

Nicotinamide in a model of osteoarthritis – mechanism of analgesic action and its effect on cognition and reduction of oxidative stress in the rat heart

Radica Stepanović-Petrović

radica@pharmacy.bg.ac.rs

Department of Pharmacology, University of Belgrade – Faculty of Pharmacy

Katarina Nastić

Department of Pharmacology, University of Belgrade – Faculty of Pharmacy

Uroš Pecikoza

Department of Pharmacology, University of Belgrade – Faculty of Pharmacy

Miroslav Dinić

Group for Probiotics and Microbiota-Host Interaction, Laboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering (IMGGE), University of Belgrade, Belgrade, Serbia

Emilija Brdarić

Group for Probiotics and Microbiota-Host Interaction, Laboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering (IMGGE), University of Belgrade, Belgrade, Serbia

Milica Labudović-Borović

Institute of Histology and Embryology "Aleksandar Đ. Kostić", University of Belgrade – Faculty of Medicine

Jelena Kotur-Stevuljević

Department of Medical Biochemistry, University of Belgrade – Faculty of Pharmacy

Aleksandar Jovanović

Department of Basic and Clinical Sciences, University of Nicosia – Medical School

Maja Tomić

Department of Pharmacology, University of Belgrade – Faculty of Pharmacy

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Abstract

Nicotinamide has demonstrated pain-relieving effectiveness in osteoarthritis patients (OA). However, there are no data about the mechanism of its analgesic effect. We aimed to examine the behavioral/cardiac effects of nicotinamide alone and in combination with duloxetine (established antidepressant for OA) or vortioxetine (novel antidepressant) in an OA model, and its impact on the expression of central/peripheral pain mediators. In the monoiodoacetate-induced rat model of knee OA, pain behavior was assessed with weight-bearing, von Frey and acetone tests. Nicotinamide, antidepressants and their combinations were administered orally for 28 days. Transcript levels of pain-related biomarkers (*Il-1 β* , *Tnf- α* , *Ngf*, *Bdnf* and *Tac1* encoding substance P) and cardiac oxidative stress markers were determined after behavioral experiments. Burrowing and novel-object-recognition tests were used to assess the effects of drugs/drug combinations on animal well-being and cognitive performance, respectively. Nicotinamide was effective as duloxetine/vortioxetine in suppressing pain behavior in OA animals. Its antihyperalgesic effect seems to be exerted by decreasing mRNA expression of pro-inflammatory mediators (*Ngf*, *Bdnf* and *Tac1*) involved in central pain pathway sensitization. Nicotinamide enhanced cognitive performance, and did not affect burrowing in OA animals. It decreased cardiac oxidative stress parameters in OA rats. Nicotinamide in combination with duloxetine/vortioxetine did not affect significantly their behavioral effects. In combination with duloxetine, nicotinamide attenuated cardiac oxidative stress parameters in comparison with those in duloxetine treatment alone. Our results clarify the mechanism of nicotinamide analgesic effect in OA, and support its use as a cardioprotective adjuvant to duloxetine in prolonged treatment.

1. Introduction

Osteoarthritis (OA) is a progressive disorder characterized by the loss of hyaline articular cartilage and underlying bone remodeling with local inflammation^{1,2}. Commonly affected joints are those of the hand, knee and hip, but the knee is one of the most frequently involved³. It has been reported that approximately one-third of people over the age of 65 worldwide have some form of OA, and the disease is more prevalent in women than in men, although the mechanism remains unclear¹. The main symptoms of knee OA are joint pain, swelling, stiffness, and mobility impairment, which collectively reduce the quality of life^{1,4}. Inflammation of the synovium is strongly associated with knee pain, and possibly with peripheral and central sensitization that may predispose individuals to chronic pain^{4,5}. Tumor necrosis factor α (TNF- α) and interleukin-1 β (IL-1 β) are considered to be the major pro-inflammatory cytokines involved in the pathophysiology of OA pain⁶. In addition to these cytokines, nerve growth factor (NGF) is the main mediator that activates and sensitizes nociceptors, and contributes to central sensitization in the pathogenesis of OA pain (by increasing the expression of substance P and brain-derived neurotrophic factor; BDNF)⁷.

Chronic pain in OA and advanced age, a major risk factor that contributes to the development and progression of OA, usually share common comorbidities, which can be psychiatric (anxiety and

depression), neurological (cognitive decline), and cardiovascular (coronary/heart insufficiency) in nature^{8,9,10}. Although non-steroidal anti-inflammatory drugs (NSAIDs) are a cornerstone of OA treatment, their effectiveness in suppressing pain is limited, with possible serious adverse effects (gastrointestinal, cardiovascular, hepatic and renal), especially with chronic use in elderly patients¹¹. In addition, these drugs do not affect concomitant diseases, although it is assumed that inflammation is a common pathophysiological substrate for OA, depression, dementia, and cardiovascular disorders^{10,12,13}. The antidepressant drug duloxetine (serotonin and noradrenaline reuptake inhibitor, SNRI) is conditionally recommended as an alternative treatment, alone or in combination with NSAIDs, because it modulates descending pain regulation, since chronic pain in OA is associated with dysfunction of central pain pathways¹¹. However, duloxetine also has certain side effects, such as palpitations, hypertension, orthostatic hypotension, and concentration impairment, which can further worsen cardiovascular and cognitive diseases in the elderly population^{11,14}. When medical therapies have failed, total joint arthroplasty is indicated¹.

New therapeutic approaches that would be at least as effective as NSAIDs/duloxetine in alleviating OA pain, but with fewer side effects, and with therapeutic/protective effects on comorbidities, are greatly needed. Nicotinamide (niacinamide), an amide derivative of nicotinic acid (niacin, vitamin B₃) and a precursor of nicotinamide adenine dinucleotide (NAD⁺), is almost devoid of adverse effects and has proven effective in enhancing pain relief in patients with OA^{4,15}. However, there are no data about the mechanism of its analgesic effect in OA. In animal studies, it has been suggested that nicotinamide harbors neuroprotective and cardioprotective potential through a complex mechanism that involves modulating metabolic and cellular signaling pathways critical for neuronal survival and synaptic plasticity, as well as heart resistance to severe hypoxia/cardiac remodeling^{16,17,18,19}. On the other hand, vortioxetine, a relatively novel antidepressant, is a well-tolerated drug with effectiveness in the treatment of chronic pain, including OA, in preclinical and clinical studies^{20,21,22,23}. Moreover, vortioxetine, probably via its polymodal mechanism of action (inhibition of serotonin reuptake and agonism/partial agonism at 5-HT_{1A}/5-HT_{1B}, and antagonism at 5-HT_{1D}, 5-HT₃, and 5-HT₇ receptors, leading to modulation of neurotransmission in noradrenaline, acetylcholine and glutamate systems), could improve cognitive dysfunction, which overall supports its potential use as a long-term treatment of chronic pain in the elderly^{24,25}.

