FINASTERIDE IMPROVES MOTOR, EEG AND CELLULAR CHANGES IN RAT BRAIN IN THIOACETAMIDE-INDUCED HEPATIC ENCEPHALOPATHY

Dušan Mladenović¹, Dragan Hrnčić², Nataša Petronijević³, Gordana Jevtić³, Tatjana Radosavljević¹, Aleksandra Rašić-Marković², Nela Puškaš⁴, Nebojša Maksić⁵, Olivera Stanojlović²*

¹Institute of Pathophysiology "Ljubodrag Buba Mihailovic", Faculty of Medicine, University of Belgrade, Belgrade, Serbia
²Institute of Medical Physiology "Richard Burian", Faculty of Medicine, University of Belgrade, Belgrade, Serbia
³Institute of Clinical and Medical Biochemistry, Faculty of Medicine, University of Belgrade, Belgrade, Serbia
⁴Institute of Histology and Embryology, Faculty of Medicine, University of Belgrade, Belgrade, Serbia
⁵Centre for Medical Biochemistry, Clinical Centre of Serbia, Belgrade, Serbia

Running head: Finasteride in thioacetamide-induced hepatic encephalopathy

*Corresponding author
Prof. dr Olivera Stanojlović, M.D., Ph.D.
Institute of Medical Physiology "Richard Burian"
Faculty of Medicine, University of Belgrade
Višegradska 26/II,
11000 Belgrade, Serbia
Phone/fax: ++381 11 3607 106
e-mail: solja@afrodita.rcub.bg.ac.rs
ABSTRACT

Neurosteroids are involved in the pathogenesis of hepatic encephalopathy (HE). This study evaluated the effects of finasteride, inhibitor of neurosteroid synthesis, on motor, EEG and cellular changes in rat brain in thioacetamide-induced HE. Male Wistar rats were divided into following groups: 1. control; 2. thioacetamide-treated group, TAA (300 mg/kg/day); 3. finasteride-treated group, FIN (50 mg/kg/day); 4. group treated with FIN and TAA, FIN+TAA. Daily doses of TAA and FIN were administered in three subsequent days intraperitoneally and in FIN+TAA group FIN was administered 2h before every dose of TAA. Motor and reflex activity was determined 0, 2, 4, 6 and 24h, while EEG activity was registered about 24h after treatment. The expressions of neuronal (NeuN), astrocytic (GFAP), microglial (Iba1) and oligodendrocyte marker (MOG) were determined 24h after treatment. While TAA decreased all tests, FIN pretreatment (FIN+TAA) significantly improved equilibrium, placement test, auditory startle, head shake reflex, motor activity and exploratory behavior vs. TAA group. Vital reflexes (withdrawal, grasping, righting and corneal reflex) together with mean EEG voltage were significantly higher (p<0.01) in FIN+TAA vs. TAA group. Hippocampal NeuN expression was significantly lower in TAA vs. control (p<0.05). Cortical Iba1 expression was significantly higher in experimental groups vs. control (p<0.05), while hippocampal GFAP expression was increased in TAA and decreased in FIN+TAA group vs. control (p<0.05). Finasteride improves motor and EEG changes in TAA-induced HE and completely prevents the development of hepatic coma.

Key words: hepatic encephalopathy; finasteride; motor tests; electroencephalography; cellular markers
INTRODUCTION

Hepatic encephalopathy (HE) represents a clinical syndrome characterized by neurological and psychiatric disturbances that develop as a result of acute or chronic liver failure (21). Various mechanisms are involved in the pathogenesis of HE, including changes in neurotransmission (17), oxidative stress, bioenergetic failure, mitochondrial permeability transition (49), inflammatory response and immune dysfunction (14,52), due to accumulation of various toxins in the organism, principally ammonia (4).

Changes in glutamatergic and GABAergic transmission have an important role in the development of HE (13,16). Both acute and chronic liver failure were found to be associated with increased GABAergic activity due to increased neurosteroid synthesis. Neurosteroids are steroid compounds, synthesized in mitochondria of glial cells and neurons from cholesterol (2). Cholesterol is uptaken into mitochondria via the activation of translocator protein (TSPO), a multimeric complex that spans both outer and inner mitochondrial membrane. Ammonia and manganese, toxins accumulated in HE, and proinflammatory cytokines up-regulate components of TSPO, and increase cholesterol uptake into the mitochondrion (2,4). Cholesterol serves as a substrate for increased synthesis of neurosteroids, including allopregnanolone (ALLO) and 3α-5α-tetradehydrodeoxy-corticosterone (THDOC), through series of enzymatic steps that include: cytochrome P450 side chain cleavage, 3β-hydroxysteroid dehydrogenase, 21-hydroxylase, 5α-reductase and 3α-hydroxysteroid dehydrogenase. Brain accumulation of ALLO and THDOC potentiates GABAergic transmission by positive allosteric modulation of GABA A receptor function and contributes to the imbalance between excitation and inhibition in HE (2,54,55). ALLO and THDOC are known to induce significant alterations of the sleep/wake cycle (44), as
well as to decrease cognitive performance in rodents (33), changes that are also present in human HE.

