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Increased Permeability of the Blood-Brain Barrier Following Administration of Glyceryl Trinitrate in Common Carp (*Cyprinus carpio* L.)

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A B S T R A C T

Nitric oxide (NO) has been implicated in the pathogenesis of migraine and treatment with its exogenous donor glyceryl trinitrate (GTN) represents widely accepted experimental »migraine model«. In this study, glyceryl trinitrate was administered intraperitoneally to carps, serum nitrite and nitrate levels were determined, permeability of blood-brain barrier was investigated, and histological changes of brain tissue were analyzed. Serum nitrite and nitrate levels displayed characteristic biphasic pattern with moderate initial increase and maximal terminal increase, suggesting the GTN-induced endogenous NO synthesis. Increased permeability of the blood-brain barrier in GTN-treated animals was determined based on Evans blue capillary leakage into the brain tissue. Histological analysis revealed changes consistent with vasodilatation and oedema. Our study strongly supports the importance of the NO role in the pathogenesis of migraine attacks and increase in blood-brain barrier permeability during the attack. The study has also provided evidence that this mechanism of action is conserved to the lower vertebrate.

Key words: *migraine, glyceryl trinitrate, permeability, blood-brain barrier*

Introduction

The free radical nitric oxide (NO) has been implicated in the pathophysiology of migraine based on several evidence derived from human and experimental animal studies. Glyceryl trinitrate (GTN) is an organic nitrate which releases NO via metabolic biotransformation in the target tissue. Intravenous infusion of nitroglycerin triggers migraine-like headaches, suggesting a specific role in primary headache pathogenesis[1–3]. Furthermore, inhibition of NO-synthase relieves acute migraine attacks[4]. More indirect evidence is provided by studies showing that platelet levels of cyclic GMP, the second messenger of NO, as well as NO metabolites nitrate/nitrite, are increased in migraineurs and that they rise further during attacks[5,6].

Nitric oxide is an important biological endogenous synthesized messenger molecule, playing a role in a variety of biological processes including neurotransmission, immune defense and the regulation of cell death (apoptosis)[7]. It is produced in neurons, glia, endothelial cells, and macrophages from and by various (NOS) and by sequential reduction of inorganic nitrate[8]. Multiple receptors and neurotransmitters can release NO leading to vasodilatation and possible extravasations subsequent to the secondary release of other mediators[9,10]. There is some evidence that represents NO as a key coupling compound which links cortical spreading depression (CSD) changes in cerebral blood flow and metabolism. It was

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suggested that NO synthase is up-regulates by cellular depolarisation/depression occuring during CSD[11].

It was demonstrated that glyceryl trinitrate enhances CSD-induced NO release in treated animals[12]. CSD's association with multiphasic NO release[13] was also demonstrated.

This study was designed to assess the effect of glyceryl trinitrate on blood-brain barrier changes, vasodilatation and neurogenic inflammation suggesting their role in the pathogenesis of migraine. Organic nitrate was experimentally administered to carps in order to elicit the NO formation.

Material and Methods

Animals

A year old forty eight carps, free of major diseases, were brought to the Department for Biology and Pathology of Fish and Bees, Veterinary Faculty, University of Zagreb from the fish farm »Topličica«, Novi Marof, Croatia. The fish weighted 574–908 g. During 4-weeks of adaptation preceding to the experiment, the fish were kept in a 660–l plastic pool under steady, slow circulation of dechlorinated tap water and were fed commercial food.

Measurement of serum nitrite, nitrate and total nitrite/nitrate

One mg/kg b.w. of glyceryl trinitrate (Trinitrosan amp., Merck Pharma GmbH) was intraperitonealy injected to carps (n=35). Caudal vessels were punctured with disposable plastic injectors, immediately after injection (0 h) and after 1, 3, 6 and 8 hours. The samples were centrifuged for 15 min at 2500 x g at room temperature and serum was filtered through a 10 kDa filter (Millipore Co Ltd., MA, USA). Serum samples were kept at –20 °C prior to the nitrite/nitrate analysis.

