Protective Effect of Vitamin K2 (Menaquinone-7) On Sodium Nitrite-Induced Neuroinflammation Apoptosis and Oxidative Stress in Rats' Brain Tissue

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Abstract

Background: Extensive use of sodium nitrite (NaNO2) as a food additive induces severe side effects, including oxidative stress, fibrogenesis, and apoptosis in active human organs, particularly affecting the brain. Antioxidants are needed to mitigate these effects.

Objectives: This study evaluates oxidative stress markers and the molecular and histopathological changes induced by NaNO2 in rat brains and assesses the presumed protective role of menaquinone-7 (Vit. K2).

Materials and methods: Forty adult rats were divided into four groups of ten each: Group I (control), Group II (30 mg/kg bwt Vit. K2 intake), Group III (60 mg/kg bwt NaNO2 intake), and Group IV (both Vit. K2 and NaNO2). Brain tissues were collected post-treatment to measure oxidative stress markers, fibrosis, and apoptosis. Histopathological examination and immunohistochemical staining for VEGF and caspase 3 were conducted.

Results: Vit. K2 improved body and brain weights compared to NaNO2-intoxicated rats. It reduced fibrosis and apoptosis, significantly increased antioxidant enzymes SOD and CAT, and enhanced total antioxidant capacity (TAC). Vit. K2 restored normal histological structures in the brain. VEGF and caspase-3 immunoreactivity in the brain tissues also shifted from strong positivity with NaNO2 to near normal with Vit. K2.

Conclusion: NaNO2 induces pathological and biochemical changes in the brain, which can be mitigated by Vit. K2. Vit. K2's protective effects are attributed to its antioxidant, antifibrotic, and antiapoptotic activity.

Keywords: Histopathology; Neuroprotection; Antioxidant Therapy; Fibrogenesis Prevention; Apoptosis Inhibition.

DOI: 10.21608/SVUIJM.2024.338424.2025

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Received: 1 October,2024.

Revised: 27 November,2024.

Accepted: 8 December,2024.

Published: 9 December, 2024

Cite this article as: Yasir Hassan Elhassan .(2024). Protective Effect of Vitamin K2 (Menaquinone-7) On Sodium Nitrite-Induced Neuroinflammation Apoptosis and Oxidative Stress in Rats' Brain Tissue. *SVU-International Journal of Medical Sciences*. Vol.7, Issue 2, pp: 892-906.

Introduction

The interaction between the functional status of the liver and brain is already settled, as liver diseases are frequently associated with secondary neurological manifestations such as anxiety and depression **(Huang et al., 2017)**. The most widely endorsed explanation of this relation is that simultaneous brain dysfunction occurs secondary to an acute or chronic liver injury-produced broad spectrum of neurologic toxins **(Jiménez-Torres et al., 2021)**. These circulating neurotoxins are blamed for impaired neurotransmission and subsequent brain pathology (**Wendon et al., 2017**). Hyperammonemia was the most accused inducer as it is the most commonest event trigger of nervous tissue affection through stimulating neuroinflammation and oxidative stress condition in the brain **(Khan et al., 2019)**.

As an example of hepatotoxins, nitrites have numerous uses in numerous human foodstuffs to manufacture unique flavors and colors of preserved food, especially meat, fish, and dairy products **(Ansari et al., 2017; McNally et al., 2016)**. Microorganism in preserved food products, as well as in the environment, provides another source through the ability to synthesize nitrites via nitrifying and denitrifying mechanisms **(Lauer, 1991)**. Even inside the human body, certain bacteria within the digestive tract and during urinary tract infection can synthesize nitrosamines from both secondary amines and nitrite **(Mensinga et al., 2003)**. Similarly, nitrates in a low dose offer a beneficial role as an antidote for poisonous chemicals or as bronchodilators and/or vasodilators **(Lawniczak et al., 2016)**. However, the existence of nitrite in our food-induced toxicities in several organs following exposure to it at lower or higher doses initiating acute or chronic toxicity **(Gui et al., 2016; Kiani et al., 2017)** varied from liver toxicity, respiratory tract impairment, neurological injury, and oxidative stress to more complicated carcinogenicity and mutagenicity **(Zhou et al., 2016)**.

Previous reports certified that nitrate in higher meditations suggestively reduces cellular oxygen and facilitates the overproduction of cellular oxidative radicals with consequent provoked lipid peroxidation, which leads to harmful effects on different tissues, particularly those of the liver and the brain **(Freire et al., 2006; Rocha et al., 2012)**.

Due to the human health issue, a plethora of work has been done, especially in the areas of antioxidants, focusing particularly on natural sources as possible solutions against the toxicity posed by food additives **(Zheng & Wang, 2001)**. Vit. K is a fat-soluble vitamin, which is critical in the blood clotting process. It exists in two primary forms: phylloquinone, or Vit. K1, is abundant in spinach and other green leaves, and menaquinone, or Vit. K2, is made by bacteria and animal tissues and found in dairy products and other fermented foodstuff **(Halder et al., 2019)**. Vit. K1 and K2 are both derived from menadione (2-methyl-1,4-naphthoquinone), with variations in the number of carbons and the number of double bonds present in the 3-polyisoprenoid side chains attached to the core structure **(Bentley et al., 1982)**. Representative types vary with respect to the phenyl units and contain menaquinone-4 (MK-4; also termed Vit. K2), MK-7, MK-8, and MK-9 **(Vos et al., 2012)**.