Although there is no ideal model of HE, it is well known that thioacetamide (TAA), a sulphur-containing compound, may induce acute liver failure with potentially reversible brain damage (38,39,41,46). TAA was widely used for investigation of the role of glutamine (50), oxidative stress (40), disturbances in glutamatergic and GABAergic neurotransmission (38,45) in the pathogenesis of type A HE.

Finasteride (FIN), 5α-reductase inhibitor, is used for the treatment of prostatic hyperplasia and androgenetic alopecia. In addition, FIN was found to inhibit neurosteroid synthesis in the brain, since 5α-reductase is an enzyme necessary for conversion of progesterone and deoxycorticosterone to the 5α-pregnane steroids, intermediates which are further reduced to ALLO and THDOC (6,43). On the other hand, the treatment with FIN may induce alternative metabolic pathways in the progesterone metabolism, such as 3α-reduction, 20α-reduction and 21-hydroxylation. These effects of FIN on neurosteroid metabolism were found to modulate the emotional state (43), exacerbate lithium-pilocarpine-induced seizures (34), increase formalin-induced pain in rats (42), as well as to alter ethanol intake pattern in C57BL/6J mice (20). In addition, 5α-reductase inhibitors exert antipsychotic-like properties in rats and are proposed as a putative novel target in the management of psychotic disorders (11,47).

Since neurosteroids have an important role in the pathogenesis of HE, it may be suggested that FIN may alleviate manifestations of HE and positively modulate its course. Neuroprotective effects of FIN could possibly improve the therapy and life quality of patients with HE. Based on this background, the aim of our study was to investigate the effects of FIN on motor and
electroencephalographic changes, as well as on the expression of cellular markers in the brain, in thioacetamide (TAA)-induced HE in rats.

**MATERIALS AND METHODS**

**Animals**

Experiments were performed on adult male Wistar rats, weighing 170-200 g, that were raised on Military Medical Academy in Belgrade. Animals were kept in individual cages (55x35x30 cm) under standard laboratory conditions (ambient temperature 22 ± 2°C, relative humidity 50%, 12/12 h dark/light cycle with lights turned on at 9:00 AM) with free access to pelleted food and tap water. All experimental procedures were in full compliance with Directive of the European Parliament and of the Council (2010/63/EU) and approved by The Ethical Committee of the University of Belgrade (Permission No 1891/2).

All animals were divided into following groups: 1. control, saline- and 2-hydroxypropyl-β-cyclodextrin (20% wt/vol)-treated group (n=8); 2. thioacetamide-treated group, TAA (300 mg/kg/day; n=18); 3. finasteride-treated group, FIN (50 mg/kg/day; n=8); 4. group treated with finasteride (50 mg/kg/day) and thioacetamide (300 mg/kg/day), FIN+TAA (n=8). Daily doses of TAA and FIN were administered intraperitoneally in three subsequent days and in FIN+TAA group FIN was administered 2 h before every dose of TAA. This dose of TAA was chosen, since in our previous study it has been confirmed to induce all stages of HE, including hepatic coma (41). The dose of FIN was selected according to previous investigations that confirmed a decrease in plasma and brain ALLO levels by 66% and 80% respectively at a 24-h postinjection time point (19). Before administration, TAA (Sigma Aldrich Co., USA) was dissolved in saline in concentration of 100 mg/mL, while FIN (Sigma Aldrich Co., USA) was solubilized in 20%
wt/vol 2-hydroxypropyl-β-cyclodextrin (Sigma Aldrich Co., USA) and administered in a stock concentration of 5 mg/mL (injection volume was 0.01 mL/g animal body weight). Motor and EEG changes were followed within 24 h after administration of the last dose of TAA. Since hypothermia may be a side effect of TAA treatment, that may protect the development of HE in acute liver failure (46,57), rectal temperature was monitored during the same period.

Animals were sacrificed 24 h after third dose of TAA and liver samples for pathohistological analysis, blood for determination of ammonia concentration and brain samples for Western blot were collected. Blood ammonia concentration in the samples collected from the right side of the heart was determined by enzymatic test (BioMerieux Lab., France). The expressions of neuronal (NeuN), astrocytic (glial fibrillary acidic protein, GFAP), microglial (Iba1) and oligodendrocyte marker (myelin oligodendrocyte glycoprotein, MOG) were determined by Western blot as described below.

