia nigra

The mean activation of total COX is shown in Figure 3. Injection of 6-OHDA significantly increased the activation levels of COX in 6-OHDA group. The activity of COX is not significantly changed by sulphite treatment in 6-OHDA+Sulphite group compared to 6-OHDA group.

Prostaglandin E2 (PGE2) tissue content in the substantia nigra

The mean concentration of PGE2 is shown in Figure 4. Injection of 6-OHDA significantly increased the level of PGE2 compared to the control group. The level of PGE2 is not significantly changed by sulphite treatment in 6-OHDA+Sulphite group compared with 6-OHDA group.

Nuclear factor Kappa-B (NF-κB) p65 protein levels in the substantia nigra

NF-κB values are shown in Figure 5. NF-κB protein levels were found to be significantly increased as a result of 6-OHDA injection. However, NF-κB level was unaltered in 6-OHDA+Sulphite group compared to 6-OHDA group.

Tyrosine hydroxylase (TH) immunohistochemistry in the substantia nigra

The number of TH-immunoreactive cells in the 6-OHDA group was significantly lower compared to the control group. However, TH (+) dopaminergic neuron numbers were not significantly altered in 6-OHDA+Sulphite group compared with the 6-OHDA group (Figure 6).

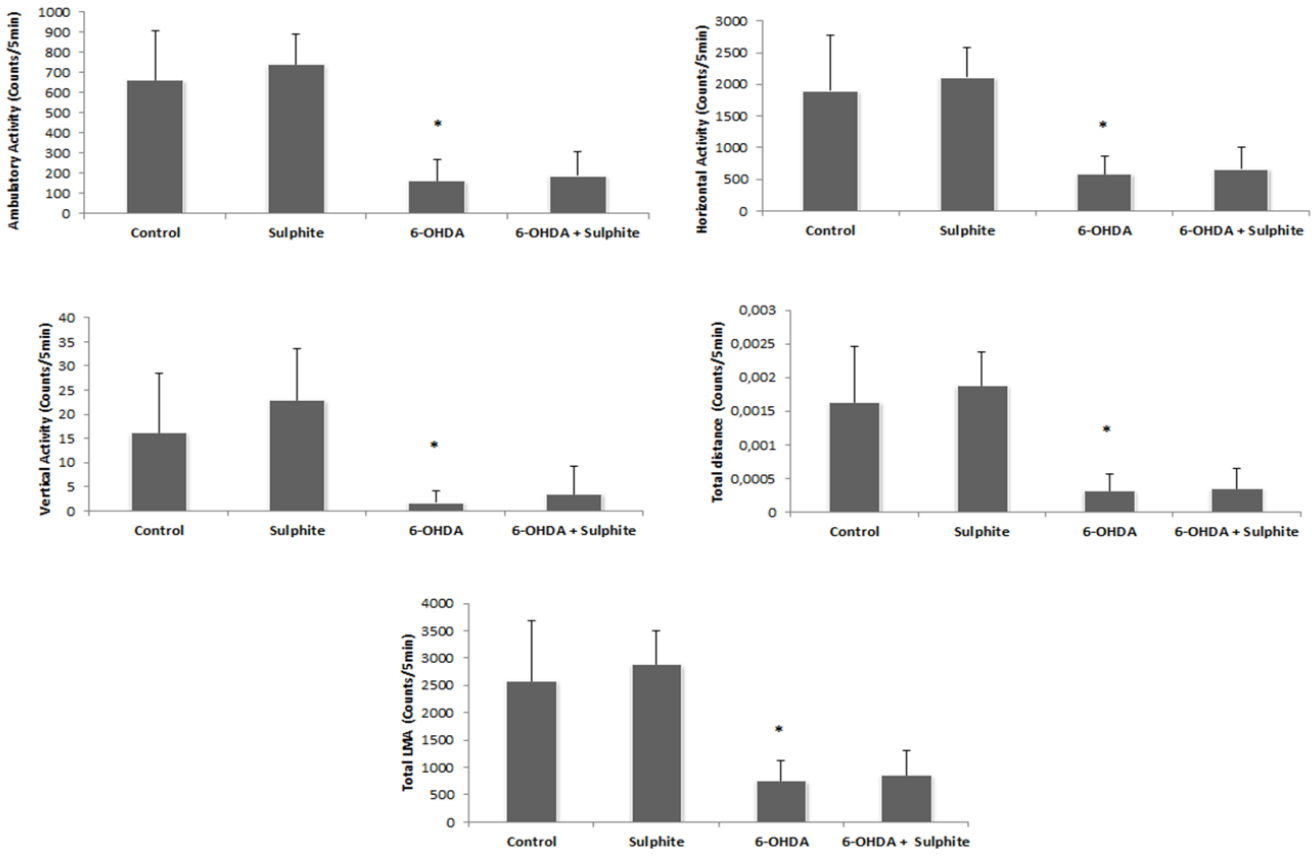

Figure 1: The effect of sodium metabisulphite on locomotor activities: Ambulatory activity (A); Horizontal activity (B); Vertical activity (C); Total distance (D); Total locomotor activity (E) in experimental groups. Data are mean values ± SD. *p <0.05 *vs.* control group.