The aim of our study was therefore to examine the effectiveness of nicotinamide, alone or in combination with vortioxetine/duloxetine, in reducing hypersensitivity in a rat model of knee OA, as well as its mechanism of action through influence on the expression of pain mediators (TNF- α , IL-1 β , NGF, BDNF, substance P) in the knee, corresponding DRGs, and spinal cord. In addition, we sought to investigate whether nicotinamide, alone or in combination with vortioxetine/duloxetine, could exert beneficial effects on cognition and burrowing behavior (animal surrogate of well-being) in animals with OA. Furthermore, we explored the effects of nicotinamide, alone or in combination with duloxetine, on heart redox status/structure. Having in mind that OA is more common in women, we conducted

experiments with nicotinamide in both male and female rats in order to enhance the clinical translatability of our results.

2. Materials and Methods

2.1. Animals

Male and female Wistar rats weighing between 170 and 250 g obtained from the breeding farm of the Military Medical Academy (Belgrade, Serbia) were used in this study. For experiments where we examined the effects of nicotinamide in combination with duloxetine and vortioxetine only female rats were used, considering that: 1. there was mainly no sex difference in the antihyperalgesic effect of nicotinamide in MIA-induced OA, 2. this protocol requires fewer animals, and 3. OA is more prevalent in women. Animals were housed in groups of five in a 12-hour light/dark cycle and were provided with food and water *ad libitum*. All experiments were conducted with approval from the Institutional Animal Care and Use Committee of the Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia (approval number: 323-07-09456/2020-05), and in accordance with the guidelines set by EU Directive 2010/63/EU for animal experiments. Adherence to ARRIVE guidelines is ensured for the reported animal studies.

2.2. Induction of OA

To induce OA, monoiodoacetate (MIA; Sigma-Aldrich Chemie GmbH, Munich, Germany) was injected into the right knee joint of anesthetized animals. Anesthesia was achieved using sevoflurane (3% sevoflurane in O₂; Sevorane®, Abbvie s.r.l., Italy) until withdrawal reflexes were absent. The knee joint was then shaved, swabbed with 75% ethanol, and positioned at a 90° flexion. Animals received a single intra-articular injection of MIA (2 mg/25 µL in 0.9% saline) into the right knee (through the infrapatellar ligament) via a 26-gauge needle. Control animals were given an intra-articular injection of sterile saline (25 µL). In both instances, the solution was injected slowly, and the joint was gently massaged after needle removal.

The room used, time of testing, and experimenters present were kept constant during the study to reduce confounding effects on observed behavior. All behavioral procedures were conducted between 10:00 and 16:00 h.

2.3. Weight-bearing test

Joint discomfort in MIA-treated animals was quantified by observing shifts in hind paw weight distribution between the right (MIA) and left (contralateral) limbs²⁶. Weight asymmetry was measured using an incapacitance tester (Incapacitance Tester Librae for Mice and Rats, Ugo Basile, Milan, Italy). All rats were placed in an angled plexiglass chamber, oriented forward, with each hind paw on a separate force plate. The force from each hind limb was recorded for 3 seconds. Each data point represents the average of five measurements. The results were presented as the percentage of weight distributed on

the ipsilateral hind paw, calculated as: $[\text{weight on the right hind paw}/(\text{weight on the right hind paw} + \text{weight on the left hind paw})] \times 100$.

Behavioral testing was conducted at baseline (day 0) and on days 7, 14, 21, and 28 after OA induction to monitor pain-related behavior in MIA-induced OA over time.

2.4. Mechanical hypersensitivity (von Frey test)

To assess the development of mechanical hypersensitivity in the hind paw on the same side as the MIA-injected knee, paw withdrawal thresholds (PWT), expressed in grams, were measured using an electronic von Frey anesthesiometer (IITC Life Science, Woodland Hills, CA)²⁶. Animals were placed in individual plexiglass enclosures on an elevated wire mesh grid and underwent a 15-minute acclimatization period to become accustomed to both the environment and the experimenter's presence. After habituation, a semiflexible plastic filament was applied with increasing force to the plantar surface of the ipsilateral hind paw until a withdrawal response occurred. The device automatically recorded the force required for this response. At each time point, six to eight PWT measurements were taken, and their mean was used for subsequent calculations.

2.5. Cold hypersensitivity (acetone drop test)

Cold hypersensitivity was assessed in the same plexiglass chambers used for the von Frey test, after assessment of mechanical sensitivity. To evaluate cold hypersensitivity, an acetone drop was applied to the plantar surface of the paw using a plastic syringe without mechanical contact with the skin, and subsequent nociceptive behavior was recorded for 60 seconds. Pain-like (nociceptive) behaviors were defined as flinching, licking, or shaking of the acetone-treated paw. Application and assessment were performed twice for each animal, with a 5-to 10-minute interval, and the average of the two measurements was used.

2.6. Burrowing behavior

Burrowing experiments were conducted as previously described²¹. For these experiments, long plastic tubes (320 × 100 mm; open end elevated by 60 mm) filled with 2000 g of substrate (gravel with individual pieces 2–6 mm in diameter) were placed in an empty cage. Training was carried out in two phases: social facilitation and individual training. Initial training involved placing two rats together in a cage with a burrowing tube overnight (from approximately 16:00 h on the first day) to use social facilitation to encourage burrowing activity. On days two and three, individual rats were housed in the same way overnight. The mean amount of gravel burrowed during these second and third training days was used as the baseline. After OA induction, burrowing tests were conducted on days 8, 13, 20, and 27 following MIA injection. For each test, animals were placed in the cages at 16:00 h and allowed to burrow until the following morning, when the amount of burrowed gravel was measured.

2.7. Novel object recognition test

To assess the impact of OA induction and prolonged treatment with nicotinamide, alone or combined with vortioxetine and duloxetine, on the cognitive performance of rats, the novel object recognition test (NORT) was employed, as previously described²¹. This test was conducted in a rectangular chamber (65 × 45 × 45 cm) under dim lighting (approximately 20 lux) on days 25, 26, and 27 after MIA injection. The rats were left in the box for 10 minutes without any objects on the first day (day 25, habituation phase). On the next day (day 26, familiarization phase), two objects (15 cm from the chamber sides and 25 cm apart) were placed in the testing box, and the rats were given 5 minutes to investigate them. Exploration was defined as looking at, licking, sniffing, or touching the object while sniffing, but not leaning against, standing, or sitting on the object. Object exploration time was recorded. After a 24-hour retention interval (day 27), the testing phase involved replacing one familiar object with a novel one in the same box. Rats explored for 5 minutes, and the time spent with each object was recorded. Exploration time was measured using the Any-maze® video tracking system (Stoelting Co., USA). The objects used were metal cans and glass cylinders filled with sand and gravel of various shapes and sizes. The objects were heavy enough not to be displaced by the animals. The discrimination index was calculated as $(N - O)/(N + O) \times 100\%$, representing the ratio of the difference between the time spent exploring the novel object (N) and the familiar object (O) to the total time spent exploring both objects, and was used to evaluate cognitive function.

2.8. Tissue isolation

On the 28th day following the MIA/saline injection the rats were fully anesthetized with a sevoflurane-oxygen combination (5% sevoflurane in O₂) and euthanized by cervical dislocation. For tissue collection, the entire knee joint, ipsilateral lumbar L3–L5 dorsal root ganglia (DRG), lumbar spinal cord (L3–L5), and heart tissue were quickly dissected. DRGs were isolated using the last rib (T13) as a landmark. Soft tissue was carefully removed from the knee, preserving the joint capsule and subchondral bone. All tissue samples were then flash-frozen in individual tubes and stored at – 80°C until further analysis.