**Pathohistological and stereological analysis**

Livers were removed from rats immediately after sacrifice, and fixed in 10% buffered formalin for histological examination. The tissue samples were processed by standard protocol, stained with hematoxylin and eosin (H/E), and analyzed by light microscope Olympus BX41 with digital camera and softver system Olympus DP-soft 5.0. Number of hepatocytes in mitosis (mitotic figures) was expressed per 1000 hepatocytes. Mitoses were counted in 7 randomly selected visual fields with minimal hepatocyte damage. Only cells with clear morphological characteristics of metaphase, anaphase, and telophase were counted. Stereological analysis for quantification of liver damage was performed by the point counting technique using a test system of 36 points, at a magnification x100. Volume density of damaged tissue (Vvd) was calculated from equation Vvd=Pd/Pt, where Pd is a number of points falling on damaged area and Pt is a
total number of test points. For each animal one slide from each liver lobe, and five randomly
chosen areas with most extensive damage in each slide were used for analysis.

**Motor testing**

Motor testing of experimental animals was performed by using test battery according to
Norton et al. (46). This battery includes the following tests: withdrawal reflex, auditory startle
reflex, head shake reflex, corneal reflex, righting reflex, equilibrium test, grasping, placement,
general motor activity and exploratory signs/nose pokes. Animals were allowed to perform
righting reflex, equilibrium test, grasping and placement up to 300 s. General motor activity was
determined in special cages (55x35x30 cm) which floor was divided into nine quadrants. Motor
activity was expressed as a number of animal crosses from one quadrant to another during 10
minutes (each time the rat crossed into a different quadrant, a value of one was tallied).
Exploratory behavior was determined as a number of nose pokes during the same time interval
(each time the rat raised its body and poked its nose, a value of one was tallied). All the tests
were performed 0, 2, 4, 6 and 24 h after administration of the last dose of TAA and the total time
required for the performance of all tests was maximally 20 min.

For each individual motor test animal could score up to 4 points. A score 4 was given if
animal performed test at 75-100% of the control value; score 3: at 50-75% of the control value;
score 2: at 25-50% of the control value; score 1: at 1-25% of the control value, while score 0
indicated that animal showed no response in the test. Control value was considered to be the
velocity of performance of the individual test by control animals (46).

Total behavioral score of the animal was calculated as the sum of all test scores (maximal
total score=40 and minimal total score=0). Coma was defined as an absence of corneal,
withdrawal, auditory startle and head shake reflex (46).
For EEG recordings, rats were anesthetized with ketamine (100 mg/kg i.p.) and three gold-plated electrodes were implanted over frontal, parietal and occipital cortices by stereotaxic method. The animals were left to recover 7 days after the surgery. An 8-channel EEG apparatus (RIZ, Zagreb, Croatia) was used. The signals were digitized using a SCB-68 data acquisition card (National Instruments Co., Austin, Texas, USA). A sampling frequency of 512 Hz/channel and 16-bit A/D conversion were used for the EEG signals. The cut-off frequencies for EEG recordings were set at 0.3 Hz and 100 Hz for the high-pass and low-pass filters, respectively. Ambient noise was eliminated using a 50 Hz notch filter. Data acquisition and signal processing were performed with LabVIEW platform software developed in the Laboratory NeuroSciLaBG (26,53). EEG was analyzed every 30 min in the period 22-24 h after the administration of the last dose of TAA (at time points 22.5, 23 and 23.5 h). According to modified method of Amodio et al. (5), for each time interval when EEG was analyzed, artifact-free recording was divided into 8 epochs each lasting 12 s. The mean power spectra density, as a measure of the mean voltage of EEG waves, was calculated with a software by using fast Fourier transformation method and the integrated energy signals were expressed as $\mu V^2/Hz$. Upon completion of the 2-hour recording sessions, the animal would be removed from the recording chamber and returned to its home cage.

**Western blot analysis**

For Western blot analysis animals were sacrificed by rapid cervical dislocation and decapitation without anesthesia. The brains were removed and two regions were dissected-dorsolateral frontal cortex (dlFC) including motor cortex, and hippocampus. dlFC and hippocampi were homogenized in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1%
IGEPAL CA-630, 1 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich, P7626), protease inhibitor cocktail (Sigma-Aldrich, P8340), 200mM sodium orthovanadate (Sigma, Germany) and 1M NaF (Merck, USA) on ice for 30 min, followed by centrifugation (14,000 g for 15 min at 4 °C), and the supernatants were collected as the cell lysates. Protein concentrations were determined using bovine serum albumin as a standard (Sigma Aldrich Co., USA) (12). Equal amounts of protein (50 μg) from each sample were separated by SDS-PAGE on 10% and 12% gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked at room temperature for 1 h in 5% nonfat dry milk in Tris-buffered saline/0.1% Tween 20 (TBST). The following primary antibodies were used in this study: monoclonal mouse anti-NeuN (1:1000, Chemicon, USA), polyclonal rabbit anti-GFAP (1:2000, DAKO, Denmark), polyclonal goat anti-Iba1 (1:250, Abcam, UK), monoclonal mouse anti-MOG (1:1000, Abcam, UK). After incubation with primary antibodies the membranes were incubated with the horseradish peroxidase (HRP) labeled secondary anti-rabbit antibody (1:2000, Southern Biotech, USA), anti-goat antibody (1:2000, Southern Biotech, USA) or anti-mouse antibody (1:2000, Southern Biotech, USA) in TBST for 1 h at room temperature. Five subsequent washes with 0.1% TBST were performed between each step. All membranes were stripped and re-probed with anti-actin antibodies (1:10000, mouse monoclonal, Sigma, USA) to ensure that all wells were equally loaded. The signal was detected by enhanced chemiluminescence and subsequent exposure on an X-ray film. Western blot were scanned and densitometric analysis were performed using *ImageQuant 5.2*.