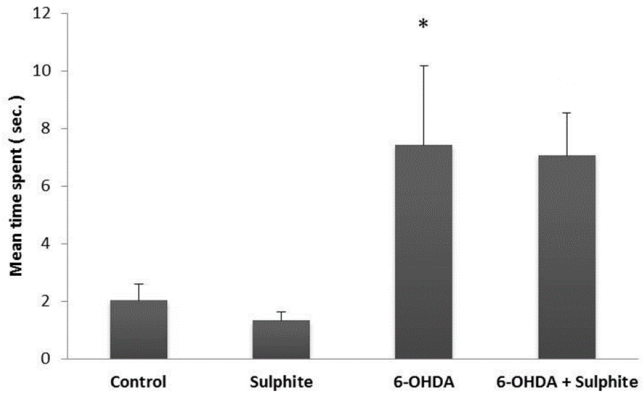

Figure 2: The effect of sodium metabisulphite on catalepsy in experimental groups. Data are mean values ± SD. *p < 0.05 *vs.* control group.

Discussion

The effects of sulphite on locomotor activity and dopaminergic neurons in 6-OHDA injected rats (as an experimental model of PD) were examined in this study. To explore the sulphite's mechanism of effect, we measured PGE2, NF-KB levels and the activity of COX. Since the implementation of experimental PD on human beings is not possible, animal models of PD have been used in the search for clues to the underlying cause of disorder and in the discovery of novel treatments. The neurotoxic chemicals which are used in the animal models of PD are 6-OHDA, MPTP, paraquat, maneb and rotenone [45,46]. For this study, 6-OHDA (a hydroxylated form of dopamine) was the preferred reagent for inducement of the experimental PD. Since 6-OHDA can not pass the blood brain barrier, unilateral medial forebrain bundle lesion model was applied stereotaxically. This model is used to mimic the mid-stage PD [40].

Sulphite and sulphite salts such as sodium metabisulphite are added to foods. These are currently used for a variety of preservative properties that include controlling microbial growth and spoilage. The acceptable daily intake (ADI) for sulphites was established as 0-0.7 mg/kg body weight by The Joint FAO/WHO Expert Committee on Food Additives [47]. The ADI value was based on long-term studies in rats, including a three-generation work of reproductive toxicity, with a NOEL (no-observed-effect level) of 0.25% $Na_2S_2O_5$ in the diet, equivalent to 70 mg/kg body weight per day of sulfur dioxide equivalents [48] By applying the typical 100-fold safety factor, ADI value was determined for humans as 0-0.7 mg/kg. However, the daily intake of sulphite may not be in agreement with this value in many cases. Studies have shown that it is possible to consume 180-200 mg of sulphite from foods and beverages in a single day or meal [18,49]. Considering that many foods such as sausage, dried fruit, beer and wine contain sulphite, the daily level of normal sulphite intake can easily be exceeded.

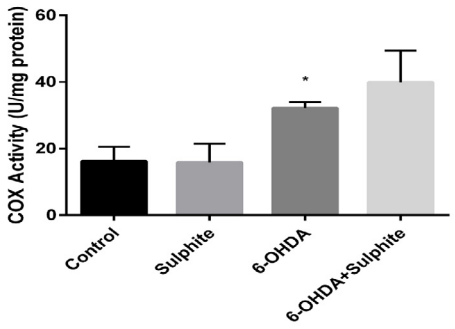

Figure 3: The effect of sodium metabisulphite on COX activity in SN of experimental groups. Data are mean values ± SD. ˚p < 0.05 *vs.* control group.

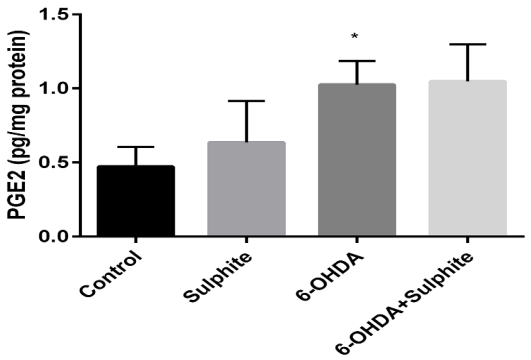

Figure 4: The effect of sodium metabisulphite on PGE2 production in SN of experimental groups. Data are mean values ± SE. *p < 0.05 *vs.* control group.

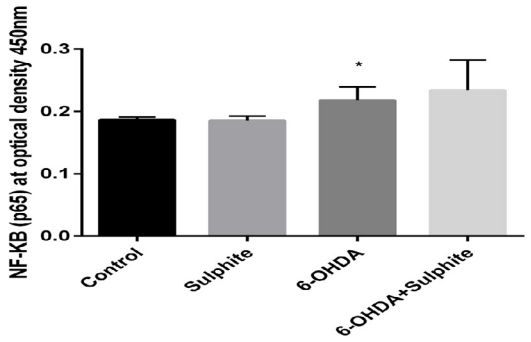

Figure 5: The effect of sodium metabisulphite on NF-κBp65 protein levels in SN of experimental groups. Data are mean values ± SD. ˚p < 0.05 *vs.* control group.

