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The antidepressant drugs vortioxetine and duloxetine differentially and sex-dependently affect animal well-being, cognitive performance, cardiac redox status and histology in a model of osteoarthritis

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ABSTRACT

Osteoarthritis represents a leading cause of disability with limited treatment options. Furthermore, it is frequently accompanied by cardiovascular and cognitive disorders, which can be exacerbated by osteoarthritis or drugs used for its treatment. Here, we examined the behavioral and cardiac effects of the novel antidepressant vortioxetine in an osteoarthritis model, and compared them to duloxetine (an established osteoarthritis treatment). Osteoarthritis was induced in male and female rats with an intraarticular sodium-monoiodoacetate injection. Antidepressants were orally administered for 28 days following induction. During this period the acetone, burrowing and novel-object-recognition tests (NORT) were used to assess the effects of antidepressants on pain hypersensitivity (cold allodynia), animal well-being and cognitive performance, respectively. Following behavioral experiments, heart muscles were collected for assessment of redox status/histology. Antidepressant treatment dose-dependently reduced cold allodynia in rats with osteoarthritis. Duloxetine (but not vortioxetine) depressed burrowing behavior in osteoarthritic rats in a dose-related manner. Osteoarthritis induction reduced cognitive performance in NORT, which was dose-dependently alleviated by vortioxetine (duloxetine improved performance only in female rats). Furthermore, duloxetine (but not vortioxetine) increased oxidative stress parameters in the heart muscles of female (but not male) rats and induced histological changes in cardiomyocytes indicative of oxidative damage. Vortioxetine displayed comparable efficacy to duloxetine in reducing pain hypersensitivity. Furthermore, vortioxetine (unlike duloxetine) dose-dependently improved cognitive performance and had no adverse effect on burrowing behavior (animal surrogate of well-being) and cardiac redox status/histology. Our results indicate that vortioxetine could be a potential osteoarthritis treatment (with better characteristics compared to duloxetine).

1. Introduction

Osteoarthritis represents the most prevalent chronic rheumatic disease which affects approximately 250 million patients globally and is one of the leading causes of disability. This disorder most commonly affects knee joints and is associated with the primary symptom of joint pain, with consequent limitation of daily activities and reduction in the quality of life [1]. Furthermore, osteoarthritis is increasingly being recognized as a disease with significant systemic complications. Most notably, observational studies have linked osteoarthritis to an increased

Abbreviations: AOPP, advanced oxidation protein products; AUC, area under the curve; GABA, gamma-aminobutyric acid; MDA, malondialdehyde; MIA, monoiodoacetate; NMDA, N-methyl-D-aspartate; NORT, novel object recognition test; NSAID, non-steroidal anti-inflammatory drug; PAB, pro-oxidant-antioxidant balance; SHG, SH groups; SNRI, serotonin and noradrenaline reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; SOD, superoxide dismutase; TOS, total pro-oxidant status; VTA, ventral tegmental area.

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risk of cardiovascular disorders (such as hypertension, myocardial infarction and stroke) and cognitive decline [2,3]. A complex link exists between osteoarthritis and these comorbidities; they share common risk factors (e.g. aging and obesity), but also some studies have suggested that osteoarthritis may be an independent risk factor for cognitive decline and cardiovascular disorders (*via* reduction in physical activity and increase in certain inflammatory mediators) [2,3].

Despite its high prevalence, the available pharmacologic options for treating osteoarthritis are limited, and the majority of available drugs only alleviate joint pain [1]. Most professional guidelines recommend the use of non-steroidal anti-inflammatory drugs (NSAIDs) as the treatment option with the best documented efficacy [4]. However, some patients do not respond to NSAIDs, and their prolonged use is associated with serious gastrointestinal, renal and cardiovascular adverse effects, especially in elderly patients in whom osteoarthritis is more common [4,5]. In cases where NSAIDs are ineffective/have serious side effects, alternative pharmacological options can be used to relieve joint pain. One of these options is the antidepressant drug duloxetine (a serotonin and noradrenaline reuptake inhibitor; SNRI), which is thought to primarily target central pain modulatory mechanisms that contribute to pain chronification and NSAID inefficacy [4,6,7]. However, duloxetine also has limited efficacy and certain adverse effects (e.g., hypertension, orthostatic hypotension, tachycardia) that are best avoided in the typical osteoarthritis patient [8,9].

Vortioxetine is a novel antidepressant drug that acts as a serotonin reuptake inhibitor and directly modulates the activity of several serotonin receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_3 and 5-HT_7) [10,11]. Because of its unique mechanism of action, vortioxetine possesses two advantages over other antidepressant drugs, a better safety profile (e.g., lower incidence of weight gain and cardiovascular adverse effects) and consistent pro-cognitive effects [11], which could be of particular benefit in osteoarthritis patients who frequently have comorbid cardiovascular and cognitive disorders. Previous preclinical studies have found that vortioxetine effectively reduces pain hypersensitivity in neuropathic/inflammatory pain models, and there is also some clinical data that vortioxetine possesses analgesic properties (in the treatment of burning mouth syndrome) [12–14].

Based on previous (pre)clinical findings [12–14], we speculated that vortioxetine could be effective in reducing pain hypersensitivity associated with osteoarthritis. Furthermore, given vortioxetine's unique mechanism of action [10,11] we theorized that it could exert beneficial effects on other behavioral endpoints (indicative of cognitive function and animal well-being) in animals with osteoarthritis, without adversely affecting cardiac structure. In order to increase the translational value of our results, we decided to examine vortioxetine's effects in both male and female rats, seeing as osteoarthritis is more prevalent in female patients [1] and to compare them to duloxetine, an established osteoarthritis treatment option [4].

2. Materials and methods

2.1. Animals

The experiments were performed on male and female Wistar rats, weighing 180–220 g (male) or 150–200 g (female) at the beginning of the study. The animals were acquired from the Military Academy Breeding Farm (Belgrade, Serbia) and housed under standard laboratory conditions: temperature of 22 ± 1 °C, relative humidity of 60% and a 12/12 h light/dark cycle. Food and water were freely available, except during experimental measurements. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia (permit number: 323–07–09456/2020–05) and were conducted in accordance with EU Directive 2010/63/EU for animal experiments. The following numbers of animals were used in individual groups: saline control groups (10 male and 9 female rats), monoiodoacetate control groups (10 male and 10 female rats), vortioxetine-treated animals (19 male and 18 female rats) and duloxetine-treated animals (19 male and 19 female rats).

2.2. Induction of osteoarthritis and experimental design

Osteoarthritis was induced with a single intraarticular (i.a.) injection of sodium monoiodoacetate (MIA; Sigma-Aldrich Chemie GmbH, Germany) into the knee joint of the right hind limb. We used 2 mg of MIA (dissolved in saline) which was delivered in a volume of 25 μL, using a microliter syringe and a 26 G needle [7]. Before the i.a. injection, the animals were briefly anesthetized using sevoflurane (Sevorane®, Abbvie s.r.l., Italy), following which the area around the right knee was shaved and disinfected with 70% ethanol. Then, the needle was inserted through the infra-patellar ligament of the knee joint, and the MIA solution was slowly injected. After the injection, the needle was gradually retracted and the knee was gently bent several times to allow MIA distribution. Saline control animals received the same volume of saline into the knee joint of the right hind limb.

After osteoarthritis induction, the animals received daily treatments (antidepressant drugs or vehicle) for 28 consecutive days (Fig. 1). Vortioxetine (Brintellix, H. Lundbeck A/S, Denmark) or duloxetine (Taita®, Hemofarm AD Vršac, Serbia) were suspended in distilled water and applied orally (p.o.) in a volume of 2 mL/kg using an oral gavage for rats. During these 28 days the animals were subjected to different behavioral tests (see below; Fig. 1). The doses of duloxetine (15 and 25 mg/kg/day) and vortioxetine (2 and 10 mg/kg/day) were chosen based on our preliminary results and literature data [6,13,15]. A.M. and U.P. randomly allocated individual animals to different treatment groups and applied the treatments, whereas K.N. performed the behavioral experiments and was blinded to the treatment the animals received. On day 28 after osteoarthritis induction (approximately 24 h after the last oral treatment) the animals were deeply anesthetized with sevoflurane and sacrificed with cervical dislocation. At this time point we collected the heart muscles. One part of heart muscle tissue of each animal was frozen (−80°C) for assessment of redox parameters, and the other part was placed in formaldehyde solution used for histological examination (see below for further information). Heart muscle tissue for the biochemical and morphological analysis was obtained through complete transverse sections of the ventricle at regular 4 mm intervals from apex to mid-ventricular level [16].