2.8.1. RNA isolation and quantitative real-time PCR (qRT-PCR)

Tissue samples from the knee joint, dorsal root ganglia (DRG), and spinal cord were homogenized using a TissueLyser II system (Qiagen, Germany) equipped with stainless-steel beads. Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. To remove residual genomic DNA, the isolated RNA was treated using the RapidOut DNA Removal Kit (Thermo Fisher Scientific).

For reverse transcription, 0.5 µg of purified RNA was used as a template with RevertAid Reverse Transcriptase (Thermo Fisher Scientific) in the presence of random hexamer primers and RiboLock RNase inhibitor (Thermo Fisher Scientific), according to the supplier's recommendations.

Quantitative real-time PCR (qPCR) was performed using the IC Green qPCR Universal Kit (NIPPON Genetics, Düren, Germany) on a LineGene 9600 Plus Real-Time PCR System (Hangzhou Bioer

Technology, Hangzhou, China). The amplification protocol consisted of an initial activation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s.

Gene expression levels were normalized to the housekeeping gene *Gapdh*, and relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences for *Ngf*, *Il-1 β* , *Tnf- α* , *Bdnf*, and *Tac1* (Tachykinin 1, a gene encoding substance P), as well as *Gapdh*, were previously published in Tomić et al.²⁶. All primers were obtained from Thermo Fisher Scientific.

2.8.2. Evaluation of cardiac muscle redox status

Samples of rat cardiac muscle were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4) at a 1:9 (w/v) ratio using a T10 basic Ultra-Turrax homogenizer (IKA, Germany). The obtained homogenates were centrifuged for 10 min at 800 × g to remove cellular debris, followed by an additional centrifugation for 20 min at 9500 × g to obtain post-mitochondrial supernatants²⁷.

In the resulting supernatants, markers of oxidative stress were analyzed according to previously described protocols^{27,28}. The examined pro-oxidant parameters included superoxide anion radicals ($O_2^{\cdot-}$), total oxidant status (TOS), pro-oxidant–antioxidant balance (PAB), advanced oxidation protein products (AOPP), and malondialdehyde (MDA).

Biochemical results were normalized to the protein concentration of the supernatants, determined by the Bradford method²⁹. An ILAB 300 Plus analyzer (Instrumentation Laboratory, Italy) was used to measure $O_2^{\cdot-}$ and TOS activity while spectrophotometric methods (Pharmacia LKB, UK) were employed to determine PAB, AOPP, MDA, and total protein content.

2.8.3. Evaluation of heart histology

Histological analysis of heart muscle followed established protocols³⁰. In summary, heart apex tissue samples were fixed by immersion in 4% neutral-buffered formaldehyde (FNB4–10 L, BioGnost Ltd., Croatia) for 24 hours. After fixation, a standard dehydration protocol with increasing ethanol concentrations was carried out, followed by paraffin embedding.

Tissue processing was conducted in an automated modular tissue processor (Leica TP1020, Leica Biosystems Nussloch GmbH, Germany) adhering to the subsequent protocol: fixation in 4% neutral-buffered formaldehyde (2 × 1.5 h – 2 × 2 h), dehydration in 70% ethanol (1.5–3 h), 96% ethanol (1.5–3 h), and absolute ethanol (3 × 1.5 h), clearing in xylene (2 × 1.5 h; Ksilen pro analysis, Zorka Pharma, Serbia), and infiltration with paraffin (Biowax Plus 56/58, BWPLUS-1, BioGnost Ltd., Croatia; 3 × 1.5 h).

Samples were embedded with the Leica HistoCore Arcadia H system (Leica Biosystems, Nussloch GmbH, Germany), and serial sections were cut at 4 μ m thickness with a Leica RM2125RTS rotary microtome. For histomorphological evaluation, sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome. Examination was performed by Leica DM2000 LED light microscope equipped with a Leica ICC50 E digital camera and LAS V4.12 software.

Morphological assessment followed criteria defined by The Royal College of Pathologists (The Royal College of Pathologists). Analysis involved four transverse sections per animal, taken from two heart levels separated by a minimum of 2 mm.

The systematic histological assessment of cardiomyocytes focused on fiber orientation, presence of cross striations, intercalated disc formation, nuclear morphology, and signs of cellular atrophy, hypertrophy, vacuolization, intracellular edema, or contraction band necrosis. Separate evaluations were also conducted for interstitial tissue morphology, microvascular structures, and components of the coronary circulation.

2.9. Statistical analysis

Statistical analyses and plotting were performed using GraphPad Prism (version 10.1.0; GraphPad Software, Inc., La Jolla, CA). For the behavioral analysis, statistical significance for time-course data was analyzed using a two-way repeated measures analysis of variance (ANOVA) with repeated measures followed by Tukey's *post-hoc* testing, with treatment as the between-subjects factor and time as the within-subjects factor. The area under the curve (AUC) was calculated for each animal using the trapezoidal method to quantify the overall impact of MIA-induced changes and the effects of drug/drug combination treatments on pain behavior and burrowing tests over time. Comparisons of AUC treatment groups within the same sex were performed using a one-way ANOVA. For NORT data, a paired *t*-test was used to analyze the time spent exploring novel or old objects, while a one-way ANOVA with a *post-hoc* Tukey's test addressed differences in discrimination indexes among distinct experimental groups. Statistical significance for relative mRNA expression levels and markers of redox status parameters was determined using a one-way ANOVA followed by *post-hoc* Tukey's test. Statistical significance was defined as a P-value less than 0.05.

3. Results

3.1. Nicotinamide reduces pain behavior in male and female rats with OA induced by MIA

Repeated administration of nicotinamide (100 mg/kg/day) for 28 days, illustrated in Fig. 2A, improved weight-bearing deficits in rats with MIA-induced OA compared with MIA control group (*post-hoc* $P < 0.001$ for male rats, while *post-hoc* $P = 0.014$ for female rats for factor treatment in the time-course data; Fig. 2B and 2C). Nicotinamide increased AUC values compared to MIA controls (*post hoc* $P < 0.001$ for male rats and *post hoc* $P = 0.15$ for female rats; Fig. 2D). Compared to the AUC values of MIA controls, nicotinamide improved weight-bearing deficits by 56.95% in male rats and 31.61% in female rats.

Repeated administration of nicotinamide (100 mg/kg/day) over a 28-day period significantly reduced mechanical hypersensitivity in OA-affected male and female rats. This effect was demonstrated by increases in PWT and AUC values (all *post hoc* $P < 0.001$ for the treatment factor in the time-course data; Fig. 2E and 2F and *post hoc* $P < 0.001$ for male rats and *post hoc* $P = 0.001$ for female rats for AUC

values; Fig. 2G). When comparing AUC values as a percentage increase relative to MIA control, nicotinamide achieved a 40.44% effect in male rats and a 34.26% effect in female rats.

A 28-day regimen of nicotinamide (100 mg/kg/day) significantly lowered the time animals spent exhibiting nociceptive behavior in the acetone test in male and female rats with OA induced by MIA (*post-hoc* $P < 0.001$ for factor treatment in time-course data for animals of both sexes; Fig. 2H and 2I) and reduced AUC values compared to MIA controls (*post-hoc* $P = 0.02$ for male rats and *post-hoc* $P = 0.003$ for female rats; Fig. 2J). When comparing AUC values as a percentage decrease relative to MIA control, nicotinamide achieved a 68.08% effect in male rats and a 69.08% effect in female rats.