Total nitrite/nitrate in in the carp serum was determined using the nitrite/nitrate colorimetric assay kit (Cayman Chemical Company, MI, USA) according to the manufacturer's instructions. Forty μl of the serum was briefly mixed with 40 μL of assay buffer, 10 μL of enzyme cofactor and 10 μL of nitrate reductase, and incubated at room temperature for 3 h (conversion of nitrate to nitrite). After 10 min of incubation in Griess reagent at room temperature the absorbance was measured at 560 nm using Tecan Ultra Fluorescence Spectrophotometer (Tecan Co., USA). Total nitrite/nitrate in serum of carps was determined using the nitrite/nitrate colorimetric assay kit (Cayman Chemical Company, MI, USA) according to manufacturer instruction. Briefly, 40 μL of serum was mixed with 40 μL of assay buffer, 10 μL of enzyme cofactor and 10 μL of nitrate reductase, and incubated at room temperature for 3 h (conversion of nitrate to nitrite). After 10 min of incubation in Griess reagent at room temperature the absorbance was measured at 560 nm using Tecan Ultra Fluorescence Spectrophotometer (Tecan Co., USA).

The level of total nitrite/nitrate and nitrite was calculated from standard curves of increasing nitrate and nitrite concentrations. Each experiment was performed in triplicate. The final results are expressed as μM of the total nitrite/nitrate and nitrite.

Measurement of Evans blue Dye (EBD) for BBB integrity

Permeability of Evans blue Dye was evaluated for BBB integrity in accordance with a modified method described by Rapoport et al.[14] BBB integrity was measured in 25 animals, 15 from the treatment group and 10 from the control group.

Carps were intraperitonealy injected with 1 mg/kg b.w. glyceryl trinitrate (Trinitrosan amp., Merck Pharma GmbH). Immediately after injection, carps were returned to the plastic pool. Carps in the control group were intraperitonealy injected with the saline. Approximately 6 hours later carps were anesthetized with MS-222 (Sigma Aldrich Chemie Gmbh, Steinheim) and 2% Evans blue Dye saline (Sigma Aldrich Chemie Gmbh, Steinheim) 2 mL/kg body weight was injected intracardialy, allowed to circulate for a couple of minutes. Pericardial cavity of the carps was opened and perfused with saline solution via bulbus arteriosus followed by 4% formaldehyde in PBS. After the experiment, anesthetized carps were bled by cutting the gill arches. Their brains were removed immediately after sacrifice and post-fixed in 4% formaldehyde in PBS solution. All brains were photographed and the degree of Evans blue staining was graded on a scale of 0–3 as previously described[14]. Zero was assigned for no stain uptake, 1 for light staining of the surface and cortical layers, 2 for darker diffuse cortical staining and light staining of the white matter, and 3 if deep blue staining of grey and white matters was observed.

Histopathology

The brains of 23 fish (13 treated with GTN and 10 controls) were fixed in 10% buffered formalin for the purpose of histological examination. The fixed material was embedded in paraffin wax and serially sectioned. The 6-μm thick sections were stained with hematoxylin and eosin (HE).

Statistical analysis

The data were statistically analyzed in accordance with the standard statistical programme (SPSS, EXCEL for Windows). The total nitrate/nitrite and nitrite levels in the serum were statistically analysed by means of one-way analysis of variance (ANOVA), followed by a multiple comparison procedure (Tukey test). The level of $P<0.05$ was considered statistically significant. The results were represented as mean±SEM.

The Mann-Whitney test was used for within-group comparisons. $P<0.05$ was considered significant.

Evans blue scores were statistically evaluated using a Mann-Whitney test. $P<0.05$ was interpreted statistically significant.

Results

There were no mortalities in any of the experimental groups. Three animals (1 from the treatment and 2 from the control group) from those that were subjected to the Evans blue dye (EBD) measurement for BBB integrity were excluded from the experiment. In case that blue coloration of the gill has not been observed immediately after injection of the dye the heart puncture in these animals was considered unsuccessful.

The total nitrite/nitrate level at 0 hours was 187.7±19.51 μmol (mean±SD), significantly higher than in 3-hours group (111.7±12.88 μmol), and significantly lower than in 6-hours (251.3±63.99 μmol) and 8-hours (255.2±25.81 μmol) groups. In the 1st hour of GTN administration the level was 185.3±15.89 μmol, insignificantly lower than in the 0-group, significantly higher than in 3-hours group, and significantly lower than in 6- and 8-hours groups. The 3-hours group showed a marked level reduction, while 6- and 8-hours groups had maximal nitrite/nitrate levels (Figure 1).