According to **Yang et al. (2020),** Vit. K is one of the most significant vitamins since it is responsible for the metabolism and synthesis of sphingolipids that are important in numerous brain functions, such as microbial neuron proliferation, differentiation, communication, and aging. Moreover, Vit. K assists in the enzymatic activation of two important proteins that maintain equilibrium in the brain. Gas-6,

which is also known as the growth-arrest specific 6 protein, possesses anti-apoptotic, mitogenic, and myelination activity, while protein S is known to be neuroprotective and promotes the stability of the blood-brain barrier **(Alisi et al., 2019)**.

Although multiple types of research on nitrates proved their toxic effect on the nervous system, there is no approved information regarding whether this effect is direct or secondary to liver or other organ toxicity **(Jiménez-Torres et al., 2021; Jalili et al., 2007)**. In this study, the nitrate induced oxidative, molecular, and histopathological changes in the brain, particularly neuroinflammation, oxidative stress markers, and apoptosis. In addition, the hypothesized protective role of Vit. K2 was acknowledged.

Material and methods

Chemicals

$NaNO_2$ and Vit. K2 were purchased from Aldrich Chemical Co. Inc. (St. Paul Avenue, Wisconsin, USA). Aqueous solutions of the $NaNO_2$ compound were prepared freshly every day. All Chemical kits were obtained from Biosystems S.A. (Spain) and Diamond (Germany).

Experimental Design

A total of 40 healthy male albino Wistar rats weighed 180–200 g was included in this study. Under a controlled environment of 12 h cycle of light and dark cycle at room temperature, the animals were provided with normal basal diets containing 21.1% of protein, 5.1% of fat, 60.0% of carbohydrates, 3.9% of fiber, 7.9% of minerals and 2.0% of vitamins, and water ad libitum throughout the experimental period. All experimental procedures were approved by the Research Ethics Committee at the Faculty of Medicine, Mansoura University, Mansoura, Egypt. The rats were randomly categorized into four groups (n=10).

Group I is the control group; healthy rats were orally given 0.9% saline.

Group II comprised healthy rats that were administered Vit. K2 at a dose of 30 mg/kg body weight orally once per day, five days a week for two months. This dosing regimen and the overall experimental methodology were adapted from a previous study (Zhu et al., 2017).

In Group III, rats were given 60 mg/kg body weight of NaNO2 daily via gastric tube once per day, five days a week for two months.

Group IV received a combination therapy where rats were administered 60 mg/kg body weight of NaNO2 daily along with Vit. K2 at a dosage of 30 mg/kg body weight orally once daily, five days a week, and continued over two months.

At the end of the experiment, the animals underwent weighing before being euthanized with an overdose of thiopental sodium. Blood samples were then collected and centrifuged to separate serum for biochemical evaluations. Immediately following this, the entire brain was dissected, rinsed in a cold-buffered saline solution (0.9%), weighed, and divided into two hemispheres. One hemisphere, which included the frontal cortex and hippocampus, was prepared for biochemical analyses focusing on oxidative stress and assessed using enzyme-linked immunosorbent assay (ELISA). The other hemisphere, containing the frontal cortex and hippocampus, was fixed in buffered formalin for later histopathological and immunohistochemical examinations. Serum and tissue samples from all experimental groups were collected for subsequent histopathological evaluation.

Evaluations of Fibrosis Markers

In this section, collagen and hydroxyproline levels were measured in brain tissues by the following procedures:

Measurement of Hydroxyproline (Hyrox): To measure tissue hydroxyproline, the acid-hydrolysis process outlined by **Gabr et al. (2019)** was employed. Acidified brain tissues were treated with 6 ml of 6 N HCl and heated at 130 degrees Celsius for 3 hours. Neutralization

was achieved using 2.5 N NaOH. Brain tissue samples were then subjected to Chloramine-T oxidation for 20 minutes at room temperature. The oxidation reaction was terminated using 0.4M perchloric acid. Color development was achieved by adding 1 mL of Ehrlich's reagent and heating at 60 degrees Celsius in a water bath for 20 minutes. The resulting mixture was analyzed using a Systronics-2203 ultraviolet spectrophotometer, measuring absorbance at 557 nm. For L-hydroxyproline determination, the quantity was calculated against a standard curve.

Measurement of Hyaluronic Acid (H.A.): The concentration of hyaluronic acid (H.A.) in tissue homogenates from the brain tissues of all rat groups was estimated using an enzyme-linked immunosorbent assay (ELISA) with HA-binding protein (Corgenix), as reported by **Legouffe et al. (2022)**. Samples from treated and untreated rats were neutralized to pH 7.0 using 2.5 N NaOH before undergoing Chloramine-T oxidation for 20 minutes at room temperature. After five minutes, the reaction was stopped with 0.4 M perchloric acid. Subsequently, 1 mL of Ehrlich's reagent was added, and the tubes were shaken and placed in a water bath at 60°C for 20 minutes to develop color. After cooling under running tap water for five minutes, absorbance was measured at 557 nm using a Systronics-2203 ultraviolet spectrophotometer.

Assessment of oxidant-antioxidant status

The homogenates of brain tissues of all control, Vit. K2 $NaNO_2$ and Vit. K2- $NaNO_2$ rats were subjected for the estimation of oxidative stress and antioxidant system markers, including malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), and total antioxidant capacity (TAC) levels as previously analyzed **(Gabr et al., 2019; Legouffe et al., 2022**).