3.2 The effects of nicotinamide on the mRNA expression of pain-related biomarkers in knee tissue, dorsal root ganglia (DRG) and spinal cord from male and female rats with MIA-induced OA

Intra-articular injection of MIA significantly increased *Tnf- α* and *Ngf* mRNA levels in knee tissue of rats of both sexes compared to saline-injected groups (tissue isolation was illustrated in Fig. 3A; all *post hoc* $P \leq 0.03$; Fig. 3B). *Il-1 β* mRNA levels were also elevated, but only reached significance criteria in males (*post hoc* $P = 0.03$ for males, *post hoc* $P = 0.06$ for females; Fig. 3B).

Nicotinamide administered daily at 100 mg/kg for 28 days did not significantly affect the mRNA levels of *Il-1 β* , *Tnf- α* , and *Ngf* in knee tissue of either male or female rats when compared to the MIA control group (all *post hoc* $P \geq 0.18$; Fig. 3B).

Compared to saline controls, intra-articular MIA injection resulted in a significant increase in the mRNA levels of *Ngf*, *Bdnf*, and the *Tac1* gene (encoding substance P) in the ipsilateral L3–L5 DRGs in males (all *post hoc* $P \leq 0.02$; Fig. 3C) and *Il-1 β* , *Ngf*, *Bdnf* and *Tac1* in females (all *post hoc* $P < 0.001$; Fig. 3C). Nicotinamide treatment significantly decreased mRNA expression of *Ngf*, *Bdnf*, and *Tac1* in the DRGs of male and female rats (all *post hoc* $P \leq 0.02$; Fig. 3C).

Similarly to the DRGs, elevated expression of *Tnf- α* , *Bdnf*, and substance P (*Tac1*) in the L3–L5 segment of the spinal cord was detected in MIA-treated animals of both sexes compared with saline controls (all *post hoc* $P \leq 0.04$; Fig. 3D), whereas in females the mRNA expression of *Il-1 β* was also increased (*post hoc* $P = 0.04$; Fig. 3D). Treatment with nicotinamide (100 mg/kg/day for 28 days) significantly decreased *Bdnf* and substance P (*Tac1*) mRNA levels in the spinal cord of male and female rats (all *post hoc* $P < 0.001$; Fig. 3D).

3.3 The influence of nicotinamide in combination with vortioxetine and duloxetine on pain-related behavior in female rats with OA induced by MIA

We have previously reported that vortioxetine and duloxetine reduce pain-related behavior in rats with MIA-induced OA^{21,26}. This study also demonstrated (in female rats) that prolonged administration of vortioxetine (2 mg/kg/day) and duloxetine (15 mg/kg/day), illustrated in Fig. 4A, improved weight-bearing asymmetry, increased PWT in the von Frey test and reduced the nociceptive behavior duration in the acetone test compared to the MIA control group (all *post hoc* $P \leq 0.0075$ for time-course data; Fig. 4).

Co-administration of vortioxetine (2 mg/kg/day) and nicotinamide (100 mg/kg/day) for 28 days reduced the MIA-induced weight-bearing impairment in the affected limb (*post hoc* $P = 0.004$ for factor treatment in time-course data and *post hoc* $P = 0.53$ for AUC values; Fig. 4B and 4D). The combination of duloxetine (15 mg/kg/day) with nicotinamide (100 mg/kg/day) also improved weight-bearing deficits (*post hoc* $P < 0.001$ for factor treatment in time-course data and $P = 0.38$ for AUC values; Fig. 4C and 4D). When comparing AUC values as a percentage increase relative to MIA control, vortioxetine-nicotinamide combination produced an effect of 29.22%, whereas duloxetine-nicotinamide combination produced an effect of 43.41% in the weight-bearing test in female rats with MIA-induced OA.

The combination of vortioxetine (2 mg/kg/day) with nicotinamide (100 mg/kg/day) decreased mechanical hypersensitivity in the von Frey test and increased AUC values compared with the MIA control group (*post hoc* $P < 0.001$ for both factor treatment in time-course data and AUC data; Fig. 4E and 4G). Similarly, a combination of duloxetine (15 mg/kg/day) with nicotinamide (100 mg/kg/day) decreased pain hypersensitivity in the von Frey test and increased AUC values compared with the MIA control (*post hoc* $P < 0.001$ for both factor treatment in time-course data and AUC data; Fig. 4F and 4G). When comparing AUC values as a percentage increase relative to MIA control, vortioxetine-nicotinamide combination produced an effect of 41.32%, whereas duloxetine-nicotinamide produced an effect of 55.77% in female rats with MIA-induced OA.

The combination of vortioxetine (2 mg/kg/day) and nicotinamide (100 mg/kg/day) significantly reduced nociceptive behaviour time in the acetone test in female rats with MIA-induced OA (*post-hoc* $P < 0.001$ for time course data; Fig. 4H) and reduced AUC values compared to MIA controls (*post-hoc* $P < 0.001$; Fig. 4J). The combination of duloxetine (15 mg/kg/day) and nicotinamide (100 mg/kg/day) also reduced nociceptive behavior time in the acetone test (*post-hoc* $P < 0.001$ for time-course data; Fig. 4I) and reduced AUC values (*post-hoc* $P < 0.001$; Fig. 4J). When comparing AUC values as a percentage decrease relative to MIA control, the vortioxetine-nicotinamide combination produced an effect of 75.79%, whereas the duloxetine-nicotinamide combination produced an effect of 95.36% in female rats with MIA-induced OA.

3.4 The effects of nicotinamide and its combination with vortioxetine and duloxetine on burrowing activity in male and female rats with MIA-induced OA

Our previous research showed that OA induction does not significantly affect the amount of gravel burrowed overnight; both saline-treated and MIA control animals, regardless of sex, displayed similar

burrowing activity throughout the study period (experimental timeline was illustrated in Fig. 5A). We confirmed this in this experiment (all *post-hoc* $P \geq 0.4568$ for the treatment factor in the time-course data and all *post-hoc* $P \geq 0.54$ for the AUC data; Fig. 5).

Nicotinamide (100 mg/kg/day) slightly reduced burrowing behavior in male rats. This effect was not statistically significant, but was close to statistical significance (*post-hoc* $P = 0.0645$ for the time-course data and $P = 0.15$ for the AUC data; Fig. 5B and 5D). In female rats, nicotineamide (100 mg/kg/day) had no significant effect on the amount of gravel the rats burrowed compared with female MIA control rats (*post-hoc* $P = 0.213$ for the treatment factor in the time-course data and *post-hoc* $P = 0.77$ for the AUC data; Fig. 5C and 5D).

Vortioxetine (2 mg/kg/day) slightly decreased burrowing behavior in female rats (*post-hoc* $P = 0.014$ for time-course data and *post-hoc* $P = 0.31$ for AUC data; Fig. 5E and G). Duloxetine (15 mg/kg/day) led to a significant decrease in the burrowing activity in female rats (*post-hoc* $P < 0.001$ for time-course data and *post-hoc* $P = 0.03$ for AUC data; Fig. 5F and G).