**Statistical analysis**

Blood ammonia concentration, general motor activity, exploratory movements and mean power spectra density were expressed as means ± SEM. Total behavioral score, volume density
of damaged tissue and number of mitoses were expressed as medians with 25th and 75th percentils in parenthesis. Statistical significance of the difference in ammonia concentration was estimated by one-way ANOVA with Fisher’s post-hoc test, while the significance of the difference between total behavioral scores was assessed by Friedman nonparametric ANOVA. The significance of the difference in volume density of damaged tissue and number of mitoses was estimated using Kruskall-Wallis test followed by Mann-Whitney U post-hoc test.

For evaluation of the difference in motor tests, mean power spectra density and expression of cellular markers two-way ANOVA with Tuckey post-hoc test and chi-square test were used. The significance was considered significant if p<0.05. For statistical analysis computer software SPSS15.0 was used.

RESULTS

Blood ammonia concentration was significantly higher in TAA (86.7 ± 7.6 µmol/L) and FIN+TAA group (79.3 ± 10.1 µmol/L) by comparison with control group (38.8 ± 5.1 µmol/L) (p<0.01). FIN alone did not induce a significant change in ammonia level vs. control group (p>0.05). In addition, no significant change was evident between TAA and FIN+TAA group (Fig. 1). No change in rectal temperature was found between experimental and control groups within 24 h after treatment (data not shown).

Pathohistological and stereological analysis

In control and FIN group there were no pathological changes in the liver parenchyma (Fig. 2A and C). Lobular architecture and radial arrangement of hepatocytes were preserved in both groups and no significant difference in the number of mitoses was evident between FIN and control group (p>0.05; Table 1). On the other hand, histopathological analysis of liver
parenchyma in TAA group revealed confluent centrilobular or bridging necrosis, while in portal areas mild inflammatory infiltrate and increased ductular structures can be noticed. Mitoses of viable hepatocytes were also evident (Fig. 2B). Volume density of damaged tissue and number of mitoses were significantly higher in TAA by comparison with control group (p<0.01; Table 1).

In FIN+TAA group focal or confluent centrilobular necrosis, and rare bridging necrosis were found (Fig. 2D). Although FIN pretreatment did not prevent TAA-induced hepatocyte necrosis, volume density of damaged tissue was significantly lower in FIN+TAA when compared with TAA group (p<0.01; Table 1). Ballooning hepatocytes were more frequent and inflammation in necrotic areas was milder in FIN+TAA compared with TAA group. In portal areas mild inflammatory infiltrate and increased ductular structures can be observed (Fig. 2D). Mitoses were also noticed, but the number of mitoses was not significantly different in FIN+TAA vs. control group (p>0.05; Table 1).

**Motor tests**

All animals from control, FIN and FIN+TAA group survived 24 h after treatment, while in TAA group mortality in this period was 50% (9/18). Mortality started 8 h following the last dose of TAA and was evident within the whole observation period. Total behavioral score was significantly lower in TAA group vs. control at all time points (p<0.01), with the lowest median 24 h after treatment (4 (0, 4)). Although total behavioral score was significantly lower in FIN+TAA vs. control group at all time points (p<0.05), the score in this group was significantly higher by comparison with TAA group whenever the tests were performed (p<0.01). FIN alone did not induce significant changes in total behavioral score when compared with control group (p>0.05) (Table 2).
The incidence of preserved vital reflexes (withdrawal, corneal, grasping and righting reflex) was significantly lower in TAA by comparison with control group at all time points (p<0.01) with the lowest incidence 24 h after TAA administration (10%). While vital reflexes were significantly diminished in TAA group, these reflexes were preserved in all animals in FIN and FIN+TAA group (Fig. 3A).

Head shake and auditory startle reflex were significantly diminished in TAA vs. control group at all time points (p<0.01). These reflexes were absent 24 h after TAA administration. Although the incidence of preserved head shake and auditory startle reflexes was lower in FIN+TAA than in control group at all time points (p<0.01), the extent of a decrease was significantly lower than in TAA group (p<0.01). The lowest incidence of preserved head shake and auditory startle reflex in FIN+TAA group was evident 4, 6 and 24 h after treatment (50%). All animals from FIN group had completely preserved these reflexes (Fig. 3B). Coma was evident in 77.8% (7/9) of animals from TAA group 6 h after treatment and in 88.9% (8/9) of animals 24 h after treatment, while in FIN+TAA group no animal (0/8) was comatose within 24 h after administration of the last dose of TAA.