The nitrate concentration curve is similar to the total nitrite/nitrate. At 0 hours it was 180.2±25.76 μmol, significantly higher than in the 3-hours group (124.6±7.13 μmol), and significantly lower than in 6-hours (232.4±63.84 μmol) and 8-hours (236.9±89.12 μmol) groups. Nitrate level in the 1st hour group was 182.9±15.39 μmol, which is insignificantly higher than in the 0-hour group and significantly higher than in the 3-hours group but significantly lower than in 6- and 8-hours groups. The level was significantly reduced in the 3-hours group, while 6- and 8-hours groups demonstrated maximal nitrite/nitrate levels (Figure 2).

Nitrite levels were markedly lower than nitrate levels and reached maximal values in the 3-hours group (14.28±9.627 μmol) and 8-hours group (16.17±5.935 μmol), which had levels statistically higher than all other groups

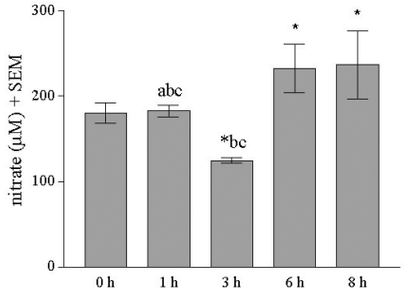

*Fig. 1. Serum levels of total nitrate/nitrite at 0, 1, 3, 6 and 8 hours after treatment with glyceryl trinitrate (1 mg/kg b.w.). Mean± S.E.M. (n=7) are given. * (p<0.05), as compared to 0 hours of treatment; a) (p<0.05), as compared to 3 hours of treatment; b) (p<0.05) as compared to 6 hours of treatment; c) (p<0.05), as compared to 8 hours of treatment.*

TABLE 1
EVANS BLUE SCORING IN THE GTN TREATMENT GROUP AND THE CONTROL GROUP

Evans Blue Score	Control Group (n=10)	Treatment Group (n=15)
»0«	8	0
»1«	2	0
»2«	0	6
»3«	0	9

* See the text on scoring

(0-hour group 8.11±3.529 μmol, 1-hour group 5.452±2.507 μmol, 6-hours group 4.014±2.252 μmol) (Figure 3).

Eight of control animals had an Evans blue score of 0 and two control animals were given a score of 1. Nine of the treated animals were given the score of 3 and the remaining six animals the score of 2 (Table 1).

Evans blue scores for the treatment group were significantly higher then for the control group ($p<0.05$). Our results did not include leakage of the dye in the areas of circumventricular organs (Figure 4).

Findings of the histological brain examination of all GTN treated animals in the study had characteristics of

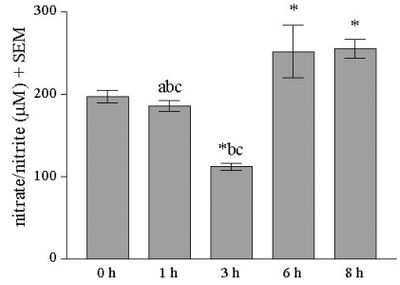

*Fig. 2. Serum nitrate levels at 0, 1, 3, 6 and 8 hours after treatment with glyceryl trinitrate (1 mg/kg b.w.) Mean±S.E.M. (n=7) are given. * (p<0.05), as compared to 0 hours of treatment; a) (p<0.05), as compared to 3 hours of treatment; b) (p<0.05) as compared to 6 hours of treatment; c) (p<0.05), as compared to 8 hours of treatment.*

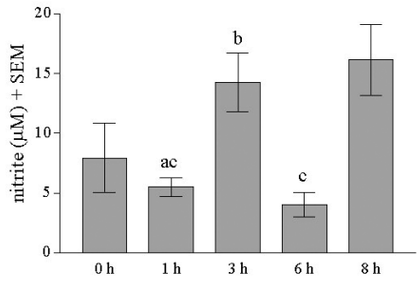

Fig. 3. Serum nitrite levels at 0, 1, 3, 6 and 8 hours after treatment with glyceryl trinitrate (1 mg/kg b.w.) Mean±S.E.M. (n=7) are given. a) (p<0.05), as compared to 3 hours of treatment; b) (p<0.05) as compared to 6 hours of treatment; c) (p<0.05), as compared to 8 hours of treatment.