The combination of vortioxetine (2 mg/kg/day) with nicotineamide (100 mg/kg/day) had no significant effect on the amount of gravel burrowed by the female rats (*post-hoc* $P = 0.8587$ for the time-course data and *post-hoc* $P > 0.99$ for the AUC data; Fig. 5E and G). On the other hand, the combination of duloxetine and nicotineamide reduced burrowing behavior in the female rats (*post-hoc* $P < 0.001$ for the treatment factor in the time-course data and *post-hoc* $P = 0.02$ for the AUC data; Fig. 5F and 5G).

3.5 The influence of nicotineamide alone or combined with vortioxetine/duloxetine on cognitive ability in the novel object recognition test (NORT) in male and female rats with OA induced by MIA

Our prior findings indicate that OA induction impairs cognitive function. Specifically, saline control rats of both sexes demonstrated a significantly longer exploration time for new object, a pattern also confirmed in the present study (experimental timeline was illustrated in Fig. 6A; $P = 0.002$ for male and $P = 0.005$ for female rats; Fig. 6B and 6C). Conversely, OA-affected rats spent similar amounts of time investigating the old and novel object ($P = 0.23$ for male rats and $P = 0.30$ for female rats; Fig. 6B and 6C). Accordingly, MIA control animals exhibited significantly lower discrimination indexes than those determined for saline control animals, irrespective of sex (*post-hoc* $P = 0.002$ for male rats and $P < 0.001$ for female rats; Fig. 6D).

Prolonged 28-day nicotineamide (100 mg/kg/day) treatment improved the cognitive ability of rats of both sexes with OA; animals demonstrated significantly greater exploration time for novel objects than for familiar ones (the *post-hoc* P values were 0.04 for male and 0.003 for female rats; Fig. 6B and 6C) and showed a higher discrimination index compared to MIA control animals (*post-hoc* $P = 0.03$ for male and *post-hoc* $P = 0.004$ for female rats; Fig. 6D).

Twenty-eight days of vortioxetine (2 mg/kg/day) did not elicit a significant effect in the NORT. Specifically, female animals showed no preference between novel and familiar objects during exploration ($P = 0.22$; Fig. 6E), and their discrimination index remained similar to that of female MIA control animals (*post-hoc* $P = 0.31$; Fig. 6G).

Prolonged treatment with duloxetine (15 mg/kg/day) in female rats produced a significant effect in NORT; animals dedicated significantly more time to exploring the novel object compared to the familiar one ($P = 0.005$; Fig. 6F) and their discrimination index was significantly elevated relative to that of female MIA control animals (*post-hoc* $P = 0.01$; Fig. 6G).

Female rats receiving both vortioxetine (2 mg/kg/day) and nicotinamide (100 mg/kg/day) did not show improved cognitive performance compared to the MIA control group; they spent comparable amounts of time exploring novel and familiar objects ($P = 0.31$; Fig. 6E) and discrimination index values were also not different (*post-hoc* $P > 0.99$; Fig. 6G).

On the other hand, the combination of duloxetine (15 mg/kg/day) and nicotinamide (100 mg/kg/day) improved cognitive performance in NORT in female rats; a significantly greater exploration time was observed for the novel object compared to the familiar one ($P < 0.001$; Fig. 6F) and their discrimination index was significantly superior to that of the female MIA control animals (*post-hoc* $P = 0.02$; Fig. 6G).

3.6 The effects of nicotinamide and its combination with duloxetine on oxidative stress markers in the cardiac muscle tissues of male and female rats with MIA-induced OA

MIA-induced OA did not lead to significant alterations in cardiac oxidative stress parameters in either male or female rats, i.e. the levels of parameters indicative of oxidative damage (TOS, $O_2^{\bullet-}$, PAB, AOPP and MDA) were comparable between MIA and saline control animals (all *post-hoc* $P \geq 0.10$ for male rats and all *post-hoc* $P \geq 0.13$ for female rats; Fig. 7).

Prolonged treatment with nicotinamide (100 mg/kg/day) led to a significant decrease in the levels of TOS, AOPP and MDA in male rats compared with MIA control animals (all *post-hoc* $P \leq 0.008$; Fig. 7A). Similarly, nicotinamide decreased the level of AOPP and MDA in the heart muscle of female rats compared with MIA control group (all *post-hoc* $P \leq 0.01$; Fig. 7A).

In accordance with our previously published results, prolonged treatment with duloxetine (15 mg/kg/day) produced a significant increase in the levels of TOS, $O_2^{\bullet-}$, PAB and the level of AOPP (all *post-hoc* $P \leq 0.08$; Fig. 7B) in the heart of female rats, compared to MIA-injected counterparts.

On the other hand, the combination of duloxetine (15 mg/kg/day) and nicotinamide (100 mg/kg/day) significantly decreased the level of AOPP and MDA in female rats (all *post-hoc* $P \leq 0.006$; Fig. 7B)

compared to MIA control group. In addition, the combination of duloxetine with nicotinamide significantly decreased the level of TOS, $O_2^{\bullet-}$, PAB, AOPP and MDA compared to duloxetine treatment alone (all *post-hoc* $P \leq 0.02$; Fig. 7B).

3.7 Nicotinamide prevented histological changes that duloxetine produced in the cardiac muscle of female rats with OA

Cardiac muscle histology remained unchanged following OA induction, showing no differences from saline controls in either sex (not shown). Prolonged nicotinamide (100 mg/kg/day) treatment also did not alter cardiomyocyte morphology, but duloxetine (15 mg/kg/day) caused histological changes in the cardiac muscle of OA-affected rats of both sexes (Fig. 7a-d). Specifically, there were hypercontraction of sarcomeres, condensation of eosinophilic cytoplasm and intracellular edema, as well as perivascular edema. In the heart preparations of female rats treated concomitantly with nicotinamide and duloxetine, all these changes were not seen (Fig. 7e).

4. Discussion

The present study shows that prolonged administration of nicotinamide leads to a significant reduction of pain hypersensitivity in rats of both sexes with OA induced by MIA. This was evidenced by measuring paw withdrawal thresholds as an expression of mechanical hyperalgesia, and time spent in nociceptive behavior as an expression of cold allodynia. In addition, nicotinamide improved weight-bearing deficits in both male and female rats with OA, but with significant improvements in male rats only. All three pain tests employed in our rat OA model align with the clinical manifestations in knee OA patients, who typically experience pain when loading the osteoarthritic joint, with certain patients also developing hyperalgesia in distant tissues (spreading or referred hyperalgesia)^{1,31}. Our results demonstrate that nicotinamide produces antihyperalgesic activity in an experimental MIA-induced model of knee OA. When the dose of nicotinamide (100 mg/kg/day) we used is converted to a human dose (equivalent to 973 mg/day for humans³²), it seems to be well below the dose of 3000 mg/day that is considered to be safe and virtually devoid of adverse effects³³. Indeed, Sahin et al.³⁴ showed in the same rat model of OA that nicotinamide (40 mg/kg and 200 mg/kg) ameliorated joint pain behavior measured as stride lengths, paw areas and widths, and Kellgren-Lawrence Mankin scores.