Similar to head shake and auditory startle reflex, the incidence of preserved equilibrium and placement test was significantly lower in TAA than in control group at all time points (p<0.01). No animal had normal performance of these tests 24 h after TAA administration. All animals in FIN+TAA group had preserved normal equilibrium and placement test 2 h after TAA treatment, while the incidence of preserved tests was significantly lower 4, 6 and 24 h after treatment in FIN+TAA vs. control group (p<0.01). However, at all time points the incidence of preserved equilibrium and placement test was significantly higher in FIN+TAA when compared with TAA group (p<0.01). All animals from FIN group normally performed these tests (Fig. 3C).
General motor activity and exploratory behavior were significantly lower in TAA and FIN+TAA groups by comparison with control group at all time points (p<0.01). However, animals from FIN+TAA group had significantly greater motor activity when compared with TAA group 4, 6 and 24 h after administration of the last dose of TAA (p<0.01; Fig. 4A). Exploratory behavior was, also, significantly higher in FIN+TAA vs. TAA group 6 and 24 h after treatment (p<0.01; Fig. 4B). No significant difference in the performance of these tests was evident between FIN and control group (p>0.05).

**EEG analysis**

FIN induced a significant increase in mean power spectral density by comparison with control group (p<0.01). While mean power spectral density was significantly lower in TAA by comparison with control group at all time points when EEG was registered (p<0.01), no significant change in mean power density was evident between FIN+TAA and control group at all time points (p>0.05; Fig. 5).

**Western blot analysis**

NeuN expression was significantly lower in the cortex of animals from FIN group and in the hippocampus of animals from TAA group when compared with control (p<0.05; Fig. 6A). GFAP expression in the cortex was significantly lower in FIN vs. control group (p<0.05), while in other groups no significant difference was found by comparison with control. In contrast to cortex, GFAP expression in the hippocampus was found to be increased in TAA and decreased in FIN+TAA group when compared with control (p<0.05; Fig. 6B). While FIN and TAA induced no significant changes in Iba1 expression in the hippocampus, cortical Iba1 expression was significantly higher in TAA, FIN and FIN+TAA groups by comparison with control (p<0.05;
Fig. 6C). MOG expression was not significantly changed by TAA or FIN in the cortex and the hippocampus (Fig. 6D).

**DISCUSSION**

TAA-induced model of HE was described in details in our previous studies (39-41) and although initial decrease in motor activity has been evident soon after administration of the second dose of TAA, the most severe form of type A HE develops within 24h following injection of the third dose (41). Since no change in rectal temperature was evident in experimental groups, the effects of TAA, including mortality, could be attributed to the development of HE.

Currently, all therapeutic approaches to the improvement of HE have limited effects and appreciable risks and benefits associated with their use. Management of HE is directed primarily to avoidance of precipitating factors and administration of agents that increase hepatic ammonia uptake (e.g. L-ornithine, L-aspartate) or decrease ammonia production (e.g. lactulose, antibiotics) (10,48). However, these therapies have significant adverse effects. Alternative treatment options in patients with HE include: benzodiazepine receptor antagonists, branched-chain amino acids, zinc supplementation, sodium benzoate, dopamine receptor agonists, acarbose and probiotics (48). New therapeutical options are being focused on the removal of ammonia and glutamate, not only by the liver, but also by extrahepatic organs (51).

The role of increased GABAergic transmission in the pathogenesis of HE prompted the investigations of the potential therapeutic effects of modulators of GABA effects in the brain. GABA_A receptor antagonists and partial inverse benzodiazepine agonists were tested in humans and several animal models of HE and revealed contradictory findings (1,3,15). Another possible
therapeutic approach may be an inhibition of neurosteroid synthesis in order to prevent GABAergic hyperactivity. This is the first study that investigated the effects of FIN, 5α-reductase and neurosteroid synthesis inhibitor, on the course of HE.

The present study clearly showed that FIN improved motor disturbances and prevented the development of the most severe forms of type A HE and lethal outcome after TAA treatment in rats. FIN pretreatment alleviated a TAA-induced decrease in total behavioral score (Table 2) and had variable effects on individual motor tests (Figs. 3 and 4). The most prominent effect of FIN pretreatment was the prevention of coma in TAA-treated rats, thus strongly suggesting that FIN may prevent the development of the most severe forms of type A HE (stage IV).

This effect of FIN was further confirmed by EEG analysis. TAA-induced coma was associated with low-voltage activity predominantly in the delta frequency range (Fig. 5). Previously, we have found a dose-dependent effects of TAA on the brain electrical activity. Initial stages of TAA-induced HE are followed by an increase in mean EEG voltage with gradual decrease and predominance of δ waves accompanying progression to the more severe stages (39). These changes correspond to the EEG characteristics of human HE with flattening of EEG in hepatic coma (23,27). FIN pretreatment induced an increase in mean EEG voltage without changes in dominant frequency (Fig. 5), that corresponds to the milder forms of HE (23).