In animals, in order to detect the development of PD, locomotor activity, and the severity of bradykinesia have been studied. In the experimental PD model, locomotor activity is known to be impaired. Administration of sulphite was shown to have no additional worsening effect on motor activity. 6-OHDA causes a particular locomotor activity loss by damaging the dopaminergic neurons. This effect has been suggested to be related to the degree of functional impairment of the dopaminergic neurons and motor performance disorder [50]. Locomotor activity is known to be sensitive to DA transmitter function; an increase in DA is known to cause hyperactivity and a decrease causes hypoactivity [51]. In our study, the locomotor activity was determined as the characteristic parameter of the formed lesion model; therefore, all groups were monitored in the locomotor activity cages. The locomotor activity test includes parameters such as the locomotion rest, stereotypic behaviour and the distance travelled [52,53]. In our study, measurement of the locomotor activity was performed on the 7th day following formation of the experimental PD model. The automatic navigation of the animals in the cages, the ambulatory test, the vertical and horizontal movements, the total locomotor activity and the walking distances have been measured. The number of beams stepped on by the animals in 5 minutes is perceived with photocells and counted with a device automatically. In our study, the locomotor activity test was evaluated and the locomotor activity in the rats with lesion formed with 6-OHDA were observed to decrease which is in accordance with literature [54]. No difference was observed between the 6-OHDA and the 6-OHDA+Sulphite groups.

According to some studies, it has been detected that sulphite treatment increased the motor activity by effecting the sodium current which leads to positive membrane potential. (Vural 2008, DU meng 2004). Previous studies indicated that sulphite administration altered the transmission of excitatory amino acids, which effect the Na channels that cause the enchancement of motor activity [55,56].

Catalepsy is a motor symptom defined as a loss of movement. In our study, the wire test was preferred and the certain catalepsy has been observed after the administration of 6-OHDA. Catalepsy has been suggested to be associated with dopaminergic cell loss. Indeed, catalepsy has been observed with the administration of DA antagonist to the striatum. It was shown that the amount of striatal DA decreased with 6-OHDA-induced dopaminergic neuron loss, and this led to catalepsy. A decrease in DA was observed in 78% of the cases [57]. In the 6-OHDA group, catalepsy was increased, but no difference was observed between the 6-OHDA+Sulphite group and the 6-OHDA group.

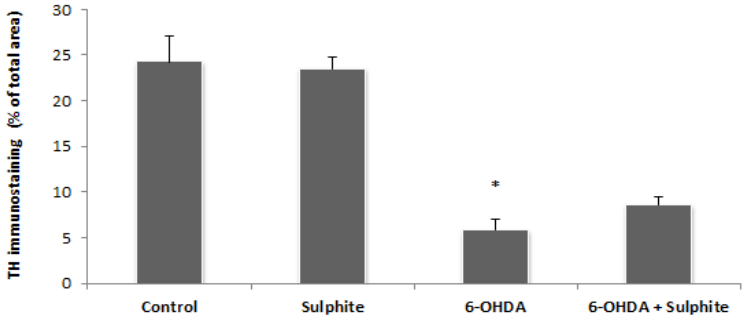

Figure 6: Quantitative values for TH immunostaining. Data are mean values ± SD. ˚p <0.05 *vs.* control group.

We observed an increase of COX-2 in 6-OHDA-treated group in our study. In particular, the COX-2 activity stimulates the inflammation process, thereby causing neurodegeneration. The PGE2 level is seen to increase, depending on the increase in catalytic activity of COX. The catalytic activity of the COX enzyme has been increased in experimental PD models [4,10]. PGE2 reflects the increased COX-2 activity. Measurement of the total COX activity shows the total activity. Regulation of the COX protein expression is processed with NF-κB. The amount of NF-κB p65 in the SN has been determined to increase 24 hours after the administration of 6-OHDA. This information is consistent with the literature [37]. In previous studies, the neurotoxicity that was formed in the dopaminergic neurons in SN has been shown to affect the nuclear location [14-17]. In experimental PD, sulphite has been observed not to have an additional effect.

However, in previous studies, inhalation of SO_2 and high-dose sulphite administration in the hippocampal cell cultures have been shown to increase the COX (COX2-COX1) protein expression, and the levels of PGE2 and NF-κB p65 [27,28,58]. These studies suggested that, SO_2, applied on olfactory cells, did not affect the level of COX. In our study, the absence of any effect of sulphite on these parameters may be due to sulphite administration as sodium metabisulphite through the digestive tract, or due to the inability to reach levels that induce changes in cell cultures. At low doses indeed, it is seen that it has no impact on cell cultures [28,29].

As a result, our findings show that the levels of PGE2, COX and NF-κB is not affected by treatment of sulphite in the mice model of PD. However, these findings do not indicate the absence of the effects of sulphite. Since the activity of SOX enzyme in rats is 20 times higher than in human, sulphite may be detoxified by the SOX enzyme. Therefore, it is necessary to study on animals which have the deficiency of SOX activity in order to predict its effect on human beings.

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