2.3. Assessment of pain-hypersensitivity (cold allodynia)

Sensitivity to cold stimuli was assessed using the acetone test [13]. Animals were placed in plastic boxes set on top of an elevated metallic mesh floor. A single drop of acetone was applied to the right hind paw following which we measured time spent in nociceptive behavior (licking, flicking and/or shaking of the acetone-treated paw) during a 60 s observation period. Sensitivity to cold stimuli was assessed before osteoarthritis induction (basal measurement), and on days 4, 7, 10, 14, 21 and 28 following MIA injection (Fig. 1). These measurements were performed before the daily application of antidepressant treatments.

2.4. Assessment of animal well-being (burrowing test)

To examine the influence of osteoarthritis induction and prolonged antidepressant treatment on animal well-being, we assessed the burrowing behavior of experimental animals. Burrowing represents an innate, self-rewarding behavior and is thought to represent a rodent correlate of human activities of daily living (a measure of quality of life) [17,18]. Additionally, depression of burrowing behavior is thought to represent a measure of spontaneous pain and has been found in animal models of joint pain [19,20].

For assessing burrowing behavior, animals were trained for 3 days before osteoarthritis induction in order to establish baseline values and

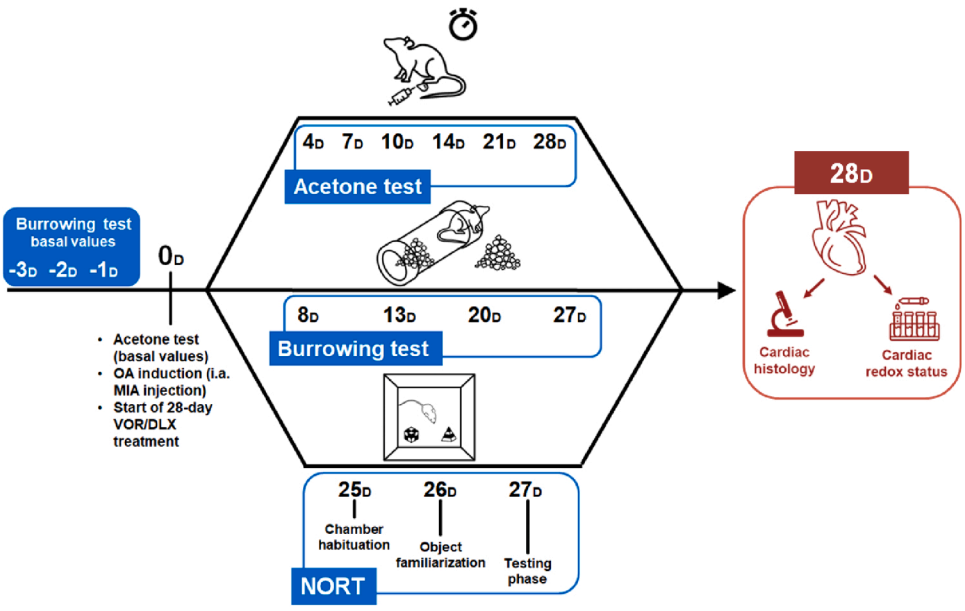

Fig. 1. Protocol for examination of the effects of prolonged vortioxetine (VOR) and duloxetine (DLX) treatment in the model of osteoarthritis induced with monoiodoacetate (MIA). The drugs were administered orally for 28 days, starting on the day of MIA intraarticular (i.a.) injection (day 0). Baseline values for behavioral tests (acetone and burrowing test) were obtained before MIA injection. Behavioral assessments were performed multiple times on the days indicated in the figure. Twenty-nine days following MIA injection (day 28) the rats were sacrificed and their heart muscles were harvested for examination of cardiac redox status and histological assessment.

to exclude animals which are poor burrowers (i.e., burrowed less than 500 g of burrowing material) (Fig. 1). Long plastic tubes (32 cm in length and 10 cm in diameter) were filled with 2000 g of burrowing material (gravel with individual pieces which were 2–6 mm in diameter) and placed in cages so that the opening of the tube was elevated by around 6 cm from the bottom of the cage. On the first training day, 2 rats were placed into each cage with the burrowing tube (around 16.00 h) and were allowed to burrow for the entire night; this was done because social facilitation is thought to positively contribute to development of burrowing behavior [17]. On the second and third day, only one rat was placed per cage and allowed to burrow the entire night. The average amount of gravel burrowed during the second and third day of training was used as a baseline value. After osteoarthritis induction, the burrowing test was repeated on days 8, 13, 20 and 27 post-MIA injection (Fig. 1). The animals were always placed at 16.00 h and left overnight to burrow. The amount of gravel burrowed during the night was measured in the morning on the following day.

2.5. Novel object recognition test (NORT)

The influence of osteoarthritis induction and prolonged antidepressant treatment on cognitive performance of rats was evaluated with the novel object recognition test (NORT) as previously described [21]. The test was performed in a rectangular chamber (65 ×45 ×45 cm) under dim illumination (approximately 20 lux). Rats were first habituated for 10 min to the test chamber, 24 h before the beginning of the experiment (25 days post-MIA injection; Fig. 1). On the next day (familiarization phase) two identical objects were placed in the chamber (15 cm from the sides of the chamber and 25 cm apart). Each animal was placed into the chamber (between the two objects and facing the opposite wall) and allowed to explore the objects for 5 min (after which they were returned to their home cages). Twenty-four hours after the familiarization phase (day 27 post-MIA injection), object recognition (testing phase) was evaluated by replacing one object used during the familiarization phase with a novel object and the animal was returned to the chamber and allowed to explore the familiar and novel object. The time spent examining each object was recorded during 5 min and was monitored with Any-Maze video-tracking software (Stoelting Co., USA). The rats were considered to be exploring the object if they were in close proximity (2 cm) and oriented to the object, or if they sniffed/pawed the object. The following objects were used in the NORT: metal cans and glass cylinders of various shapes and sizes (each object was sufficiently large to prevent animals from displacing it). After each trial, the apparatus and objects were cleaned with 70% ethanol to reduce olfactory cues. We calculated the time spent exploring the familiar object (O) and novel object (N), as well as the discrimination index which was calculated according to the following formula: (N-O)/(N + O) x 100 (%).

2.6. Analysis of the redox status of heart muscle

Samples of the rat heart muscles were homogenized in ice cold 0.1 M phosphate buffer (pH = 7.4) in a 1:9 weight-to-volume ratio with the use of the T10 basic Ultra-Turrax homogenizer (IKA, Germany). After homogenization, the samples were placed in a cooling centrifuge for 10 min at 800 x g, and then for 20 min at 9500 x g to obtain post-mitochondrial supernatants [22]. In supernatants, we measured the levels/activity of several pro-oxidant markers and products of their activity, as well as enzymatic and non-enzymatic antioxidants using previously described methods [22,23]. These included the levels of superoxide anion radicals, total pro-oxidant status (TOS), pro-oxidant-antioxidant balance (PAB), levels of advanced oxidation protein products (AOPP) and malondialdehyde (MDA). The activity of superoxide dismutase (SOD) and level of SH groups (SHG) were measured to assess enzymatic and non-enzymatic antioxidant activity, respectively. All results from the analysis of heart redox parameters were normalized to the protein concentration of supernatants, which was determined using the Bradford method [22]. The level of superoxide anion, TOS and SOD activity were determined on the ILAB 300 Plus analyzer (Instrumentation Laboratory, Italy), whereas PAB, AOPP, MDA, SHG and protein concentrations were evaluated on a continuous spectrophotometer (Pharmacia LKB, UK).