It is evident that nicotinamide prevents hyperactivity and sensitization of pain pathways in the MIA inflammatory model of OA, but it was of interest to elucidate the underlying mechanism and sites of its antihyperalgesic effect. Neuroinflammation at the peripheral and central part of pain transmission/modulation is recognized as one of the mechanisms contributing to the development of chronic pain, including OA pain. In our study, intra-articular injection of MIA produced a significant increase in *Tnf- α* and *Ngf* mRNA levels in the knees of the animals of both sexes, compared with the knees of sham-injected animals. Levels of *Il-1 β* mRNA were also markedly increased. This is in

agreement with the previously published data, which showed an increase in the concentration or gene expression of pro-inflammatory mediators in rodents with knee OA^{26,35}. The elevation of the *Ngf* level is of particular interest because that neurotrophic factor is one of the most important mediators in the pathogenesis of OA pain³⁶. In clinical studies, it has been shown that monoclonal antibodies directed against NGF significantly reduce pain and improve joint function in patients with OA⁷. NGF is released by immune and non-immune cells, such as chondrocytes and synoviocytes, in response to joint damage. Stimulation of affected cells with various pro-inflammatory mediators, including IL-1 β , or mechanical stress, has been shown to increase NGF gene expression³⁷. In our study, nicotinamide did not significantly reduce either *Ngf* or *Tnf- α* and *Il-1 β* mRNA levels in knee tissue compared to the MIA control in both sexes of animals. These data could at least partly explain the absence of a significant antihyperalgesic effect of nicotinamide in female rats in the weight bearing test, suggesting little or no effect of nicotinamide on nociceptive or peripheral sensitization^{38,39}. On the other hand, the presence of a significant antihyperalgesic effect of nicotinamide in male rats in the weight bearing test could be at least partly attributed to increased serum testosterone levels induced by nicotinamide⁴⁰, seeing as testosterone has been shown to provide protection against inflammatory articular pain⁴¹.

MIA injection significantly increased the gene expression of pro-inflammatory cytokines and neuromodulators in the L3–L5 ipsilateral DRGs and spinal cord, compared with sham controls. More specifically, significantly increased mRNA levels of *Tnf- α* , *Ngf*, *Bdnf*, *Tac1* (encoding substance P) were recorded in both sexes in the L3–L5 ipsilateral DRGs and spinal cord (except for *Ngf* in the spinal cord and *Tnf- α* in DRGs), whereas *Il-1 β* was increased only in females in both DRGs and the spinal cord. These results are consistent with previous studies which also showed increased levels of pro-inflammatory mediators in the central sensory system in the same model^{26,42}. It has been shown that all these mediators derive not only from neurons, but also from non-neuronal cells in the DRG (e.g., macrophages, T-cells) and spinal cord (e.g., glial cells)^{43,44}. Nicotinamide significantly reduced the expression of *Bdnf* and *Tac1* (substance P) in the DRG and spinal cord in male and female rats, and *Ngf* in the DRG in both sexes too, compared with MIA controls. Recently, silent information regulator 1 (Sirtuin 1, SIRT1, one of the seven members of the SIRT family), a multifunctional nicotinamide adenine dinucleotide (NAD⁺) dependent histone deacetylase, has been reported to be involved in the development of chronic pain⁴⁵. DRG and spinal SIRT1 expression is downregulated in chronic pain, whereas activation of SIRT1 might be a potential strategy for treating chronic pain^{45,46}. Nicotinamide activates SIRT1, leading to the deacetylation/suppression of a broad range of target proteins involved in pain sensitization⁴⁷. SIRT1 stimulates the deacetylation/inhibition of NF- κ B. Inflammation, as well as mechanical loading of the joint in OA, lead to the activation of NF- κ B, a transcription factor that increases gene expression for various pro-inflammatory mediators⁴⁸. The reduction of *Ngf* mRNA level in DRG induced by nicotinamide could be related to the inhibition of NF- κ B. NGF in complex with its tyrosine kinase receptor A (TrkA) can cause transcriptional changes in the DRG that lead to the synthesis of substance P and BDNF, which facilitate the transmission of nociceptive information in the primary synapse⁷. Thus, through the reduction of NGF in the DRG, nicotinamide can indirectly exert the reduction of *Tac1* and BDNF at the central level, at least

partially. However, significantly decreased mRNA levels of *Tac1* and *Bdnf* by nicotinamide can also be NF- κ B-mediated directly, since there is a positive feedback loop between NF- κ B and *Tac1/BDNF* gene expression^{49,50}. The efficiency of nicotinamide in reducing mechanical hypersensitivity (von Frey test) and cold allodynia (acetone test) in both female and male rats could be explained by the inhibition of central sensitization⁵¹.

Since OA is a complex condition for which there is no effective treatment, a combination of drugs targeting different mechanisms/sites involved in modulating pain pathways could be a promising approach to improve efficacy and reduce adverse effects in the treatment of OA. In our previous studies^{21,26} we demonstrated that duloxetine (an established drug for OA) and vortioxetine reduced pain hypersensitivity in animals with MIA-induced OA in all three pain-related tests and in both sexes. Nicotinamide may be as effective as duloxetine and vortioxetine in reducing pain hypersensitivity in rats with MIA-induced OA. The combinations of nicotinamide and duloxetine or vortioxetine produced no greater effects in reducing mechanical/cold hyperalgesia or weight-bearing asymmetry in comparison with individual components. One of the factors that may control the nature of drug interactions in an animal pain model is stimulus intensity. Kissin et al.⁵² demonstrated that, at low stimulus intensities, the interaction between morphine and barbiturate is synergistic, while at higher stimulus intensities the interaction is additive or less. In our experiments, OA was induced with 2 mg of MIA dissolved in saline. An intra-articular MIA injection to the rat joints induces different OA changes dose-dependently: 0.25 mg and 0.5 mg MIA cause fewer OA changes than 2.0 mg and 4.0 mg MIA⁵³. Consequently, it remains to be explored whether lower concentrations of MIA would reveal additive or greater than additive effects between nicotinamide and duloxetine or vortioxetine.

In a further set of experiments, we confirmed our previous finding that MIA-treated rats did not decrease burrowing behavior in comparison with saline control animals of both sexes²¹. This is consistent with the results obtained by Bryden et al.⁵⁴, who also found no changes in burrowing behavior observed in rats with unilateral MIA-induced knee OA. It therefore seems that burrowing, as a self-rewarding behavior in rodents, is not necessarily impaired in animals experiencing pain. Nicotinamide had no significant effect on the amount of gravel burrowed by rats of either sex compared with MIA controls. Brain regions involved in motivation and reward processing are extensive and complex, but the ventral tegmental area and nucleus accumbens form a crucial brain dopamine pathway known as the mesolimbic pathway⁵⁵. It has been shown that drugs of abuse limit adenosine receptor (A_{2A}) activity in favour of increased activity of dopamine receptors (D_2) in the mesolimbic system⁵⁶. By increasing extracellular NAD⁺ levels and its degradation into adenosine, which activates adenosine receptors, nicotinamide can counteract the effects of dopamine⁵⁷. This might provide an explanation for the insignificant effect of nicotinamide on burrowing as a self-motivated behavior. Recently, our research group has reported that duloxetine significantly, and vortioxetine slightly but insignificantly, reduced burrowing behavior in female rats²¹. In the present experiments, we provided the same evidence, and further demonstrated that nicotinamide in combination with these antidepressants did not change the burrowing behavior pattern in rats.