Assessment of apoptosis

Bcl-2 concentrations were determined in brain tissues of all control, Vit. K2 $NaNO_2$ and Vit. K2- $NaNO_2$ groups using a commercially available, non-isotropic, enzyme-linked immunosorbent assay (Oncogene Research Products, bcl-2 ELISA, Cat#QIA23) and an enzyme-linked immunosorbent sandwich assay Kit (Zymed® Cytochrome c ELISA Kit Cat. No. 99-0040) was used to identify cytochrome c concentrations in the brain homogenates as well **(Sun et al., 1988)**. In addition, commercially available colorimetric kits (Gen Script, Piscataway, NJ, USA) were used to identify caspase-3 enzyme activity in the brain tissues **(Al-Gayyar et al., 2016).**

Histopathological Examination of brain tissues

The brain samples were fixed in 10% buffered formaldehyde, and a rotary microtome was used to collect five micrometer-thick paraffin sections. Paraffin sections were processed by the routine methodology and stained with hematoxylin and eosin (H&E) and immunohistochemical staining for detection of vascular endothelial growth factor (VEGF) and caspase 3. Using the strept avidin-biotin technique, Paraffin sections were deparaffinized in xylene, rehydrated, rinsed in tap water, and embedded in 3% Hydrogen peroxide (H_2O_2) in phosphate buffer solution (PBS) for 10 min to block endogenous peroxidase. Sections were incubated overnight at 4°C with the respective primary antibodies. A monoclonal mouse anti-human antibody was used to detect VEGF-A (Clone No. SP28, Catalog No. M3281, Spring Bioscience, ready to use). A polyclonal rabbit anti-active caspase-3 (Clone No C92-05, Catalog No 55955, PharMingen, San Diego, CA, at 1:500 dilution) was used to detect of caspase-3. Following incubation with primary antibodies, the sections were then incubated with the appropriate secondary antibody; A biotinylated anti-mouse IgG (LSAB™2 Kit; Dako) for VEGF and a biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) for caspase 3. Sections were incubated for 30 min at room temperature in horseradish peroxidase-avidin-

biotin complex (Vectastain Elite, Vector, CA), then 3,3'-diaminobenzidine in H2O2 (DAB kit, Vector, CA), was added to visualize the reaction as a brown, insoluble product. Sections were then counterstained with hematoxylin and mounted. Negative control sections were obtained following the same staining steps but without incubation with the primary antibodies.

Statistical analysis

All data were expressed as Mean ± Standard Deviations (S.D.). The results obtained were statistically analyzed by GraphPad Prism (version 7). In addition, a one-way ANOVA test followed by Tukey's post hoc analysis was applied to compare and identify the significance between groups. The statistical significance was assigned at p-value <0.05.

Results

Body and brain weight

Table 1 presents the impact of NaNO2 and Vit. K2 on the experimental animal groups' relative body and brain weights. In the group treated with NaNO2 at a dose of 60 g/bwt, there was a statistically significant ($P < 0.05$) reduction in body weight and increased brain weight compared to the normal control group. Conversely, administering Vit. K2 at 30 mg/kg body weight to rats in the NaNO2-treated group (GIV) notably ($P < 0.001$) brought the body and brain weights back toward normal levels when compared to those treated with NaNO2 alone. Meanwhile, normal rats received Vit. K2 (GII) exhibited a non-significant ($P < 0.01$) increase in body and brain weights relative to the control group (**Table.1**).

Table 1. Rat body and brain weights

Groups	Body	Brain
GI	18.6±4.1	0.89±0.05
GII	18.4±3.7	0.86±0.04
GIII	12.4±2.5[a]	1.8±0.5[a]
GIV	15.5±3.7[b]	1.2±0.3[b]

Values are represented as mean ± SD, n=10. [a] $P < 0.05$ (GIII vs GI). [b] $P < 0.01$ (GIV vs GI).

Effect of Vit. K2 administration on tissue oxidant-antioxidant status

Table 2 highlights the oxidative stress markers used to assess the free radical initiation activity in NaNO2-induced cellular toxicity in brain tissues, including MDA, NO, SOD, CAT, and total antioxidant capacity (TAC). These parameters have an inverse relationship in comparison. In the NaNO2-treated rats, a significant increase ($P < 0.01$) in MDA and NO levels and a significant decrease ($P < 0.01$) in antioxidant activities, such as SOD, CAT, and cellular TAC, were observed when compared to the control and normal groups treated with Vit. K2 (see table 2). However, the oxidative damage effects induced by NaNO2 in brain tissues were mitigated following Vit. K2 administration in group IV rats (refer to table 2). The protective effect of Vit. K2 against oxidative damage caused by NaNO2 toxicity was corroborated by a significant increase ($P < 0.001$) in antioxidants, including SOD, CAT, and cellular TAC, and a decrease ($P < 0.001$) in MDA and NO levels in the brains of rats treated with both Vit. K2 and NaNO2, compared to the group exposed to NaNO2 alone (**Table. 2**).