2.7. Assessment of cardiac histology

Assessment of histology of cardiac muscles was performed according to previously reported methods [24]. In brief, samples of the apex of the heart muscles were fixed with 4% neutral-buffered formaldehyde (FNB4–10 l, BioGnost Ltd., Croatia) for 24 h by an immersion procedure. Afterwards, the samples were dehydrated (with increasing concentrations of alcohol) and embedded in paraffin. Histological tissue specimens were processed by using a modular automated tissue processor (Leica TP1020 Leica Biosystems Nussloch GmbH, Germany) according to following defined protocol: 4% neutral-buffered formaldehyde (4% NB Formaldehyde FNB4–10 l, BioGnost Ltd., Croatia)

2 × 1.5 h – 2 × 2 h, 70% alcohol (Histanol 70, H70–5 L, BioGnost Ltd., Croatia) 1.5–3 h, 96% alcohol (Histanol 96, H96–5 L, BioGnost Ltd., Croatia) 1.5–3 h, 100% alcohol (Histanol 100, H100–5 L, BioGnost Ltd., Croatia) 3 × 1.5 h, xylol (Ksilen pro analysis, Zorka Pharma, Serbia) 2 × 1.5 h, paraffin (Biowax Plus 56/58, BWPLUS-1, BioGnost Ltd., Croatia) 3 × 1.5 h. Tissue was embedded by using the Leica HistoCore Arcadia H Leica Biosystems (Nussloch GmbH, Germany) and serially sectioned with a microtome (Leica RM2125RTS, Leica Biosystems Nussloch GmbH, Germany). For morphological evaluation, individual 4 μm thick sections of heart muscle tissue were stained with hematoxylin and eosin and Masson trichrome staining technique and analyzed by using the Leica DM2000 LED microscope and with Leica ICC50 E camera equipped with the LAS V4.12 software. The guidelines for morphological evaluation were established according to the protocol of The Royal College of Pathologists [16]. For each animal, we analyzed a total of 4 cross sections (which were obtained from two distinct parts/levels of the heart, separated by at least 2 mm).

The following histological parameters were analyzed and included into the report on histological analysis of cardiomyocytes: orientation, cross striation, intercalated disks formation, morphology of nuclei, morphological signs of cardiomyocytes atrophy or hypertrophy, cellular vacuolization, intracellular edema, or contraction band necrosis. Separate sections included morphological reports on the morphology of interstitial tissue, interstitial blood vessels and elements of coronary circulation.

2.8. Statistical analysis

The statistical analysis was performed using SigmaPlot 11 (Systat Software Inc., Richmond, CA, USA). The results are presented as mean group values ± SEM obtained from groups of 6–10 animals (the size of groups was chosen based on our preliminary behavioral experiments in the MIA-induced osteoarthritis model). The statistical analysis was performed separately for results obtained in male and female rats. Data was assessed for normality with the Shapiro-Wilk test ($P > 0.05$). Time course data from the acetone and burrowing test were analyzed using two-way repeated measures ANOVA (type of treatment was the between-subject factor, and time was the within-subject factor) with *post-hoc* Tukey test. In addition, for data obtained in the acetone and burrowing test, we calculated the area under the curve (AUC) values, using the trapezoidal method. AUC data were statistically analyzed using one-way ANOVA with *post-hoc* Tukey test. Data from NORT were analyzed using two-way repeated measures ANOVA (time spent exploring the novel or old object; type of treatment was the between subject factor and type of object, novel or old, was the within-subject factor) or one-way ANOVA with *post-hoc* Tukey test (differences between the discrimination indexes among different experimental groups). The results for redox status parameters of heart muscles were analyzed with one-way ANOVA with *post-hoc* Tukey test. A value of P less than 0.05 was considered significant in all statistical tests that were used.

3. Results

3.1. Vortioxetine and duloxetine alleviated cold allodynia in animals with osteoarthritis

When we analyzed data from the acetone test, we found a significant effect of type of treatment on time spent in nociceptive behavior during the time course of the experiment ($P < 0.001$ for both male and female rats) and AUC data ($F_{(5,44)} = 8.241$ and $P < 0.001$ for male rats; $F_{(5,48)} = 6.996$ and $P < 0.001$ for female rats) (Fig. 2).

Animals of both sexes with MIA-induced osteoarthritis developed cold allodynia, i.e., they spent significantly more time in nociceptive behavior in the acetone test compared to saline control animals (*post-hoc* $P < 0.001$ for both time course and AUC data; Fig. 2).

Vortioxetine significantly reduced the time spent in nociceptive behavior in the acetone test in male and female rats with MIA-induced osteoarthritis. In male rats, the *post-hoc P* values (compared to the MIA control) for time course data were < 0.001 for both doses of vortioxetine (Fig. 2A), whereas the *post-hoc P* values for female rats were 0.002 (2 mg/kg/day) and < 0.001 (10 mg/kg/day) (Fig. 2B). Additionally, vortioxetine significantly reduced AUC values compared to MIA controls in both male (*post-hoc* $P = 0.001$ and $P < 0.001$ for 2 and 10 mg/kg/day, respectively; Fig. 2E) and female rats (*post-hoc* $P = 0.002$ and $P < 0.001$ for 2 and 10 mg/kg/day, respectively; Fig. 2F). Based on the AUC values (percent reduction compared to MIA control AUC values), vortioxetine's effects were dose-related: the 2 mg/kg/day dose produced an effect of 65.2% in male and 67.2% in female rats, and the 10 mg/kg/day dose produced effects of 84.4% in male and 71.9% in female rats.

Duloxetine significantly reduced nociceptive behavior in the acetone test in male rats with MIA-induced osteoarthritis. The *post-hoc P* values (compared to the MIA control) determined for time course data were 0.011 and 0.010 for 15 and 25 mg/kg/day, respectively (Fig. 2C). Similarly, AUC values of duloxetine-treated male rats were significantly lower compared to AUC values of the MIA control group (*post-hoc* $P = 0.026$ and 0.015 for 15 and 25 mg/kg/day, respectively; Fig. 2E). In female rats, only the higher tested dose of duloxetine (25 mg/kg/day) significantly reduced cold allodynia compared to the MIA control (*post-hoc* $P = 0.005$ for time course data and *post-hoc* $P = 0.005$ for AUC data), whereas the lower tested dose (15 mg/kg/day) did not significantly affect nociceptive behavior in the acetone test (*post-hoc* $P = 0.387$ for time course data and *post-hoc* $P = 0.304$ for AUC data; Fig. 2, D and F). Compared to AUC values of MIA control animals, duloxetine dose-dependently reduced cold allodynia by 48.8% and 53.7% in male rats and 34.1% and 57.6% in female rats (for the 15 and 25 mg/kg/day doses, respectively).

We did not detect a significant difference between the effects produced by duloxetine and vortioxetine in male rats in the acetone test. The *post-hoc P* values for the largest difference in antinociceptive effects (between vortioxetine 10 mg/kg/day and duloxetine 15 mg/kg/day) was 0.253 for time course data and 0.195 for AUC data. In female rats, the *post-hoc P* value for the largest difference in antinociceptive effects (also between vortioxetine 10 mg/kg/day and duloxetine 15 mg/kg/day) was 0.114 for time course data and 0.179 for AUC data.

3.2. Duloxetine depressed burrowing behavior in rats with osteoarthritis

We found a significant effect of type of treatment on the amount of gravel that rats burrowed during time course of the experiment ($P = 0.002$ for male and $P < 0.001$ for female rats), as well as on AUC data determined for the burrowing test ($F_{(5,43)} = 5.408$ and $P < 0.001$ for male rats; $F_{(5,45)} = 10.533$ and $P < 0.001$ for female rats) (Fig. 3).

In the burrowing test, osteoarthritis induction did not lead to a significant change in the amount of gravel burrowed during the night, i.e. saline and MIA control animals of both sexes burrowed similarly during the entire period of examination (*post-hoc* $P \geq 0.998$ for time course data and *post-hoc* $P \geq 0.999$ for AUC data; Fig. 3).

Vortioxetine had no significant effect on burrowing behavior of male rats (*post-hoc* $P \geq 0.999$ for time course data and *post-hoc* $P \geq 0.991$ for AUC data compared to the MIA control group; Fig. 3, A and E). In female vortioxetine-treated rats, there was a slight depression of burrowing behavior compared to MIA control rats (Fig. 3, B and F). This effect was not statistically significant when we analyzed time course data (*post-hoc* $P \geq 0.399$) or the reduction in AUC values compared to MIA control (*post-hoc* $P \geq 0.298$).