FIN improved motor activity and exploratory behavior 6 and 24 h after the administration of the last dose of TAA (Fig. 4A and B). This delay in onset of FIN effects may be explained by mechanism of its action, since enzyme inhibition requires enough time for maximal efficiency. Improvement of motor manifestations of TAA-induced HE, prevention of coma, as well as induction of EEG changes that correspond to milder forms of HE, strongly suggest that FIN exerts neuroprotective effect in TAA-induced HE and may be a potential device for the
improvement of the course of type A HE. FIN did not enable a complete recovery of motor functions, since the pathogenesis of motor disturbances in HE is complex and involves changes in various neurotransmitters, including glutamate, dopamine, acetylcholine and catecholamines (17,22,37). This possibly indicates that FIN in a combination with modulators of other neurotransmitters could exert even better protective effects in HE.

$\text{GABA}_A$ receptor antagonists were, also, found to ameliorate behavioral changes in HE. Bicuculine, a drug from this group, was found to improve learning ability in hyperammonemic rats (15), while flumazenil improved spontaneous motor activity in rats with ischemia-induced acute liver failure (58). Although flumazenil exerted beneficial effects on HE in rats, the therapeutic potential of this drug in humans still remains controversial, since only a subset of patients had benefit from this therapy (18,32). Beneficial effects of flumazenil in some patients may be attributed to the potentiating effect of neurosteroids on endogenous ligands at the benzodiazepine site, thus indicating that neurosteroids increase the sensitivity of $\text{GABA}_A$ receptor complex to low concentrations of benzodiazepines (4). This clearly indicates that low neurosteroid level is essential for flumazenil efficiency and that therapy should be preferentially directed towards blockade of neurosteroid effects, not to blockade of $\text{GABA}_A$ receptors. In accordance with this and findings of our study, FIN may have more consistent neuroprotective effects in HE than $\text{GABA}_A$ antagonists.

Partial benzodiazepine inverse agonists, such as Ro15-4513, were found to have consistent beneficial effects on the course of HE due to reduction of positive modulatory effect of neurosteroids on $\text{GABA}_A$ receptors (1). Studies in cultured hippocampal neurons confirmed that Ro15-4513 reduces GABA-induced chloride currents in a dose-dependent manner (3). However, these agonists are not useful for the treatment of human HE, since they have serious side effects,
such as convulsions and anxiety (4). This also suggests that therapeutic approach to type A HE should be directed to the modulation of neurosteroid function, which renders FIN as a candidate for this use.

The mechanisms of protective effects of FIN in HE are still not completely clear. FIN pretreatment alleviated TAA-induced necrosis of hepatocytes (Fig. 2), thus indicating a hepatoprotective effect of FIN. The mechanisms of beneficial effects of FIN on the liver are still not understood, but may be related to the inhibition of $5\alpha$-reductase in hepatocytes (28). On the other hand, blood level of ammonia, one of the major toxins in the pathogenesis of HE, was not significantly different between FIN+TAA and TAA group (Fig. 1). According to previous findings (31) blood ammonia (not pathohistological changes) correlates precisely with appearance and the stage of type A HE. This strongly suggests that beneficial effects of FIN on the liver do not contribute significantly to the improvement of TAA-induced HE. The major mechanism of neuroprotective effect of FIN is suggested to be an inhibition of hyperammonemia-induced synthesis of neurosteroids by blockade of $5\alpha$-reductase and subsequent reduction of GABAergic effects in the brain (19), but some additional mechanisms cannot be excluded.

According to the NeuN expression, FIN may prevent TAA-induced neuronal damage in the hippocampus (Fig. 6A). Acute liver failure may be associated with neuronal death due to glutamate-induced excitotoxicity (16,24), the effect that, based on our study, may be blocked by FIN.

Although not entirely clear, neurosteroids were suggested to be possibly involved in the development of neuroinflammation in HE (4). The production of cytokines, as well as the expression of microglial markers were found to be increased in experimental acute liver failure
following liver ischemia (29) or azoxymethane treatment (8). In addition, the level of peripheral
neurosteroids was found to be increased at late pregnancy (25), at the same time when the
incidence of HE rises in pregnant women with fulminant liver failure (35). Our results confirm
that TAA-induced HE is accompanied by an increased expression of microglial markers (Fig.
6C). However, since FIN pretreatment did not prevent TAA-induced increase in Iba1 expression,
our study does not support the role of neurosteroids in the inflammation and suggests that
inhibition of neuroinflammation may not be a mechanism of hepatoprotective effects of FIN in
HE.