Table 2. Effect of Vit. K2 on brain tissue oxidant-antioxidant status

Groups	Oxidative stress markers				
	MDA (nmol/g.tissue)	NO (m M/L)	SOD (U/g.tissue)	CAT (U/g.tissue)	TAC (nmol/mM Trolox eq.)
GI	3.1 ± 0.21	1.3± 0.04	45.3 ± 2.3	825.8± 12.6	31.3± 3.7
GII	8.1 ± 0.85[b]	3.8± 0.6[b]	18.7 ± 1.2[b]	345.3± 4.5[b]	10.6± 2.1[b]

GIII	2.5 ± 0.32[a]	0.85± 0.02[a]	49.3 ± 2.8[a]	865.3± 14.7[a]	35.6± 4.2[a]
GIV	4.3 ± 0.65[c]	1.6± 0.05[c]	35.7 ± 1.8[c]	696.5± 7.2[c]	25.7± 5.7[c]

Values are represented as mean ± SD, n=10. [a]P < 0.05 (GIII vs GI). [b] P < 0.01 (GII vs GI &GIII). [c] P < 0.001(GIV vs GII). MDA: malondialdehyde, NO: nitric oxide, SOD: superoxide dismutase, CAT: catalase, and TAC: total antioxidant capacity.

Effect of Vit. K2 administration on tissue fibrosis of the brain

The effect of Vit. K2 on the brain levels of fibrotic markers in $NaNO_2$-exposed rats at doses of 60mg/kg bwt for 2 months were identified (**Fig.1**). The results showed that the treatment with Vit. K2 significantly reduced the expression levels of both hydroxyproline (hydrox.) and hyaluronic acid (H.A.) compared to $NaNO_2$-treated rats with higher expression amounts of hydrox. and H.A., respectively, in the brain tissues (**Fig.1A&B**). In addition, when normal healthy rats were treated with Vit. K2 for 2 months (GII), the brain tissues showed a significant reduction in the expression levels of fibrotic markers hydrox. and H.A., which recommends the protective activity of Vit. K2 against toxicants in foods such as food additives ($NaNO_2$), proceeded via an antifibrotic mechanism.

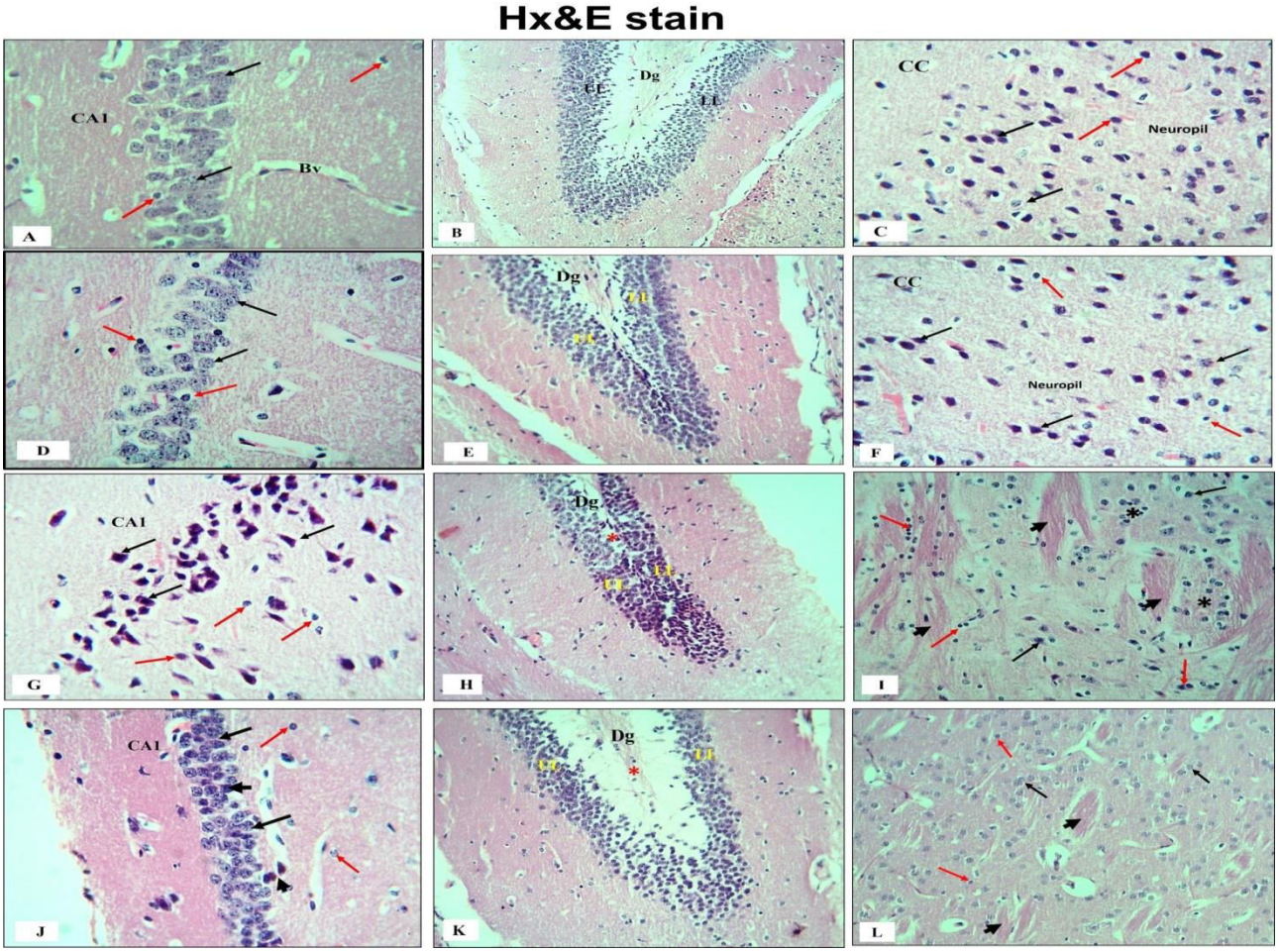

Fig. 1: A photomicrograph of GI, (A, B &C) and GII (D, E & F): A & D show normal shape arrangement and distribution of nerve cells (black arrow) which contain rounded and vesicular nuclei and glial cells (red arrows) identified by their smaller size and small rounded dark nuclei in the area A1 (CA1) of the hippocampus. B & E show the normal shape and distribution of the cells of upper (UL) and lower limbs (LL) of dentate gyrus (Dg). C & F show the normal cells and neuropil of cerebral cortex (CC). In GIII (G, H & I); G: CA1 area shows apparent reduction in its