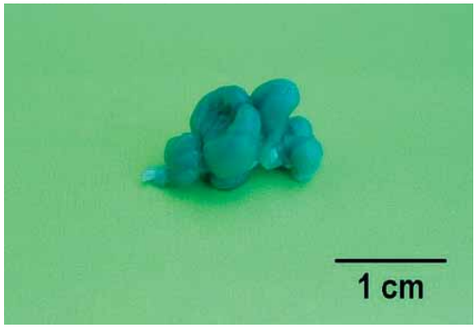

Fig. 4. Evans blue stained brain in a treated carp.

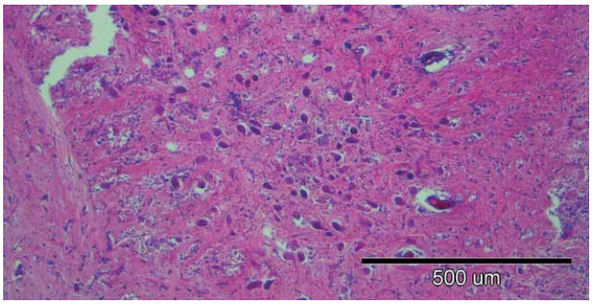

Fig. 5. H&E stained brain preparation of treated carp showing dilated blood vessels containing blood, with thinned and elongated, but regular endothelial cells. Oedema in adjacent neural tissue is also displayed.

vasodilatation and brain edema. In all treated animals we detected vacuolization or spongy change of the neuropil and pericellular vacuolization as well as pallor of the tissue and swollen astrocytes (Figure 5). Small areas of meningeal hemorrhage and leukocyte infiltration seen in one of the treated animals, was assessed as procedural complication/over-dosage of GTN. Brain parenchyma under this finding was notably edematous. Histological findings in the control group were normal.

Discussion

The exact molecular mechanism of migraine remains to be fully clarified. The NO has emerged as an important mediator of a neurogenic cranial vessel inflammatory response that ultimately might result in headache.

According to Olesen et al., nitroglycerin can trigger migraine-like headaches, suggesting a specific role in primary headache pathogenesis[1]. There is a pharmacologic but indirect clinical evidence for the NO hypothesis. Sumatriptan can terminate the nitroglycerin-induced migraine. Calcium channel blockers may work by reducing NO synthase activity and thus levels of NO. Lassen et al. published a double-blind, placebo-controlled study on L-methylarginine, a non-specific NO synthase inhibitor in the treatment of acute migraine. They found that headache was relieved in the treated group as compared to the placebo group [4].

The purpose of this study was to determine whether the increased permeability of the blood-brain barrier (BBB) after GTN-treatment is associated with over expression/induced nitric oxide biosynthesis. Because the extremely short half-life of NO makes its detection difficult, we sought to determine the predominant role of NO in blood brain barrier (BBB) reversible disruption by studying stable NO metabolites, nitrite and nitrate ions. In our study the total nitrite/nitrate and nitrate serum concentrations showed biphasic pattern with moderate increase in 0 (1) hour of GTN administration and marked increase after 6 and 8 hours. The nitrite levels were markedly lower than nitrate ones and showed maximal values in the 3- and 8-hours groups. Theoretically, the nitrate and nitrite standard curves should be identical, as nitrate is converted to nitrite, but *in vivo* slight or greater differences almost always exist due to incomplete reduction of nitrate[15]. Since according to current studies and our results the ratio of nitrite to nitrate in biologic fluids is variable and unpredictable, the best index of NO production appears to be the total of nitrate and nitrite[16]. The reason for delayed (6- and 8-hours groups) elevation in nitrite/nitrate levels probably includes GTN-evoked endogenous NO synthesis. This biphasic pattern of bioactivated NO release following GTN infusion corresponds with previous studies[17–19]. GTN is an organic nitrate with a short plasma half-life (1–4 min), although its half-life can reach 2 hours in a slice of lipophilic brain tissue[20,21]. It is metabolized to NO within cells by a combination of glutathione-S-transefrase, cytochrome P_{450} and thiol reactions. NO synthesizes endogenously by nitric oxide synthases (NOS) using L-arginine as a substrate[22]. Release of NO from blood vessels, perivascular nerve endings and brain tissue is proposed to be a molecular trigger mechanism of spontaneous migraine pain[23]. There are reports of inducible NOS (iNOS) mRNA up-regulation beginning at 2 hours and increasing at 4, 6 and 10 hours following the GTN infusion[24]. Thus, biphasic pattern must be due to a phenomenon inherent in the mechanism of the NO generation induced by GTN.