TAA has different effects on GFAP expression in various brain regions (Fig. 6B), thus
suggesting regional differences in astrocyte response to hyperammonemia. This is in accordance
with previous studies, since in portacaval shunted rat model increased GFAP immunoreactivity
was found in Bergmann glia, specialized cerebellar astrocytes (29), and decreased in frontal
cortex (7). On the other hand, GFAP expression in Bergmann glia in human liver cirrhosis was
not found to be altered, thus indicating that the course of HE affects GFAP expression (30). FIN
pretreatment reduced the expression of GFAP in the hippocampus and the cortex, further
suggesting its protective role on astrocytes in HE.

The therapeutic use of FIN could be possibly limited by its side effects. Some studies
reported persistent diminished libido, erectile dysfunction and depression in a subset of patients
with prostatic hyperplasia and androgenetic alopecia, that may be causally related to FIN therapy
(36,56). Our study found potential adverse effects of FIN on neurons in selective brain regions,
based on an increase in mean EEG voltage (Fig. 5), accompanied with a decrease in NeuN
expression in the cortex, but not in the hippocampus (Fig. 6A). An increase in EEG voltage
suggests a possible increase in neuronal synchronization after FIN treatment, due to a decrease in
GABAergic transmission and an imbalance toward excitation in the brain (2). However, the significance of this effect, as well as ratio between the therapeutic efficacy and side effects of FIN should be further investigated.

Based on our results it can be concluded that FIN improves motor, reflex and EEG changes in TAA-induced type A HE and completely prevents the development of hepatic coma. TAA-induced flattening of EEG is replaced by an increase in mean voltage after FIN pretreatment. In addition, FIN prevents the reduction of neuronal marker expression in the cortex and increase of astrocytic marker in the hippocampus after TAA treatment. Motor and EEG changes suggest that FIN may be used as a potential neuroprotective device in the treatment of type A HE, but possible adverse effects of this therapy should be further investigated.

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DISCLOSURES

No conflict of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Mladenović, Hrnčić, Petronijević, Radosavljević, Rašić-Marković, and Stanojlović participated in research design; Mladenović, Hrnčić, Petronijević, Jevtić, Radosavljević, Rašić-Marković, Puškaš, Maksić, and Stanojlović conducted experiments; Mladenović, Hrnčić,
Petronijević, Jevtić, Radosavljević, Rašić-Marković, Puškaš, Maksić, and Stanojlović contributed new reagents or analytical tools; Mladenović, Hrnčić, Petronijević, Jevtić, Puškaš, Maksić, and Stanojlović performed data analysis; Mladenović, Hrnčić, Petronijević, Jevtić, Radosavljević, Rašić-Marković, Puškaš, Maksić, and Stanojlović wrote or contributed to the writing of the manuscript; Mladenović, Hrnčić, Petronijević, Jevtić, Radosavljević, Rašić-Marković, Puškaš, Maksić, and Stanojlović approved final version of the manuscript.
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Figure captions

Fig. 1. The effects of finasteride (FIN) and thioacetamide (TAA) on blood ammonia concentration. Daily doses of FIN (50 mg/kg) and TAA (300 mg/kg) were administered intraperitoneally in three subsequent days, and FIN was administered 2 h before each dose of TAA. Blood samples from the right side of the heart were collected 24 h after the last dose of TAA.

Significance of the difference was estimated by one way analysis of variance (ANOVA) with Fisher’s post hoc test (**p<0.01 vs. control).

Fig. 2. Histopathological changes of the liver parenchyma in control and experimental groups:

A) Normal liver tissue from healthy untreated control rat,
B) Bridging necrosis in rats treated with TAA,
C) Preserved liver parenchyma in finasteride-treated rats,
D) Necrosis of liver parenchyma in rats pretreated with finasteride followed by TAA.

Arrowhead – mitoses, black asterisk – bridging necrosis, two black asterisks – mild centrilobular necrosis, arrow – necrotic field with associated hemorrhage, plus – ballooning hepatocytes, hashtag – portal mild inflammatory infiltrate (H&E)

Fig. 3. The effects of finasteride (FIN) pretreatment on thioacetamide (TAA)-induced changes in vital (withdrawal, corneal, grasping and righting) reflexes (A), auditory startle and head shake reflex (B) and equilibrium and placement test (C). Daily doses of FIN (50 mg/kg) and TAA (300 mg/kg) were administered in three subsequent days, and FIN was administered 2 h before each dose of TAA in FIN+TAA group. Motor tests were performed 0, 2, 4, 6 and 24 h after the administration of the last dose of TAA.

Significance of the difference was estimated by chi-square test (**p<0.01 vs. control, # p<0.01 vs. TAA group).
**Fig. 4.** The effects of finasteride (FIN) pretreatment on thioacetamide (TAA)-induced changes in general motor activity (A) and exploratory behavior (B).

For further information see Fig. 3.

Significance of the difference was estimated by two-way analysis of variance (ANOVA) with Tuckey’s post hoc test (**p<0.01 vs. control, # p<0.01 vs. TAA group).

**Fig. 5.** The effects of finasteride (FIN) on mean EEG power spectra density in thioacetamide (TAA)-induced hepatic encephalopathy. EEG was registered 22-24 h after treatment and analyzed by fast Fourier transformation at time points 22.5, 23 and 23.5 h. For each time point artifact-free recording was divided into 8 epochs each lasting 12 s.