Importantly, nicotinamide did not change vortioxetine burrowing behavior, having in mind the compatibility of the experimental and clinical data which demonstrate that vortioxetine improves motivation, in patients with major depressive disorder who had an inappropriate response to SNRIs, including duloxetine⁵⁸.

We verified the results from our recent study showing that MIA-induced OA exerted impaired cognitive performance in the NORT in rats of both sexes²¹. Numerous preclinical and clinical studies have provided evidence that chronic pain can lead to cognitive impairment, and one of the hypotheses of cognitive dysfunction includes structural and functional changes in the medial prefrontal cortex and hippocampus^{59,60}. It is assumed that potential factors of cognitive deficits are oxidative stress, inflammation, and mitochondrial dysfunction^{61,62}. Nicotinamide improved the cognitive performance in the NORT in rats of both sexes with MIA-induced OA. Preclinical studies have demonstrated that NAD⁺ precursors produce beneficial effects in learning and memory recovering in Alzheimer's disease, diabetes, traumatic brain injury, aging, vascular dementia, and schizophrenia¹⁷. It has been suggested that nicotinamide could decrease cognitive impairments primarily by enhancing mitochondrial function and reducing oxidative stress and inflammation¹⁷. Our results contribute to the existing data by demonstrating that nicotinamide as an NAD⁺ precursor can improve cognitive decline caused by chronic pain. Having in mind that chronic inflammation in the brain is a hallmark of many neurodegenerative diseases, including Alzheimer's disease, we might suppose that nicotinamide, through a significant reduction of pro-inflammatory mediators in the central nervous system, can provide cognitive recovery in chronic pain. Nicotinamide alleviates neuroinflammation via NAD⁺-dependent deacetylation mechanisms with activation of the sirtuin pathway^{17,63}. Similar to the findings from the burrowing test, the combination of nicotinamide with vortioxetine or duloxetine did not alter their individual effects on NORT performance in female rats. Our research group had previously shown that vortioxetine improved cognitive performance in the NORT in female rats in a dose-dependent, and duloxetine in a dose-independent manner²¹. In the present experiments, only lower doses of antidepressants were tested, so vortioxetine had no effect, while duloxetine reversed cognitive impairment in female animals with OA.

Prolonged treatment with nicotinamide significantly decreased several indicators of cardiac oxidative damage in rats of both sexes compared with MIA control animals, and did not affect cardiomyocyte morphology. As a precursor of NAD⁺, a central redox cofactor, nicotinamide contributes to the preservation of myocardial tissue by reducing oxidative stress via activating SIRT1/SIRT3 and enhancing autophagy^{47,64,65}. Moreover, nicotinamide has been shown to increase cardiac resistance to stress by upregulating the ATP-sensitive potassium channel subunit SUR2A⁶⁶. In our previous study, we demonstrated that prolonged treatment with duloxetine induced cardiotoxicity through oxidative stress and mitochondrial damage of cardiomyocytes in female rats²¹. A combination of duloxetine with nicotinamide significantly attenuated oxidative stress by decreasing the levels of all examined parameters of oxidative stress (TOS, O₂^{•-}, PAB, AOPP and MDA) compared to duloxetine treatment alone, and prevented damage of cardiomyocytes. Although translating animal data to humans is difficult, this

finding could be of interest in light of the protective effect of nicotinamide in prolonged (several years) duloxetine use, since we administered duloxetine to rats for 28 days, which is equivalent to approximately two years of treatment in humans⁶⁷. This observation may be particularly relevant in the context of OA management, since duloxetine is recommended as an alternative treatment, alone or in combination with NSAIDs, and pharmacotherapy in OA may extend for years before total knee arthroplasty.

5. Conclusions

Nicotinamide may be as effective as duloxetine (a recommended treatment of OA) and vortioxetine in decreasing pain hypersensitivity in rats with OA. Its antihyperalgesic effect seems to be exerted, at least in part, by reducing the expression of pro-inflammatory mediators and modulators that contribute to central sensitization in the pain path. Moreover, nicotinamide did not have a significant effect on burrowing as a self-motivated behavior, but it improved cognitive performance in animals with OA, and decreased oxidative stress parameters in the heart muscle of MIA-injected rats. The effects of nicotinamide were largely sex-independent. Nicotinamide did not affect significantly behavioral effects of duloxetine and vortioxetine. In combination with duloxetine, nicotinamide attenuated all measured cardiac parameters of oxidative stress in comparison with those in duloxetine treatment alone. Our results support the use of nicotinamide in OA treatment, and clarify, at least in part, its mechanism of analgesic effect. In addition, the results of the study indicate that nicotinamide could be useful as a cardioprotective agent in combination with duloxetine in prolonged treatment of OA.

Declarations

Ethics approval

All experiments were conducted with approval from the Institutional Animal Care and Use Committee of the Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia (approval number: 323-07-09456/2020-05), and in accordance with the guidelines set by EU Directive 2010/63/EU for animal experiments. Adherence to ARRIVE guidelines is ensured for the reported animal studies.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Radica Stepanović-Petrović: Conceptualization (lead), Writing- original draft (lead), Writing- review & editing (lead), Supervision (lead), Project administration (lead), Funding acquisition (lead). **Katarina Nastić:** Methodology (supporting), Investigation (lead), Formal analysis (lead), Data Curation (lead), Writing- original draft (supporting), Visualization (lead). **Uroš Pecikoza:** Investigation (supporting), Formal analysis (supporting), Resources (lead), Writing- review & editing (supporting). **Miroslav Dinić:** Investigation (supporting), Formal analysis (supporting). **Emilija Brdarić:** Investigation (supporting). **Milica Labudović-Borović:** Investigation (supporting). **Jelena Kotur-Stevuljević:** Investigation (supporting), Formal analysis (supporting). **Aleksandar Jovanović:** Supervision (supporting). **Maja Tomić:** Conceptualization (supporting), Methodology (supporting), Writing- review & editing (supporting), Project administration (supporting).

Data availability

Data will be made available on request.