In male rats, the higher tested duloxetine dose (25 mg/kg/day) significantly reduced burrowing behavior compared to MIA control rats (*post-hoc* $P = 0.005$ for time course data and *post-hoc* $P = 0.003$ for AUC data), whereas the lower tested dose (15 mg/kg/day) had no significant effect (*post-hoc* $P = 0.842$ for time course data and *post-hoc* $P = 0.741$ for AUC data; Fig. 3, C and E). AUC values obtained in male rats treated with

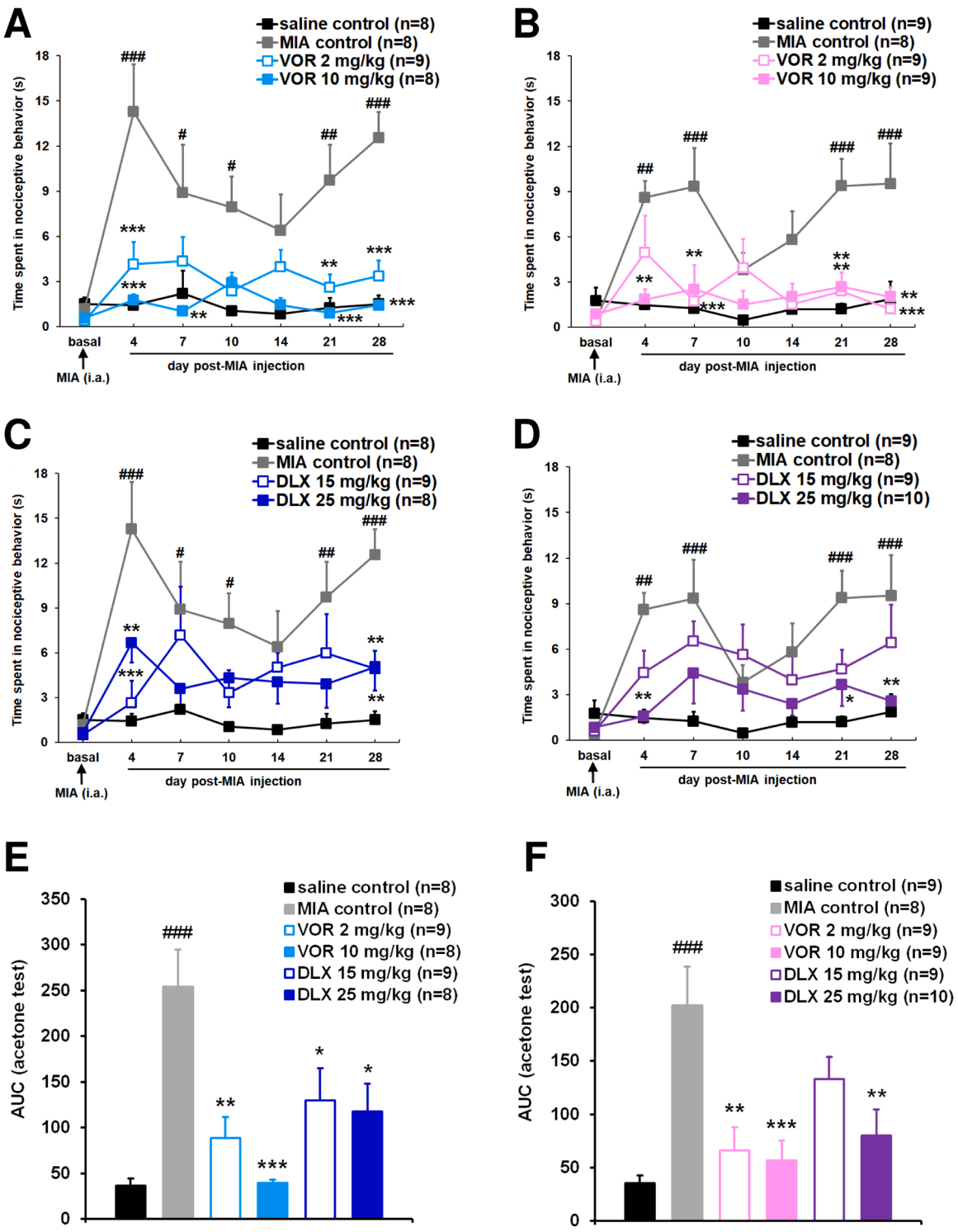

Fig. 2. Vortioxetine (VOR) and duloxetine (DLX) reduce cold allodynia in male and female rats with monoiodoacetate (MIA)-induced osteoarthritis. Results from the acetone test are presented as time course data (panels A-D - time in seconds spent in nociceptive behavior following acetone application) and area-under-the-curve (AUC) data obtained during the 28 days of the experiment (panels E and F). Results for male rats are represented with light and dark blue coloring (panels A, C and E) and for female rats with pink and purple coloring (panels B, D and F). Antidepressant drugs, VOR (panels A and B) and DLX (panels C and D), were administered orally for 28 days starting on the day of MIA intraarticular (i.a.) injection (denoted by arrow). Statistical significance was determined in comparison with the saline control group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$) or MIA control group ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) with two-way repeated measures ANOVA (time course data) or one-way ANOVA (AUC data), followed by Tukey *post-hoc* test. The number of animals per group (n) is indicated in the legends of individual panels.

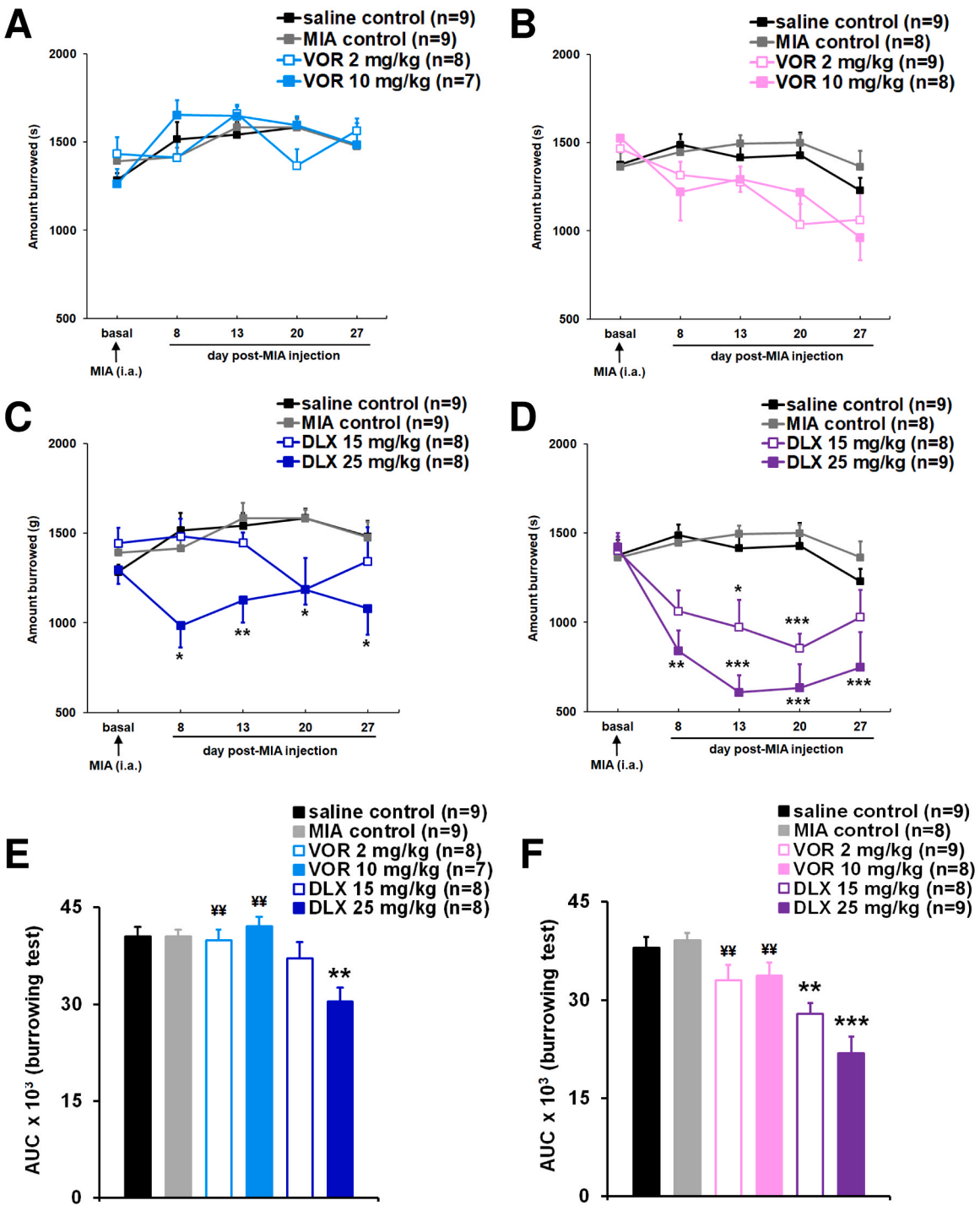

Fig. 3. The effects of vortioxetine (VOR) and duloxetine (DLX) on burrowing behavior of male and female rats with monoiodoacetate (MIA)-induced osteoarthritis. Results from the burrowing test are presented as time course data (panels A-D – amount of gravel burrowed overnight in grams), and area-under-the-curve (AUC) data obtained during the 28 days of the experiment (panels E and F). Results for male rats are represented with light and dark blue coloring (panels A, C and E) and for female rats with pink and purple coloring (panels B, D and F). Antidepressant drugs, VOR (panels A and B) and DLX (panels C and D) were administered orally for 28 days starting on the day of MIA intraarticular (i.a.) injection (denoted by arrow). Statistical significance was determined in comparison with the MIA control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) or the DLX 25 mg/kg group ($^{¥¥}P < 0.01$), with two-way repeated measures ANOVA (time course data) or one-way ANOVA (AUC data), followed by Tukey *post-hoc* test. The number of animals per group (n) is indicated in the legends of individual panels.

