Comparative Neuropathological Study of Sodium nitrite and Nisin Exposed Rats With Special Reference to Expression of Glial Fibrillar Acidic Protein and Vascular Endothelial Growth Factor

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ABSTRACT— The present study was performed to evaluate the neurotoxicity of sodium nitrite (NaNO₂) and nisin in cerebral cortex and hippocampus. A total of 27 adult female rats were divided into three groups, control group, sodium nitrite and nisin treated groups with 9 rats per each. The rats of groups 2 and 3 were given orally 30 mg/kg body weight and 50 mg/kg b.w daily of NaNO₂ and nisin respectively for 2 months by gastric intubation while rats in control group were given orally 0.9% saline. Histopathological examination of brain was performed, in addition to immune histochemical staining for glial fibrillar acidic protein (GFAP) and vascular endothelial growth factor (VEGF) were applied on brain sections of both treated and control groups. Results revealed various histopathological alterations that were more evident in deep cerebral grey matter and white matter including neuronal degeneration, astrogliosis and vascular changes in addition to reduction in cellular mass in different regions of hippocampus was detected. There was no recognized histopathological changes in nisin treated group. Increase expression of GFAP & VEGF in NaNO₂-treated group was evident compared with nisin and control groups. Conclusion: sodium nitrite has neurotoxic potential via inducing histopathological alterations that were evident in deep cerebral cortex and different region of hippocampus with increased expression of GFAP & VEGF while the nisin is non neurotoxic agent.

Key words: albino rats, sodium nitrite, , nisin, neurotoxicity, histopathology , GFAP, VEGF , immunohistochemistry.

INTRODUCTION

Sodium nitrite is one of synthetic food preservatives that become popularly used in various feed stuff in addition to biomedical application as in post hemorrhagic cerebral vasospasm (Pluta et al, 2005) and in myocardial infarction (Webb et al, 2004). Health hazards have encountered with sodium nitrite consumption. Sodium nitrates cause neurotoxicity via induction of brain hypoxia (Zaidi, 2010), exerts oxidative stress and retrogrades the endogenous antioxidants (Hassan et al, 2010). In such concern role of hypoxia and oxidative stress induced neurotoxicity are well established.

The structural and functional integrity of brain depends on regular oxygen supply (Acker T & Acker H, 2004) although the brain represents only about 2% of the body's weight, it utilizes about 20% of the body’s oxygen. As a result, the brain is especially sensitive to hypoxia. Symptoms of mild cerebral hypoxia include inattentiveness, poor judgment, memory loss, and a decrease in
motor coordination. Brain tissue exposed to the hypoxic insult responds by glycolysis, angiogenesis, vasodilatation and erythropoiesis (Harris, 2002). Vulnerability of brain tissue to hypoxia is variable. Hypoxia is found to be a potent inducer of vascular endothelial growth factor (VEGF) expression in several organs including the CNS (Minchenko et al, 1994). Hypoxia is also activates and promotes proliferation of resident astrocytes in vivo (Ridet, 1997), a process often referred to as astrogliosis or glial scarring. It is known that astrocytes are more resistant to oxidative stress than neurons due to compensation for a lack of oxygen by switching to glycolytic metabolism (Marrif H & Juurlink, 1999) that allows the constant ATP levels required to ensure cell viability during stress (Hochachka & Lutz, 2001). The glial fibrillar acidic protein (GFAP) is a sensitive and specific biomarker for astrocytes (O’Callaghan, 1991).

Nisin is a natural food preservative produced by Lactococcus lactis. It is of wide inhibition effect on Gram positive bacteria, and is widely accepted as a safe biological preservative. Nisin reduces chemical preservatives concentration required (Mobasseri et al, 2009). Also, it has biomedical application as dental care products (Turner et al, 2004) and shown to be effective in the treatment of head and neck squamous cell carcinoma (Ritchie et al, 2012). Previous studies suggested that nisin did not affect the normal physiology of body organs and had no adverse effects on the reproductive performance in nisin-treated male rats and their offspring (Gupta et al., 2008 and Reddy et al., 2012). The present study is designed to provide an new point of view in explaining the role of sodium nitrite induced neurotoxicity regarding immunohistochemical expression of glial fibrillar acidic protein and vascular endothelial growth factor in cerebral cortex and different region of hippocampus as well as to establish a comparative histopathological study between sodium nitrite and nisin used as synthetic and natural food preservatives respectively.

MATERIALS AND METHODS

Animals: A total of twenty seven female albino rats weighing about 100-120 g were used in the present study. They were obtained from the animal house belong to Ophthalmology research Institute, Giza, Egypt. Animals were fed on basal diet and watered ad-libitum. Rats were left for two weeks for acclimatization before starting the experiment. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

Chemicals:

NaNO₂ (El Gomhoria Company,Cairo,Egypt) was applied as a freshly prepared solution.

Nisin from Lactococcus lactis (N5764-1GP code:101183002) (Sigma–Aldrich, St. Louis, MO). That was dissolved in 0.9% saline.