thickness, most of its nerve cells are apoptotic with small size and small deeply stained nuclei with apparent increase in the number of glial cells. In H: both two limbs of the dentate gyrus show reduction in their thickness and shrink in the neural tissue in between them (red star). In I: Numerous areas of gliosis (arrow heads) and neuronophagia (black stars) are seen in the tissue of CC. An apparent reduction of nerve cells and an increase of glial cells is evident. In Group IV (J, K & L); (J): Preservation of the thickness of CA1 and shape of most nerve cells with fewer number of apoptotic nerve cells (arrow heads). (K): shows nearly normal thickness, shape of cells of dentate gyrus and amount of neural tissue between both limbs. Fewer areas of gliosis and preservation of the number of nerve cells in relation to number of glial cells in CC tissue are observed in (L). (Hematoxylin and stain; A, C, D, F, G & J X400; B, E, H, I, K & LX200)

Effect of Vit. K2 administration on tissue apoptosis of the brain

The potential effect of K2 administration on tissue apoptosis induced by $NaNO_2$ toxicity was estimated by evaluating the expression ratios of antiapoptotic bcl-2, cytochrome c oxidase, and capase-3 markers in the brain tissues (**Fig.2**). In sod. nitrite-treated ($NaNO_2$) rats, bcl-2 and cytochrome c oxidase (cyt. c) significantly reduced and caspase-3 significantly upregulated (increased) in the tissues of the brain (**Fig. 2B**) compared to healthy control and Vit. K2(GII) rats respectively. However, the expression of Cyto c and bcl-2 significantly increased, and the expression of caspase-3 significantly reduced in the tissues of the brain when $NaNO_2$-intoxicated rats were treated with Vit. K2 (**Fig.2A&B**). These findings significantly support the antioxidant as well as anti-apoptotic activity of Vit. K2 against $NaNO_2$ intoxication of the brain tissues.

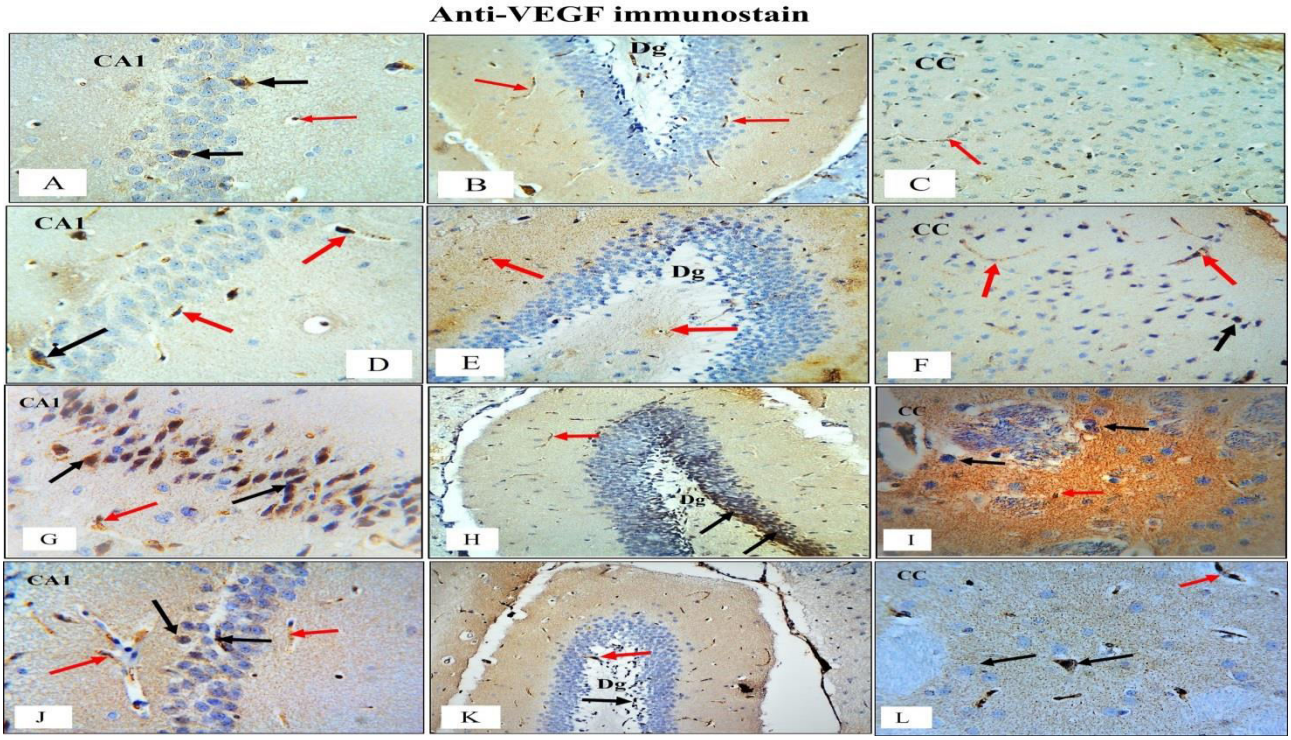

Fig.2. A photomicrograph of anti-VEGF immunostained sections in GI ; (A, B & C) and GII (D, E & F) show nearly negative reaction in nerve cells and positive immunoreaction in the endothelial cells of blood capillaries and few nerve cells in the CA1& Dg of hippocampus and