25 mg/kg/day were 24.9% lower compared to MIA control values. In female rats, both tested duloxetine doses significantly reduced burrowing behavior (*post-hoc* $P = 0.015$ and < 0.001 for 15 and 25 mg/kg/day, respectively; Fig. 3D) and significantly reduced AUC values compared to the MIA control group (*post-hoc* $P = 0.005$ and < 0.001 for 15 and 25 mg/kg/day, respectively; Fig. 3F). Duloxetine's effects in female rats were dose-related; AUC values were reduced by 28.6% (15 mg/kg/day) and 44.1% (25 mg/kg/day) compared to female MIA control AUC values.

Moreover, in both male and female rats we found that groups that were treated the higher tested duloxetine dose burrowed significantly less than vortioxetine-treated animals. Compared to the corresponding

groups treated with duloxetine 25 mg/kg/day, the *post-hoc P* values were ≤ 0.008 (vortioxetine 2 mg/kg/day) and ≤ 0.006 (vortioxetine 10 mg/kg/day) for time course data obtained in male and female rats. Similar results were obtained for AUC data and the *post-hoc P* values were ≤ 0.008 (vortioxetine 2 mg/kg/day) and ≤ 0.002 (vortioxetine 10 mg/kg/day) for male and female animals (Fig. 3, E and F).

3.3. Vortioxetine and duloxetine differentially affect cognitive performance in NORT

In NORT we found that osteoarthritis induction leads to impaired cognitive performance, i.e. saline control rats of both sexes spent significantly more time exploring the new object (*post-hoc* $P < 0.001$ for both male and female rats), whereas rats with osteoarthritis spent a similar amount of time exploring the familiar and novel object (*post-hoc* $P = 0.488$ for male rats and 0.641 for female rats; Fig. 4). Furthermore, when we analyzed the discrimination indexes we found a significant effect of type of treatment for both male ($F_{(5,46)} = 3.542$, $P = 0.009$) and female animals ($F_{(5,45)} = 3.814$, $P = 0.006$). In the *post-hoc* analysis, we found that the discrimination indexes of MIA control animals were significantly smaller than the discrimination indexes determined for saline control animals of both sexes (*post-hoc* $P = 0.015$ for male and 0.049 for female rats; Fig. 4).

Prolonged vortioxetine treatment improved cognitive performance of rats with osteoarthritis. In male rats, both tested vortioxetine doses produced a significant effect and animals spent significantly more time exploring novel objects compared to the old ones (the *post-hoc P* values were 0.001 for vortioxetine 2 mg/kg/day and $P < 0.001$ for vortioxetine 10 mg/kg/day; Fig. 4A). There was also a dose-related increase of the discrimination index as compared to MIA control animals; however, the differences were not statistically significant (*post-hoc* $P = 0.328$ for 2 mg/kg/day and *post-hoc* $P = 0.111$ for 10 mg/kg/day; Fig. 4E). Similar results were obtained in vortioxetine-treated female rats; the *post-hoc P* values for the difference in exploring the novel versus the old object were 0.052 for 2 mg/kg/day and $P < 0.001$ for 10 mg/kg/day (Fig. 4B). Furthermore, vortioxetine increased the discrimination index compared to female MIA control animals and the difference was statistically significant for the higher tested dose (*post-hoc* $P = 0.364$ for 2 mg/kg/day and *post-hoc* $P = 0.006$ for 10 mg/kg/day; Fig. 4F).

In male rats, duloxetine treatment did not produce a significant effect in the NORT and animals spent a similar amount of time exploring novel and old objects (*post-hoc* $P = 0.155$ for 15 mg/kg/day and *post-hoc* $P = 0.435$ for 25 mg/kg/day; Fig. 4C). Furthermore, the lower tested dose slightly increased the discrimination index; however, this was not significantly different from the discrimination index of male MIA control animals (*post-hoc* $P = 0.536$ for 15 mg/kg/day and *post-hoc* $P = 0.999$ for 25 mg/kg/day; Fig. 4E). In female rats, both tested doses of duloxetine produced a significant effect in NORT. Female rats treated with 15 mg/kg/day spent significantly more time exploring the novel object compared to the old one (*post-hoc* $P < 0.001$; Fig. 4D) and their discrimination index was significantly higher than the discrimination index of female MIA control animals (*post-hoc* $P = 0.009$; Fig. 4F). The higher tested duloxetine dose (25 mg/kg/day) had a smaller effect compared to the lower dose in female rats in NORT (*post-hoc* $P = 0.049$ for the difference in time spent exploring the novel *vs* the old object and *post-hoc* $P = 0.223$ for the discrimination index compared to MIA control; Fig. 4, D and F).

With regard to discrimination index data, we did not find a significant difference between vortioxetine and duloxetine-treated male (*post-hoc* $P \geq 0.193$) and female animals (*post-hoc* $P \geq 0.590$).

3.4. Duloxetine increased oxidative stress parameters in hearts of female rats with osteoarthritis

In male rats, we did not detect a significant effect of type of treatment on oxidative stress parameters that were determined in heart muscles, i.e. neither osteoarthritis induction, nor antidepressant drug treatment significantly affected oxidative stress parameters: superoxide anion radical levels ($F_{(5,50)} = 0.137$, $P = 0.983$), TOS ($F_{(5,50)} = 1.969$, $P = 0.100$), PAB ($F_{(5,50)} = 0.545$, $P = 0.741$), AOPP ($F_{(5,50)} = 1.152$, $P = 0.346$) and MDA ($F_{(5,50)} = 0.858$, $P = 0.516$) (Supplemental Fig. 1). Furthermore, treatments did not have a significant effect on SOD activity ($F_{(5,44)} = 1.748$, $P = 0.144$) and SHG ($F_{(5,50)} = 1.672$, $P = 0.159$) in heart muscles of male rats (not shown).

On the other hand, in female rats we detected a significant effect of type of treatment on superoxide anion radical levels ($F_{(5,46)} = 8.310$, $P < 0.001$) and PAB ($F_{(5,46)} = 3.037$, $P = 0.019$) determined in heart muscles. The effect on AOPP was borderline significant ($F_{(5,46)} = 2.107$, $P = 0.082$), with the largest difference being between the MIA control group and duloxetine 15 mg/kg/day group. We did not detect a significant treatment influence on TOS ($F_{(5,46)} = 1.916$, $P = 0.110$) and MDA ($F_{(5,46)} = 0.326$, $P = 0.894$) (Fig. 5). In the case of superoxide anion radical levels and PAB, *post-hoc* tests revealed that osteoarthritis induction was not associated with significant changes in these redox parameters in the hearts of female rats (*post-hoc P* values were ≥ 0.918 for the comparison between saline and MIA controls). Similarly, prolonged vortioxetine treatment did not produce a significant change in superoxide anion radical levels and PAB (*post-hoc P* values were ≥ 0.466 for both tested parameters in comparison to the MIA control). On the other hand, both tested doses of duloxetine (compared to the MIA control) significantly increased superoxide anion radical levels (*post-hoc* $P = 0.001$ and $P = 0.017$ for 15 and 25 mg/kg/day, respectively) and the lower tested dose significantly increased PAB (*post-hoc* $P = 0.036$ and $P = 0.192$ for 15 and 25 mg/kg/day, respectively). Furthermore, we found that the level of superoxide anion radicals in female rats treated with vortioxetine 2 mg/kg/day were significantly lower than levels in rats treated with duloxetine 15 mg/kg/day (*post-hoc* $P = 0.003$) and 25 mg/kg/day (*post-hoc* $P = 0.032$). Moreover, in female rats we detected a significant influence of treatment on SOD activity ($F_{(5,46)} = 2.731$, $P = 0.030$), but not on SHG ($F_{(5,46)} = 1.664$, $P = 0.162$) (not shown). However, *post-hoc* testing found that the effects of vortioxetine and duloxetine on SOD activity (compared to the MIA control) were not significant in female rats (*post-hoc* $P \geq 0.474$).