Glial fibrillar acidic protein (GFAP) and vascular endothelial growth factor (VEGF) (catalog MA1-166629,Thermo Scientific Co., UK)

Experimental design: Rats were divided into three groups with nine rats per each: G1-control groups that were received standard diet without any treatment and the orally given 0.9% saline. G2-NaNO₂-treated group; received standard diet and given orally sodium nitrite at a dose of 30 mg/kg body weight daily for 2 months as previously described by Isyaku and Joseph, (2011) and G3- nisin treated group at dose of 50mg/kg b.w that was dissolved in 0.9 % saline orally by gavages daily for 2 months as
described previously by Reddy et al,(2012).

At the end of the experimental period animals were euthanized by cervical dislocation and brains were dissected.

**Histopathological and immunohistochemical examination:**

Brain tissue specimens were collected and preserved in 10% neutral buffered formalin then routinely dehydrated in different grades of alcohol, cleared in xylene, embedded in paraffin, sectioned with microtome at 5µ thickness and finally stained with hematoxylin and eosin (H&E) according to Bancroft and M. Gamble, (2008). On the other hand, selected tissue sections were dewaxed in xylene, rehydrated and pretreated with 3 % hydrogen peroxide for blocking the activity of endogenous peroxidase. Microwave as sited antigen retrieval was done for 20 minutes. Sections were incubated overnight at 40 C° with primary antibody for Glia fibrillar acidic protein (GFAP) and vascular endothelial growth factor (VEGF) (catalog MA1-166629,Thermo Scientific Co., UK) was diluted with phosphate buffer saline (PBS) (1:50) then washed with PBS and incubated with biotinylated mouse secondary antibody (Cat No.32230,ThermoScientific Co., UK) and finally conjugated with streptavidin-peroxidase. Sections were washed with PBS and incubated with diaminobenzidid (DAB) for 5 minutes.

**RESULTS**

**Histopathological findings:**

Histopathological examination of cerebral cortex revealed individual necrosis of motor neurons that comprising the deep cerebral grey matter and congestion of blood vessel (Fig.1.a) associated with glia cell proliferation presumably astrocytes (astrogliosis) that extended into cerebral white matter. The latter showed diffuse gliosis (Fig.1.b). Gliosis was specifically concentrated around blood capillaries that showing hypertrophy of its lining endothelium with elongation of the blood vessel length (Fig1. c, d and e). In addition, focal gliosis was observed in deep white matter with perivascular glia cell aggregation associated with congestion of blood vessels (Fig.1f) compared with nisin treated and control groups which showed normal histological structure of motor neurons that have abundant eosinophilic cytoplasm, large centrally located nuclei and normal distrobution of glia cells in both cerebral grey and white matter (Fig.1. g & h). In hippocampus there was reduction in the cellular thickness comprising the different regional areas. In dentate gyrus region (DG) there was reduction in the thickness of small basophilic granular cells and cellular vacuolation (Fig. 2a) associated with individual neuronal necrosis within the molecular layer and glia cell proliferation. Partial loss of small and large pyramidal cells comprising hippocampus cornu ammonis region 1 (CA1) and cornu ammonis region 3(CA3) respectively were detected (Fig.2. c-e). In comparison with nisin treated group, the three hippocampus regions (Fig.2b,d and f) showed normal histological cellular density .

**Immunohistochemical findings:**

Immunohistochemical staining revealed relative increased expression of glial fibrillar acidic protein (GFAP) denoting positive astrogliosis that was evident in cerebral white matter (Fig.3.a & c) and DG hippocampus region (Fig.3.e) than CA1 and CA3 region compared with the nisin treated and control group that showed faint expression of astrocytic dendrites either in number or size(Fig.3. b, d & f). While the VEGF
expression showed the same pattern of positivity expression in different areas of cerebrum and hippocampus as previously described in GFAP in sodium nitrite treated group (Fig.4a, c) compared with nisin treated group (Fig.4b, d).

Legends of Figures:-

Fig.1) figures a-f illustrating sodium nitrite treated rats and g, h illustrating nisin treated rats

a-Cerebral grey matter showing necrosis with neuronophagia of individual motor neurons(H&EX400).

b-Cerebral white matter showing diffuse glia cell proliferation(H&EX200).

c- Higher magnification of the previous note the focal and diffuse glia cell proliferation in the neuropil(H&EX400).

d-Cerebral white matter showing elongation of cerebral blood capillary with increased perivascular aggregation of glia cells (H&EX400).

e- Cerebral white matter showing perivascular glial cell proliferation associated with hypertrophy of capillary endothelium (H&EX400).

f- Deep cerebral white matter showing congestion of cerebral blood vessel associated with intense perivascular aggregation of glia cells(H&EX400).

g- Cerebral grey matter showing normal motor neurons morphology with relatively large cell body and vacuolated nuclei containing prominent nucleoli(H&EX400).

h- Cerebral white matter showing normal distribution of glia cell in neuropil with normal histological structure of capillary endothelium (H&EX400).