cerebral cortex (CC). In GIII (G, H & I), marked positive immunoreaction is detected in most nerve cells and endothelial cells of blood capillaries in CA1, Dg and CC. GIV (J, K & L) shows fewer VEGF immunopositive cells in CA1, Dg and CC in comparison with some sporadic positive endothelial cells. (CA1: area 1; Dg: dentate gyrus, CC: cerebral cortex, black arrows: nerve cells, red arrows: blood capillaries). (Anti-VEGF immunostained; A, D, G, I, J & L X400; B, E, H & K X 100; C & F X200)

Histopathological results.

Hematoxylin and Eosin stain (**Fig.1**): Sections of G.I. and II hippocampi, dentate gyri, and cerebral cortices showed normal shape, arrangement, and distribution of nerve glial cells in area A1 of the hippocampus. Normal shape and distribution of the cells of upper and lower limbs of the dentate gyrus and cells and neuropil of the cerebral cortex. GIII, on the other hand, showed the hippocampal CA1 area's reduction in its thickness, with most of its nerve cells being apoptotic with small size and small, deeply stained nuclei. An evident increase in the number of glial cells was detected. The two limbs of the dentate gyrus showed a reduction in their thickness and shrinkage in the neural tissue between them. The cerebral cortices showed numerous areas of gliosis and neuronophagia with an apparent reduction of nerve cell number and an increase of glial cells. GIV, on the other hand, exhibited preservation of the thickness of hippocampi with few apoptotic nerve cells. The dentate gyrus appeared with normal thickness of both limbs. Fewer areas of gliosis with apparent preservation of the number of nerve cells were also observed.

Immunohistochemical staining

Anti-VEGF immunostained sections of the brain were done (**Fig.2**). G.I. and II showed a nearly negative reaction in hippocampi, dentate gyri, and cerebral cortices nerve cells and a positive reaction in the endothelium of the capillaries with few positive nerve cells in the hippocampus and cerebral cortex. GIII, on the reverse, showed marked positive immunoreaction in most of the neurons and endothelium. **GIV** showed fewer VEGF Immunopositive cells in comparison to the $NaNO_2$ group in addition to the positive endothelial cells.

Anti-caspase-3 immunostained sections of hippocampi, dentate gyri, and cerebral cortices.

G.I. and GII showed a nearly negative reaction in all areas. GIII had a marked positive reaction in the neuronal nuclei of all regions. At the same time, GIV showed fewer positive cells in the three regions (**Fig.3**).

Discussion

The cellular toxicity caused by $NaNO_2$ in this study, which dramatically increased cellular oxidative stress, fibrosis, and apoptosis in the brain tissues, was significantly mitigated by Vit. K2. $NaNO_2$ administration caused a considerable loss in body weight and increased brain weight, which may be related to the toxicant's cellular toxicity. This outcome is consistent with earlier findings **(Helal & Soliman, 2008; Hunault et al., 2009; Hassan et al., 2013)**. The concurrently rising protein catabolism was related to reduced body weight **(Helal et al., 2020)**. The concurrent decrease in serum and tissue proteins supported this view. On the other hand, the nitrite's vasodilatory function, which was explained by several mechanisms, including the conversion of nitrite to nitric oxide, maybe the reason for the rise in brain weight **(Jung et al., 2006)**.

However, when NaNO2-intoxicated rats were treated with Vit. K2, a considerable increase in body and brain weights was seen, indicating Vit. K2's antagonistic activity. Multiple studies reported the benefits of Vit. K2's antioxidant action, Menaquinone-7, shields osteoblasts from oxidative damage, promoting osteoblast proliferation,

differentiation, and mineralization **(Mehta et al., 2021)**. Similarly, it increases myelin synthesis with subsequent enhanced repair in the central and peripheral nervous systems **(Carrie et al., 2004)**.