3.5. Duloxetine produces histological changes in the heart muscles of animals with osteoarthritis

Osteoarthritis induction in rats of both sexes did not lead to changes in the histology of heart muscles. In saline and MIA control groups cardiomyocytes displayed regular morphology (no overt hypertrophy or atrophy), proper arrangement of myofibrils and had one or two centrally located oval euchromatic nuclei. Furthermore, we did not observe any changes in interstitial tissue, interstitial blood vessels and elements of coronary circulation (not shown).

Prolonged vortioxetine treatment was not associated with changes in cardiomyocyte morphology, cardiac interstitial tissue and interstitial blood vessels in rats with osteoarthritis (not shown). On the other hand, in duloxetine-treated rats, we found changes in cardiomyocyte morphology. Most notably we observed vacuolation of cardiomyocytes, intracellular edema, hypercontraction of sarcomeres, a certain degree of cytoplasm condensation, contraction band necrosis phenomena and, in some specimens, edema of interstitial tissue and perivascular edema. These changes were more pronounced in heart muscles of female duloxetine-treated rats and in groups that received the higher tested duloxetine dose (Fig. 6).

4. Discussion

Vortioxetine displayed comparable efficacy to duloxetine (an established osteoarthritis treatment option) in reducing pain hypersensitivity of male and female animals with MIA-induced osteoarthritis. One of the mechanisms thought to contribute to pain generation/

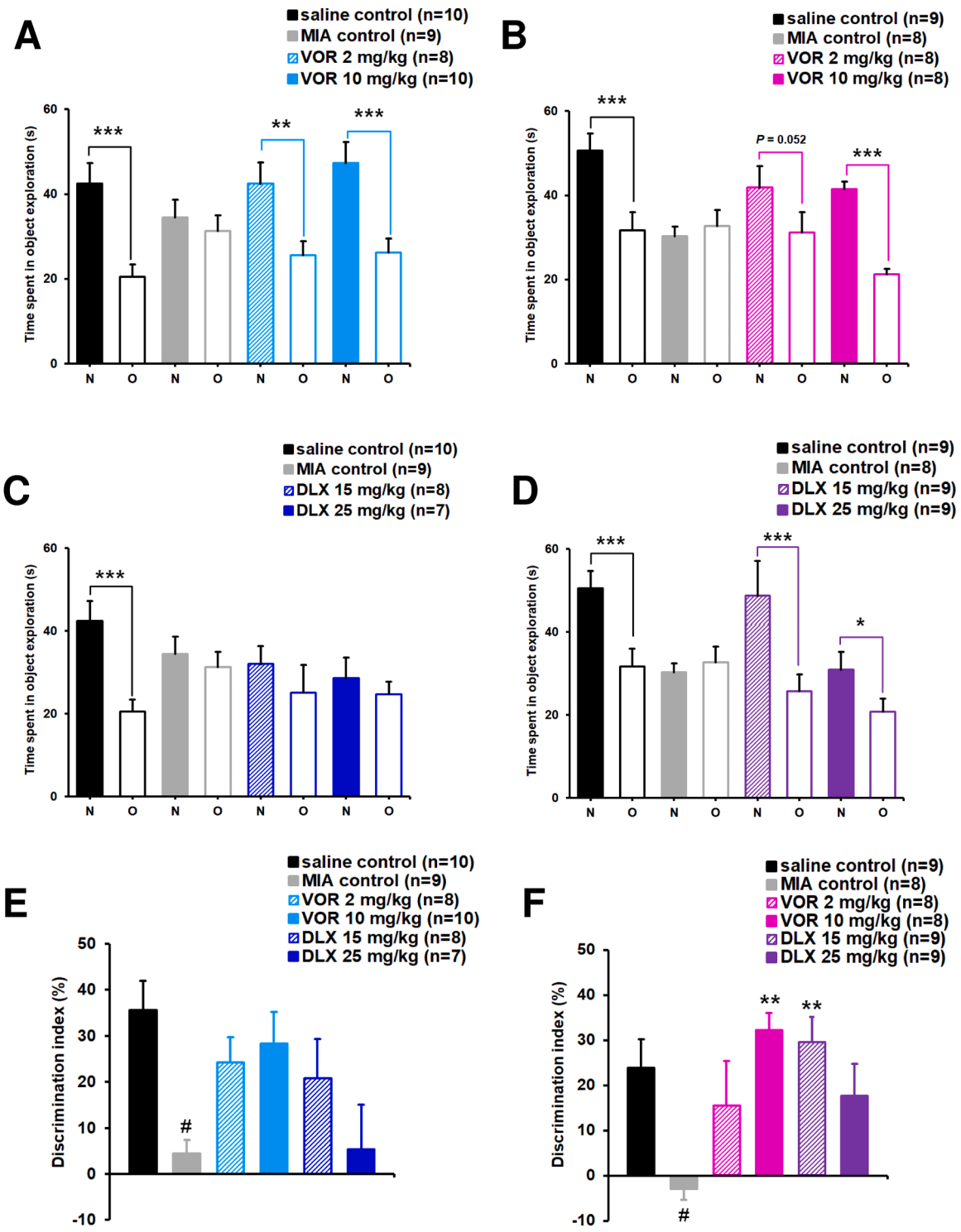

Fig. 4. The effects of vortioxetine (VOR) and duloxetine (DLX) on cognitive performance in the novel object recognition test (NORT) of rats with monoiodoacetate (MIA)-induced osteoarthritis. Results from the NORT are presented as time (in seconds) spent exploring the novel (N) and old (O) object (panels A-D) and discrimination indexes (panels E and F) which were obtained 27 days after MIA injection. Results for male rats are represented with light and dark blue coloring (panels A, C and E) and for female rats with pink and purple coloring (panels B, D and F). Antidepressant drugs, VOR (panels A and B) and DLX (panels C and D) were administered orally every day starting on the day of MIA injection. Time spent in exploring the N and O object was analyzed using two-way repeated measures ANOVA, with Tukey *post-hoc* test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for the comparison between time spent exploring the N *vs* the O object) and the differences between discrimination indexes were analyzed with one-way ANOVA, followed by Tukey *post-hoc* test ($^{\#}P < 0.05$ compared to the saline control group; $^{**}P < 0.01$ compared to the MIA control group). The number of animals per group (n) is indicated in the legends of individual panels.