For further information see Fig. 3.

Significance of the difference was estimated by two-way analysis of variance (ANOVA) with Tuckey’s post hoc test (**p<0.01 vs. control).

**Fig. 6.** The effects of finasteride (FIN) and thioacetamide (TAA) on the expression of neuronal (NeuN) (A), astrocyte (GFAP) (B), microglial (Iba1) (C) and oligodendrocyte (MOG) marker (D) in the rat cortex (Cx) and the hippocampus (Hipp). The expression of markers was determined 24 h after treatment and primary antibodies used for determination were monoclonal mouse anti-NeuN (1:1000), polyclonal rabbit anti-GFAP (1:2000), polyclonal goat anti-Iba1 (1:250), monoclonal mouse anti-MOG (1:1000). After incubation with primary antibodies the membranes were incubated with the horseradish peroxidase (HRP) labeled secondary anti-rabbit (1:2000) or anti-mouse (1:2000) antibody.

For further information see Fig. 3.

Significance of the difference was estimated by two-way analysis of variance (ANOVA) with Tuckey’s post hoc test (**p<0.01 vs. control).
Table 1. The effects of thioacetamide (TAA) and finasteride (FIN) on volume density and number of mitoses in the liver

<table>
<thead>
<tr>
<th>Group</th>
<th>Volume density of damaged tissue (%)</th>
<th>Number of mitoses/1000 hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 (0; 0)</td>
<td>0 (0; 1)</td>
</tr>
<tr>
<td>FIN</td>
<td>0 (0; 0)</td>
<td>0 (0; 1)</td>
</tr>
<tr>
<td>TAA</td>
<td>58.33 (52.78; 66.67)**</td>
<td>2 (0; 4)**</td>
</tr>
<tr>
<td>FIN+TAA</td>
<td>38.89 (13.89; 70.83)**#</td>
<td>0 (0; 1)</td>
</tr>
</tbody>
</table>

Daily doses of FIN (50 mg/kg) and TAA (300 mg/kg) were administered intraperitoneally in three subsequent days, while in FIN+TAA group FIN was administered 2 h before every dose of TAA. Liver samples were collected 24 h after treatment and stereological analysis was performed by the point counting technique using a test system of 36 points. Volume density of damaged tissue was calculated from equation $P_d/P_t$, where $P_d$ is a number of points falling on damaged area and $P_t$ is a total number of test points. Hepatocytes in mitosis were counted in 7 randomly selected fields with minimal hepatocyte damage. Only cells with clear morphological characteristics of metaphase, anaphase, and telophase were counted.

Data are presented as medians with 25th and 75th percentiles in parentheses. Statistical significance of the difference was evaluated by Kruskall-Wallis nonparametric ANOVA with Mann-Whitney $U$ post hoc test (** $p<0.01$ vs. control, # $p<0.01$ vs. TAA group).
Table 2. The effects of thioacetamide (TAA) and finasteride (FIN) on total behavioral score of experimental animals

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>FIN</th>
<th>TAA</th>
<th>FIN+TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 (40,40)</td>
<td>40 (40, 40)</td>
<td>15 (1, 22)**</td>
<td>34 (34, 35.25)* #</td>
</tr>
<tr>
<td>2</td>
<td>40 (40,40)</td>
<td>40 (40, 40)</td>
<td>10 (1, 20)**</td>
<td>33.5 (33, 34.25)* #</td>
</tr>
<tr>
<td>4</td>
<td>40 (40,40)</td>
<td>40 (40, 40)</td>
<td>10 (1, 17)**</td>
<td>33.5 (32.25, 34.25)* #</td>
</tr>
<tr>
<td>6</td>
<td>40 (40,40)</td>
<td>40 (40, 40)</td>
<td>10 (1, 17)**</td>
<td>34 (32.5, 35)* #</td>
</tr>
<tr>
<td>24</td>
<td>40 (40,40)</td>
<td>40 (40, 40)</td>
<td>4 (0, 4)**</td>
<td>34 (33, 34)* #</td>
</tr>
</tbody>
</table>

Daily doses FIN (50 mg/kg) and TAA (300 mg/kg) were administered intraperitoneally in three subsequent days, while in FIN+TAA group FIN was administered 2 h before every dose of TAA. Total behavioral score was calculated as a sum of scores that animals scored up in all performed tests. In each test animal could score up to 4 points: score 4 if animal performed test at 75-100% of the control value; score 3, at 50-75% of the control value; score 2, at 25-50% of the control value; score 1, at 1-25% of the control value while score 0 indicated that animal showed no response in the test. Data are presented as medians with 25th and 75th percentiles in parentheses. Statistical significance of the difference was evaluated by Friedman nonparametric ANOVA (*p<0.05 and ** p<0.01 vs. control, # p<0.01 vs. TAA).