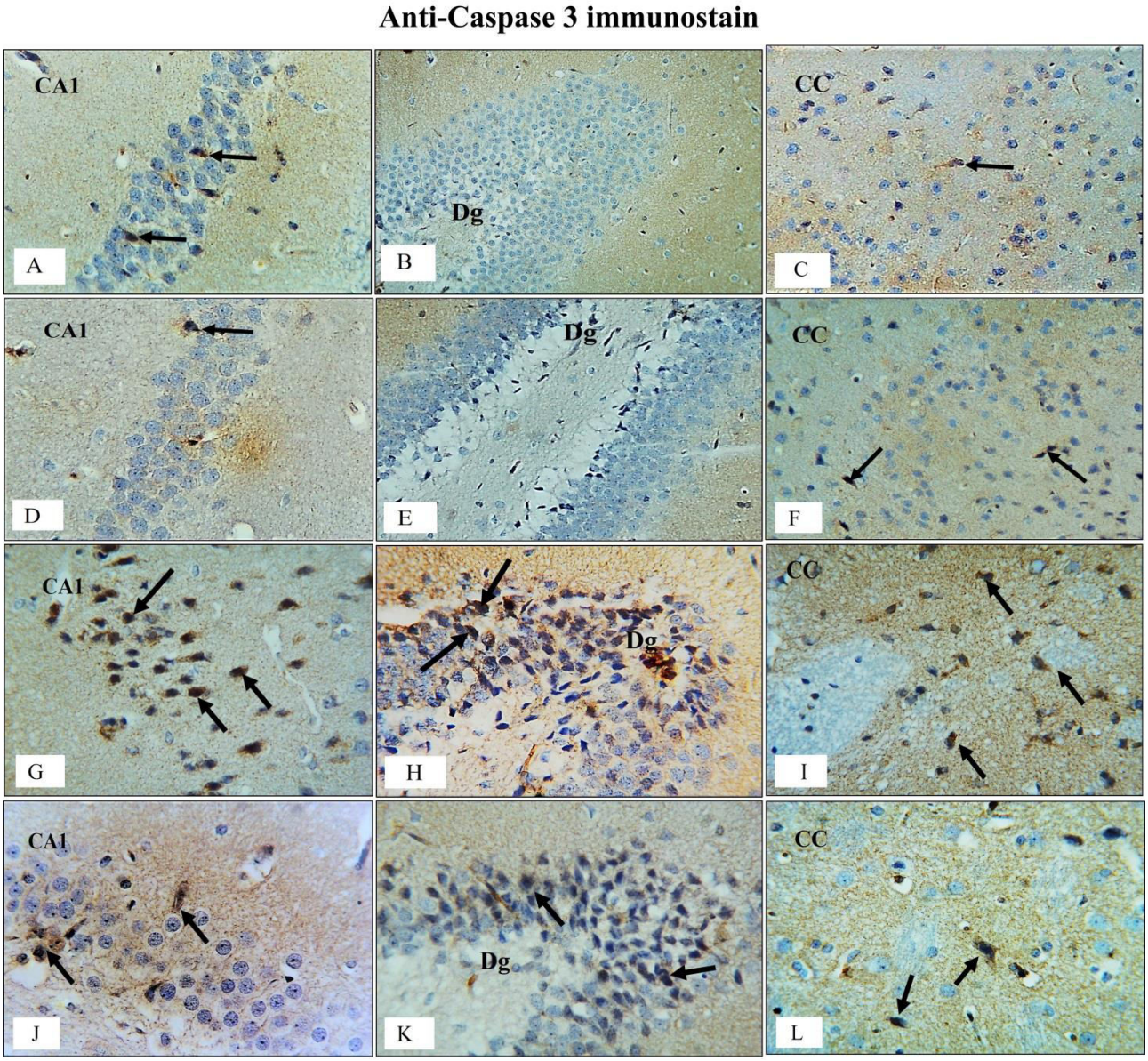

Fig.3: A photomicrograph of anti-caspase 3 immunostained sections in GI ; (A, B & C) and GII (D, E & F) show nearly negative reaction in all areas; area 1 (CA1), dentate gyrus (Dg) and cerebral cortex (CC) except one or two caspase3- immunopositive cells. In GIII, marked positive reaction is detected in nuclei of most nerve cells in CA1, Dg and CC whereas, GIV shows few positive nerve cells in comparison. (Black arrows refer to immunopositive nerve cells). (Anti-caspase 3- immune stain; A, D, G, H, I, J, K & LX400; B, C, E & F X200)

This study showed that Menaquinone-7 has a substantial antioxidant effect against NaNO2 toxicity in the brain tissues. The results demonstrated that it significantly activates cellular oxidative stress through increased MDA and NO expression, decreased activity of antioxidant enzymes, SOD and CAT, and reduced the cellular total

antioxidant capacities of the injured tissues; conversely, with Vit. K2, the antioxidant activity of the enzymes SOD and CAT is markedly increased, and the free radicals responsible for cellular oxidative stress, MDA and NO, are inhibited and returned to normal levels.

Thorough investigations inspected the antioxidant activity of Vit. K2 as a natural antioxidant. One is that it prevents the body from producing ROS by inhibiting the activation of 12-lipoxygenase in arachidonic acid-induced oxidative damage to developing oligodendrocytes **(Li et al., 2003)**. Additionally, it prevents glutathione depletion-induced oxidative damage and the production of ROS in growing oligodendrocytes and juvenile neurons **(Li et al., 2022)**. According to other studies, it serves as a mitochondrial electron carrier to safeguard compromised mitochondrial function and maintain regular ATP synthesis within the electron-transport chain in the mitochondria **(Yang et al., 2020)**. According to a different study, it can again balance ROS production and correct mitochondrial dysfunction by enhancing ATP production **(Tang et al., 2022)**.

Similar to our findings, earlier research revealed that the widespread use of considerably exposed our bodies to several long-term negative effects. According to these studies, rats exposed to nitrite had dramatically increased expression of the fibrosis markers MTC, TGF, and SMA, as well as excessive collagen deposition **(Al-Gayyar et al., 2016; Sherif et al., 2013)**.

In the current study, the levels of hydroxyproline and hyaluronic acid, fibrotic markers, increased in the brain tissues of rats given $NaNO_2$ intoxication. This finding coincided with the histological finding of gliosis in the brain tissues, particularly the frontal cortex. Similar to other natural antioxidants, Vit. K2 therapy significantly decreased the production of fibrosis-related indicators to near-normal levels. Neuroinflammation and chronic glial hyperactivation are important events in neurodegeneration **(Kaur et al., 2019; Hanslik et al., 2020)**.