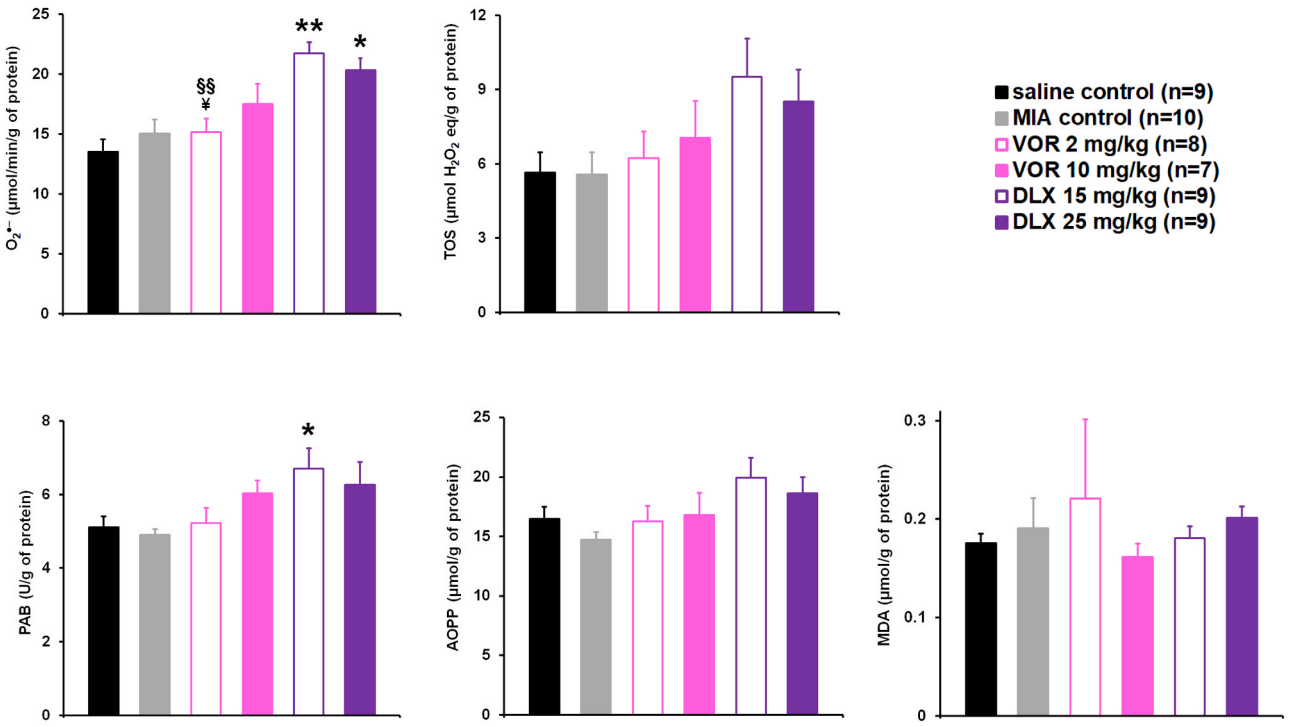

Fig. 5. Duloxetine (DLX), unlike vortioxetine (VOR), increases oxidative stress markers in the heart muscle tissue of female rats with monoiodoacetate (MIA)-induced osteoarthritis. The levels of superoxide anion radicals ($O_2^{\bullet-}$), total pro-oxidant status (TOS), pro-oxidant antioxidant balance (PAB), advanced oxidation protein products (AOPP) and malondialdehyde (MDA) were determined after prolonged (28 days) administration of VOR and DLX. Statistical significance was determined in comparison with the MIA control (*$P < 0.05$, **$P < 0.01$), DLX 15 mg/kg ($^{§§}P < 0.01$) or DLX 25 mg/kg group ($^{¥}P < 0.05$), with one-way ANOVA, followed by Tukey *post-hoc* test. The number of animals per group (n) is indicated in the legend of the panels.

chronification in this model [7,15], and osteoarthritis in humans [25], are changes in the activity of descending pain modulatory systems which project from the brainstem to the spinal cord (where they regulate spinal nociceptive transmission). Specifically, a decrease in descending noradrenergic modulation has been found to contribute to pain hypersensitivity in animals with MIA-induced osteoarthritis [7,26]. Furthermore, drugs that inhibit noradrenaline reuptake and increase extracellular noradrenaline levels (such as milnacipran, an SNRI antidepressant similar to duloxetine) have been demonstrated to reduce pain hypersensitivity in the model of MIA-induced osteoarthritis, *via* a mechanism which involves activation of spinal α_2-adrenergic receptors [26]. A similar mechanism could also explain vortioxetine's efficacy in this model, seeing as we have previously shown that vortioxetine is capable of increasing noradrenaline content in the brainstem of mice with oxaliplatin-induced neuropathic pain [13], and that activation of adrenergic α_2-receptors contributes to its antinociceptive effect in a model of inflammatory pain [14]. Furthermore, dysregulation of descending serotonergic modulation is also thought to contribute to the development of chronic pain in the MIA model, with spinal serotonin 5-HT_3 receptors playing a pain facilitatory role and 5-HT_7 receptors producing a pain inhibitory effect [7,27]. On this point, vortioxetine is a potent 5-HT_3 receptor antagonist, and this mechanism could contribute to its efficacy in relieving pain hypersensitivity [10]. Additionally, vortioxetine is a 5-HT_7 antagonist, which could potentially offset its pain relieving properties; however, it has a lower affinity for these receptors compared to the previously mentioned 5-HT_3 receptors [11].

In our study, animals with MIA-induced osteoarthritis did not develop a deficit in burrowing behavior, which is in contrast with previous studies that found depression of burrowing in models of joint pain [19,20]. However, similarly to us, Bryden *et al.* found that burrowing behavior was not depressed in animals with unilateral MIA-induced knee osteoarthritis [28]. Even though burrowing behavior is considered to be indicative of spontaneous pain [20], from our results it is clear that burrowing does not need to be reduced in animals experiencing pain. On this point, burrowing is also considered to be a self-rewarding behavior that is dependent on animal motivation and capacity to feel rewarding properties [17,18]. It could be suggested that animals, despite experiencing pain, are still capable of feeling the rewarding properties of this form of behavior.

An interesting finding was that duloxetine-treated animals of both sexes developed a significant and dose-related decrease in burrowing behavior, whereas vortioxetine slightly and insignificantly reduced this behavior only in female rats. Furthermore, vortioxetine-treated animals burrowed significantly more than animals treated with the higher tested duloxetine dose. In line with our previous discussion, these results could indicate that duloxetine may decrease the rewarding properties of burrowing. The neural mechanisms controlling motivation and reward are complex, but the key regulators are dopaminergic neurons of the ventral tegmental area (VTA) [29]. Prolonged treatment with antidepressants that inhibit serotonin reuptake has been found to depress the activity of VTA dopaminergic neurons [30]. Furthermore, both serotonergic and noradrenergic projections from brainstem neurons have been shown to decrease the activity of the VTA [31], so it is possible that duloxetine by inhibiting serotonin/noradrenaline reuptake could decrease VTA activity with consequent reduction in burrowing behavior. This assumption and our findings are in line with those of Weber et al. [32] who demonstrated that diverse antidepressants (which inhibit serotonin and/or noradrenaline reuptake) can attenuate another form of self-rewarding behavior (rodent wheel running). On the other hand, vortioxetine was found to depress VTA activity to a lower extent compared to other antidepressant drugs that block serotonin reuptake [33,34], which can provide an explanation for its lower ability to reduce self-rewarding behaviors, such as burrowing. The exact mechanism behind vortioxetine's lower propensity to inhibit VTA activity is not known, but Ebrahimzadeh et al. proposed that it could be because of vortioxetine's ability to directly activate 5-HT_{1A} receptors, which are