Histological findings revealed vasodilatation characteristics. The NO contributes to vasodilatation observed in cortical spreading depression[13]. Transient arterial dilatation during CSD was reduced by approximately 50% during application of NO-synthase inhibitor[25,26]. It is well known that NO activates cytoplasmic guanylate cyclase, hence causing an increase in cyclic guanosine monophosphate, a decrease in intracellular calcium and vasodilatation[27].

We have also detected Evans blue leakage indicating BBB opening, as well as (initially) vasogenic oedema in the brain tissue in the 6-hour group. With regard to molecular mechanisms, cortical spreading depression upregulates a variety of gene for expression of COX-2, TNF-[alpha] and IL-1[beta], galanin and metalloproteinases[28]. Besides, NO have been implicated in MMP activation. The basal lamina contains extracellular matrix molecules such as type IV collagen, laminin, and fibronectin, most of which are substrates for MMPs, especially

MMP-2 and MMP-9[29,30]. The activation of metalloproteinases leads to leakage of the blood-brain barrier, allowing potassium, (more) nitric oxide, adenosine and other products released by cortical spreading depression to reach and sensitize the dural perivascular trigeminal afferents[28]. We believe that the NO was the mediator of delayed changes in BBB permeability observed in this study. In previous studies, leakage of plasma proteins from blood vessels was first detected 4 hours after GTN infusion, and this can be suppressed by administration a specific type II NOS inhibitor[29].

On the grounds of our experiment and previous investigations of experimental migraine models, we strongly suggest the implication of the NO in the migraine pathophysiology. The precise mechanism of the NO-activated cascade remains to be clarified. The existing evidences may set novel targets for treatments that would either prevent or abort headache. This study has also shown that the mechanism of NO elicitation and BBB permeability changes are conserved to the lower vertebrate.

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POVEĆANA PROPUSNOST KRVNO-MOŽDANE BARIJERE MOZGA ŠARANA (*CYPRINUS CARPIO* L.) NAKON PRIMJENE GLICERIL TRINITRATA

SAŽETAK

Dušik (II) oksid (NO) je impliciran u patogenezi migrene, a tretiranje njegovim egzogenim donorom gliceril trinitrataom (GTN), predstavlja općenito prihvaćen eksperimetalni »model migrene«. U našem smo pokusu šarane tretirali s gliceril trinitratom intraperitonealno te smo odredili koncentraciju serumskih nitrita i nitrata, ispitali propusnost krvno-moždane barijere i analizirali histopatološke promjene u tkivu mozga. Koncentracija serumskih nitrita i nitrata imala je karakterističan bifazični obrazac s blagim inicijalnim i maksimalnim terminalnim povećanjem što sugerira GTN-induciranu endogenu NO sintezu. GTN-tretirane životinje su pokazale povećanu propusnost krvno-moždane barijere, što smo utvrdili na temelju istjecanja Evans blue boje u moždano tkivo. Histopatološkom analizom ustanovljene su promjene karakteristične za vazodilataciju i edem. Naše istraživanje snažno podupire važnu ulogu NO u patogenezi migrenskog napadaja, odnosno povećanje propusnosti krvno-moždane barijere u migrenskom napadaju. Ovo istraživanje također dokazuje da je ovaj mehanizam djelovanja konzerviran do nižih kralješnjaka i zbog toga ga možemo smatrati važnim.