When microglia are activated, they excessively produce cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), and interleukin 6, setting off an inflammatory cascade. Oxidative stress is a significant factor causing microglial activation, among other mediators **(Sánchez-Sarasúa et al., 2020).** Studies have shown that Vit. K2 can mitigate microglial inflammation in mouse microglia-derived MG6 cells when these cells are exposed to LPS (Lipopolysaccharides) **(Saputra et al., 2019).** Similarly, preconditioning with Vit. K2 has been found to suppress NF-κB signaling and decrease the production of inflammatory cytokines TNF-α, IL-1β, and IL-6. It also reversed the increase of proinflammatory cytokines triggered by glial activation in rat astrocytes **(Yang et al., 2020).**

The expression of apoptosis-related proteins (bcl-2, cytochrome c, and caspase-3 as markers of brain cellular apoptosis showed a significantly increased compared to $NaNO_2$-intoxicated rats.

Bcl-2, an anti-apoptotic protein associated with the mitochondrial outer membrane, plays a crucial role in inhibiting the release of Cytochrome C. Bax, on the other hand, is a pro-apoptotic protein that can compromise the integrity of the mitochondrial outer membrane, facilitating Cytochrome C's exit from the mitochondria. Bax can interact with Bcl-2 and suppress its function. Therefore, the ratio of Bax to Bcl-2 is a key determinant of a cell's vulnerability to apoptotic signals (Elmore, 2007). Research indicates that Bcl-2 is significantly reduced, while Bax levels increase in sensitive regions such as the hippocampus **(Wang et al., 2017)**.

Alternatively, suppressing Bax expression can shield cells from apoptosis **(Elmore, 2007).**

Our research aligns with earlier studies showing that Vit. K2 reduces the levels of the pro-apoptotic protein Bax in osteoblasts by elevating the Gas6 protein **(Villa et al., 2017).** Injection of Vit. K2 can potentially prevent ROS generation and calcium influx **(Ferland et al., 2012)**. Moreover, the anti-inflammatory aspects of Vit. K2 decreased the Bax/Bcl2 ratio and blocked necrosis and apoptosis **(Mizushina et al., 2011).**

This study demonstrated that NaNO2caused definite histopathological changes in the cerebral cortex and area A1 of the hippocampus, which were collectively characterized by a reduction in hippocampal neuronal cellular density and an increase in VEGF expression in neurons, astrocytes and vascular endothelium in the CA1 of the hippocampus and cerebral cortex, notably in areas of glial scarring and gliosis. Recent studies have proved the localization of VEGF and its receptors in neurons and astrocytes **(Rosenstein et al., 2004)**. The result that hypoxia upregulates VEGF expression in this study supported the hypoxic mechanism of sodium nitrite-induced neurotoxicity **(Neufeld et al., 1999)**. When cerebral microvascular angiogenesis in a condition of hypoxia is examined at the cellular level, it is discovered that it can be found that vascular endothelial cells are regulated by a variety of surrounding cells **(Teng et al., 2008)**, one of which is astrocytes cells. The hypoxia-regulated nature of VEGF makes it an important neovascularization factor, which can specifically bind to vascular endothelial cells and promote the growth of endothelial cells. It has been found that both hypoxia and astrocytes can promote VEGF expression **(Sinor et al., 1998; Rattner et 2019)**.

In this study, the encountered astrogliosis of the cerebral cortex and hippocampus that showed strong expression for VEGF may be attributed partly to neuronal loss in different areas of the cerebrum and hippocampus as a part of the reparative process to form glial scar or, as a consequence for increased expression of VEGF as recorded by Krum et al., **(Krum et al., 2002)** who proved that VEGF is considered as a very potent mitogen for astrocytes. Another theory is that astrogliosis results from hypoxia, as shown by Ridet et al. **(Ridet et al., 1997)**, who reported that hypoxia and ischemia activate and enhance the proliferation of resident astrocytes in vivo.

Rats treated with Vit. K2 to protect them against $NaNO_2$ toxicity showed improvements in the cerebral cortex and hippocampal structure, which were likewise linked to a decline in VEGF expression in the brain cells. The mechanism by which Vit. K2 act to decrease neuronal damage and decrease VEGF expression could be explained by the reported antagonizing effect of Vit. K2 on the upregulation of proinflammatory cytokines caused by glial activation in rat astrocytes **(Yang et al., 2020).**

An associated investigation found that hypoxia-induced IL-6 and TNF-α secretion were decreased in astrocytes pretreated with Vit. K2 regardless of their normal or hypoxic culture conditions. The anti-hypoxic effect of Vit. K2 was also proved in astrocytes that were exposed to hypoxia as its reactivity towards oxygen species (ROS) was reduced **(Hadipour et al., 2020; Huang et al., 2021).** Both studies got the same conclusion despite using different glial cell types, Vit. K2 forms, and activation techniques, that Vit. K2 diminished the level of proinflammatory cytokines. Results obtained point out the potential of Vit. K2 in the alleviation of neuroinflammation and neurodegeneration.

Conclusion

The results of this study show that the dietary supplement NaNO2 accumulated mostly in cells, where it initiated a series of actions that produced reactive oxygen species and activated a number of cellular processes,

including fibrogenesis, apoptosis, and a decrease in cellular antioxidant systems. As a result, the brain tissues experience fibrosis and apoptosis. Due to its antioxidant, antifibrotic, anti-apoptotic, and antiangiogenic action, Vit. K2 appears to have a more substantial neuroprotective effect in NaNO2-induced brain injury.

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