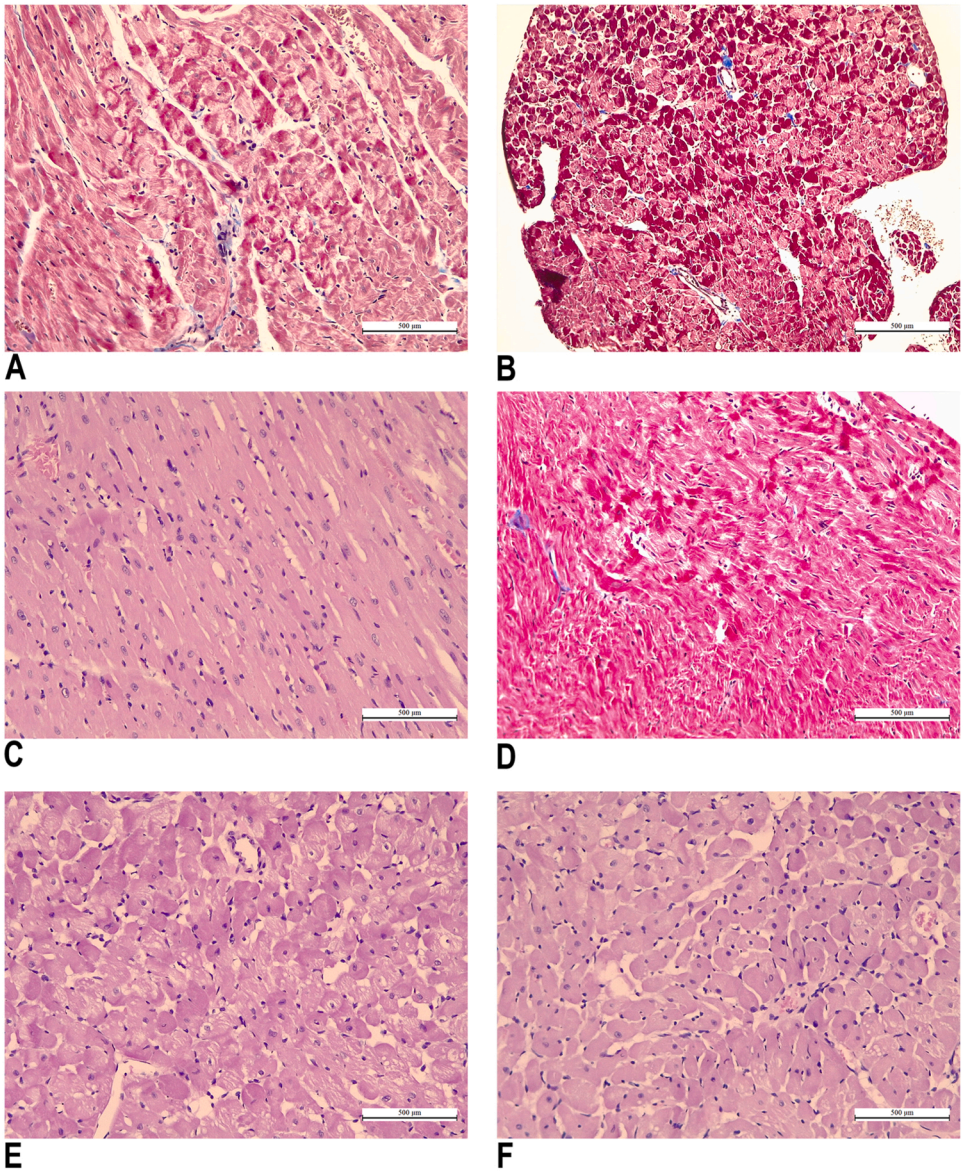

Fig. 6. Histology of the heart muscle tissue of duloxetine (DLX)-treated rats with osteoarthritis. (**A**) Male rat treated with DLX 15 mg/kg (Masson trichrome staining technique, magnification 200x). Cardiomyocytes with intracellular edema and hypercontraction of sarcomeres. (**B**) Female rat treated with DLX 25 mg/kg (Masson trichrome staining technique, magnification 100x). Subendocardial cardiomyocytes with intracellular edema and hypercontraction of sarcomeres. (**C**) Female rat treated with DLX 25 mg/kg (hematoxylin eosin staining technique, magnification 200x). Cardiomyocytes with certain condensation of eosinophilic cytoplasm and contraction band necrosis phenomena. (**D**) Female rat treated with DLX 25 mg/kg (hematoxylin eosin staining technique, magnification 100x). Subepicardial cardiomyocytes with attenuated, wavy appearance and hypercontraction of sarcomeres. (**E and F**) Female rat treated with DLX 25 mg/kg (hematoxylin eosin staining technique, magnification 200x [E] or 100x [F]). Extensive vacuolization of cardiomyocytes, intracellular edema, hypercontraction of sarcomeres, interstitial edema, condensation of cytoplasm and contraction band necrosis phenomena.

known to stimulate VTA dopaminergic neurons [33]. Our results from the burrowing test are also in line with some clinical data. Prolonged treatment with antidepressant drugs (including SNRIs) has been linked to the phenomenon of emotional blunting, i.e., a diminished emotional response to positive and aversive stimuli, which can present clinically as low motivation, apathy, emotional indifference/detachment [35,36]. Vortioxetine was found to improve the manifestations of emotional blunting (including motivation) in depressed patients who had an inadequate response to SSRI/SNRI therapy [35].

We found that rats with MIA-induced osteoarthritis displayed impaired cognitive performance in the NORT. Vortioxetine reversed this form of cognitive impairment in rats of both sexes in a dose-related manner. Previous studies have demonstrated that vortioxetine produces beneficial effects in NORT in naïve animals, and different disease models associated with cognitive impairment [37]. Here we expanded the existing data with results that vortioxetine is capable of attenuating cognitive deficits associated with chronic pain. One of the main mechanisms thought to contribute to vortioxetine's pro-cognitive properties is blockade of serotonin 5-HT_3 receptors on GABAergic interneurons and disinhibition of pyramidal neuron activity of the hippocampus, a region involved in memory processing, including object recognition [38,39]. Electrophysiological studies have found that vortioxetine enhances NMDA-mediated glutamatergic neurotransmission and long-term potentiation in the hippocampus, a NMDA receptor-dependent neural process thought to underlie memory formation [33,40]. Furthermore, vortioxetine was capable of attenuating NORT impairment induced by a NMDA receptor antagonist [41]. A similar mechanism could also account for vortioxetine's ability to reverse cognitive impairment associated with osteoarthritis, seeing as hippocampal glutamatergic dysfunction has also been demonstrated in animals experiencing chronic pain, and stimulation of glutamatergic NMDA neurotransmission can alleviate NORT deficits in these animals [42].

Unlike vortioxetine, duloxetine did not produce a dose-related effect in NORT; the lower tested dose reversed cognitive impairment to a larger extent than the higher dose in female rats with osteoarthritis. A similar pattern of results was obtained in duloxetine-treated male rats; however, the effects were not statistically significant. These dose-unrelated effects of duloxetine in NORT could be attributed to the complex influence of serotonin and noradrenaline on hippocampal function. Specifically, serotonin can both depress and stimulate the function of the hippocampus since this structure expresses diverse types of serotonergic receptors which mediate opposing functional effects,

whereas noradrenaline is mostly associated with stimulation of hippocampal function [43]. Furthermore, both depletion of serotonin and increase in serotonergic neurotransmission (with SSRIs) have been found to impair cognitive performance in NORT, while SNRIs can improve NORT performance (including in chronic pain models) [44–46]. It can be suggested that higher duloxetine doses excessively increase serotonin levels, which could be offsetting its beneficial cognitive effects observed with lower doses. Our findings on the differential effects of vortioxetine and duloxetine in the NORT are also in line with certain clinical data which show that vortioxetine produces more consistent pro-cognitive effects compared to other antidepressants [47].

Duloxetine, unlike vortioxetine, increased several parameters indicative of oxidative damage in the heart tissue of female rats, whereas these effects were not seen in their male counterparts. Furthermore, duloxetine induced complementary histological changes in the heart muscles, which were more pronounced in female rats. We observed cytoplasmic vacuolation of cardiomyocytes which is considered to develop because of mitochondrial damage and under conditions of increased oxidative stress [48,49]. As far as we are aware, vortioxetine is not associated with the capacity to induce oxidative tissue damage [11]. However, previous studies have found that duloxetine can induce oxidative damage in *in vitro* models and rodents [50,51], and this ability has been linked to duloxetine-induced hepatotoxicity, a well-known adverse effect of this antidepressant [4]. The mediators of these effects are considered to be duloxetine metabolites, which are formed by the CYP1A2 and CYP2D6 enzymes [51]. It is worth noting that one of these enzymes (CYP1A2) is also expressed in heart muscles and its expression has been shown to be higher in female compared to male rats [52]. This finding could provide an explanation why we observed changes in redox parameters only in female duloxetine-treated rats (with more pronounced histological changes), but not in male rats. To the best of our knowledge, there are no published data on the cardiotoxic properties of duloxetine in humans; however both CYP enzymes that are involved in duloxetine metabolism are expressed in the human heart which suggests that heart oxidative damage could potentially be seen in humans [53,54]. Duloxetine is considered to have an acceptable cardiovascular safety profile, with the main adverse effects being mild tachycardia and elevations in blood pressure; however, most studies that examined duloxetine's safety have been short in duration [9]. To this extent, we administered duloxetine for 28 days in rats, which is considered to be equal to a period of around 2 years in humans [55]. Even though extrapolation of data from animals to humans is difficult, our results suggest there is a potential for cardiac damage after prolonged (possibly several years) duloxetine treatment.

5. Conclusions

Vortioxetine displayed comparable efficacy to duloxetine (an established osteoarthritis treatment) in reducing pain hypersensitivity of animals with osteoarthritis, indicating it could be a potential drug for osteoarthritic pain. Our results also suggest that vortioxetine could be associated with more beneficial effects compared to duloxetine, since it had no adverse effect on burrowing behavior (animal surrogate of well-being) and was capable of dose-dependently reversing cognitive impairment in animals with osteoarthritis. Furthermore, in the tested dose range, vortioxetine (in contrast to duloxetine) does not adversely affect the heart structure/redox status.

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CRediT authorship contribution statement

Katarina Nastić: Investigation, Methodology, Formal analysis, Visualization. **Uroš Pecikoza:** Investigation, Formal analysis, Writing – original draft. **Milica Labudović-Borović:** Investigation, Methodology, Formal analysis, Visualization, Writing – review & editing. **Jelena Kotur-Stevuljević:** Investigation, Methodology, Formal analysis, Writing – review & editing. **Ana Micov:** Investigation, Formal analysis. **Aleksandar Jovanović:** Formal analysis, Writing – review & editing. **Maja Tomić:** Investigation, Formal analysis, Writing – review & editing. **Radica Stepanović-Petrović:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

None.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115360.

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