Fig.2) figures a, c, e illustrating hippocampus of sodium nitrite treated rats while b, d, f concerning nisin treated ones

a-DG area showing extensive vacuolation of granular basophilic cells with necrosis and shrinkage of neurons comprising the molecular layer with glia cell proliferation(H&EX400).

b-DG area of nisin treated rat showing compact granular basophilic cells with normal morphology of pyramidal neurons comprising the molecular layer with normal glia cell distribution (H&EX400).

c- CA1 area of sodium nitrite treated rat showing cellular reduction of small pyramidal neurons comprising the CA1 region (H&EX400).

d- CA1 of nisin treated rat showing normal compact cellular density of small pyramidal neurons (H&EX400).

e- CA3 region of sodium nitrite treated rat showing shrinkage and loss of large pyramidal neurons associated (H&EX400).

f- CA3 region showing normal large pyramidal neurons with vesicular nuclei (H&EX400).

Fig.3) GFAP stained sections

a-Cerebral white matter of sodium nitrite treated rat showing relative increased number of positive stained astrocytes(X100).

b-Cerebral white matter of nisin treated rat normal astrocytes distribution(X100).

c-higher magnification of (a) note the increased positive staining density of astrocytes processes and cytoplasm (X400).
d- higher magnification of (b) showing normal astrocytes processes and cell body (X400).

e- Hippocampus of sodium nitrite treated rat showing intense positive staining of astrocytes processes and cell body (X400).

f- Hippocampus of nisin treated rat showing normal stained astrocyte processes and body (X400).

**Fig.4) VEGF stained sections**

a- Cerebral cortex of sodium nitrite treated rat showing strong positive brown stained neurons and glia cells (X400).

b- Cerebral cortex of nisin treated rat showing negative stained neurons and glia cells (X400).

c- Hippocampus of sodium nitrite treated rat showing relative positive brown stained individual pyramidal neurons(X400).

d- Hippocampus of nisin treated rat showing negative stained neurons and glia cells (X400).
DISCUSSION

Nowadays contemporary addition of preservatives in our food stuff become popular but the trend for addition of synthetic food preservatives is more likely occurred than the natural ones. This may be due to cost issues. However recent researchers studied the deleterious hallmarks of synthetic food additives. Sodium nitrite is one of the synthetic food additives that is commonly used in cured and processed meat products. In the present study sodium nitrite as one of the synthetic food additives and nisin as one of the natural food additives were used to evaluate the histopathological changes in cerebral cortex and hippocampus with special reference to GFAP and VEGF expression in brain sections.

Results showed that sodium nitrite induced various histopathological alterations involving the deep cerebral cortex and different region of hippocampus that collectively characterized by reduction in hippocampal neuronal cellular density which associated with increased expression of GFAP & VEGF that was expressed in neurons, astrocytes and vascular endothelium. Recent studies have depicted the localization of VEGF and its receptors on neurons and astrocytes (Jeffrey and Janette, 2004).

The presented results in this work proved the hypoxic mechanism of sodium nitrite induced neurotoxicity because expression of VEGF is up regulated by hypoxia (Levy et al, 1996, Cobbs et al, 1998, and Neufeld et al, 1999) which is one of the potent inducers for VEGF expression in many organs including CNS (Minchenko et al, 1994). In the present study hypertrophy of capillary endothelium was evident in cerebral white matter and in medullary center of hippocampus of sodium nitrite treated group compared with nisin and control groups, a result which agreed with Dombrowski et al, (2008) who found that hypoxia elicited a profound increase in vascular endothelial growth factor receptor in hippocampus that corresponded to increased blood vessel density also VEGF has specific activity for endothelial cell proliferation in vivo (Plate et al, 1992). Moreover, reduction in cellular density and hippocampal neuronal loss that observed in sodium nitrite treated group agreed with Zaidi, (2010) also Biradar et al, (2013) who found that memory impairment in sodium nitrite induced neurohypoxia. In addition, it was known that hippocampus is a structure heavily implicated in memory consolidation (Guacobini, 1990). It has been reported that during hypoxia the glutamate and aspartate levels rise dramatically, which cause excitotoxicity or disrupt the major excitatory input to the hippocampus, that normally protects the hippocampus from hypoxic damage (Hagan & Beaughard, 1990), also hippocampus contains high levels of mineralocorticoid receptors, which make it more vulnerable to stress compared to most other brain areas (Plate et al, 1992).

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

Nisin treated group showing no histopathological changes in both cerebral cortex and hippocampus this agreed with Delves-Broughton, (2005) who stated that nisin is toxicologically safe. Also, Reddy et al., (2012) confirmed suitability of nisin as a safe and effective microbicide.
REFERENCES


Delves-Broughton, J. (2005):"Nisin as a food preservative" Food, Australia, 57 (12).