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Figures

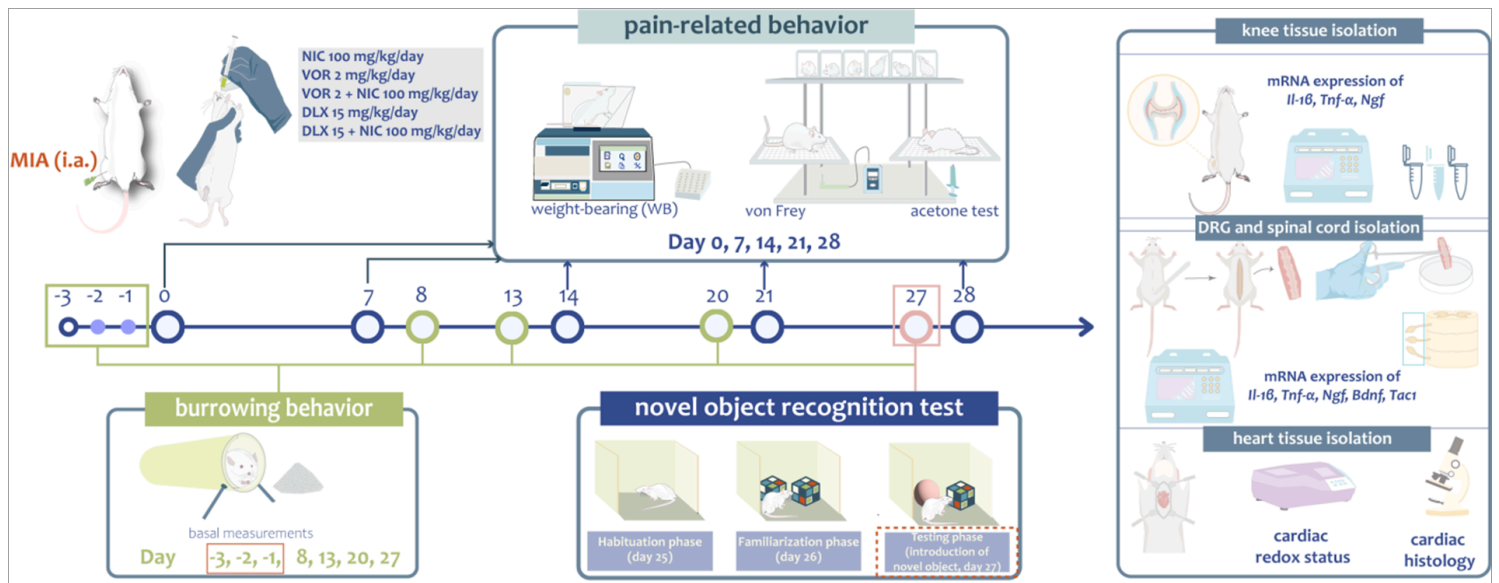


Figure 1

Schematic representation of the study design. Oral administration of nicotinamide (NIC), vortioxetine (VOR), duloxetine (DLX), and their combinations (VOR+NIC/DLX+NIC) began on day 0, the day of monoiodoacetate (MIA) intra-articular (i.a.) injection, and continued daily for 28 days. Prior to MIA injection, baseline values were recorded for behavioral assessments (weight-bearing, von Frey, acetone, and burrowing tests). Multiple behavioral assessments were conducted afterward on the specific days shown in the figure. On day 28, which was twenty-nine days following the MIA injection, the rats were sacrificed and their knee tissue, dorsal root ganglia (DRG), spinal cord, and heart muscle were harvested for examination of mRNA expression of pain-related mediators, heart redox status, and histological assessment.

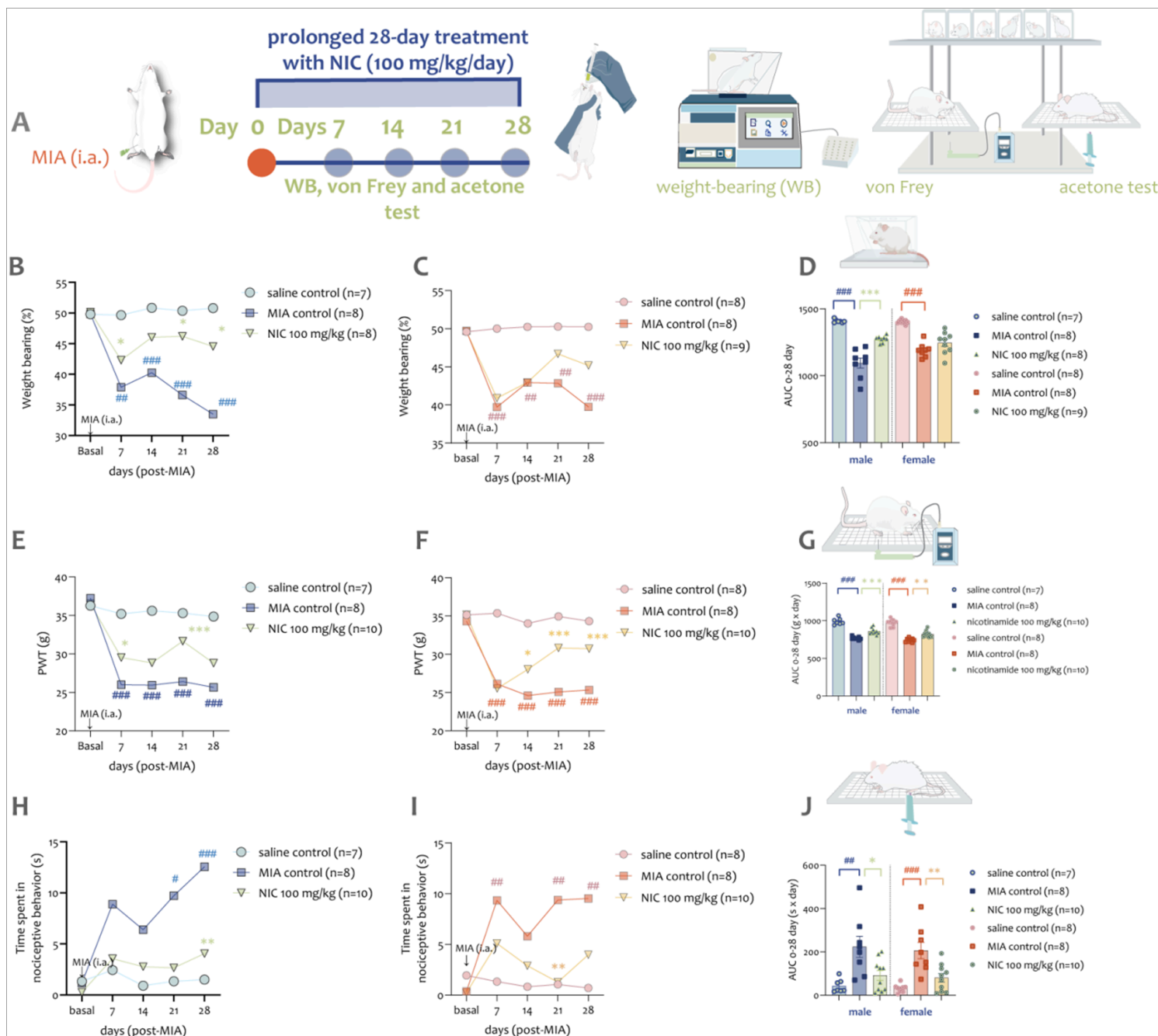


Figure 2

Nicotinamide (NIC) decreases pain hypersensitivity in male and female rats with OA induced by monoiodoacetate (MIA). (A) Schematic illustration of experimental timeline and treatment conditions. Oral administration of NIC began on the day of MIA intra-articular (i.a.) injection and continued for 28 days. (B, C and D) Effects of NIC in weight-bearing test in both male (B) and female (C) rats. (D) Area under the curve (AUC) of the weight-bearing distribution reported in panels B and C reflects data collected over the 28-day experiment. (E, F and G) Effects of NIC in the von Frey test in both male (E) and female (F) rats. (G) AUC of the paw-withdrawal threshold (PWT) reported in panels E and F reflects data collected over the 28-day experiment. (H, I and J) Effects of NIC in the acetone test in both male (H) and female (I) rats. (J) AUC of the time spent in nociceptive behavior reported in panels H and I reflects data

collected over the 28-day experiment. To determine statistical significance, comparisons were made against the saline control group (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$) and the MIA control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Two-way repeated measures ANOVA was used for time course data, and one-way ANOVA for AUC data, followed by Tukey's *post-hoc* test. Each panel legend specifies the number of animals (n) per group. Standard errors are omitted from the time-course graph for clarity.

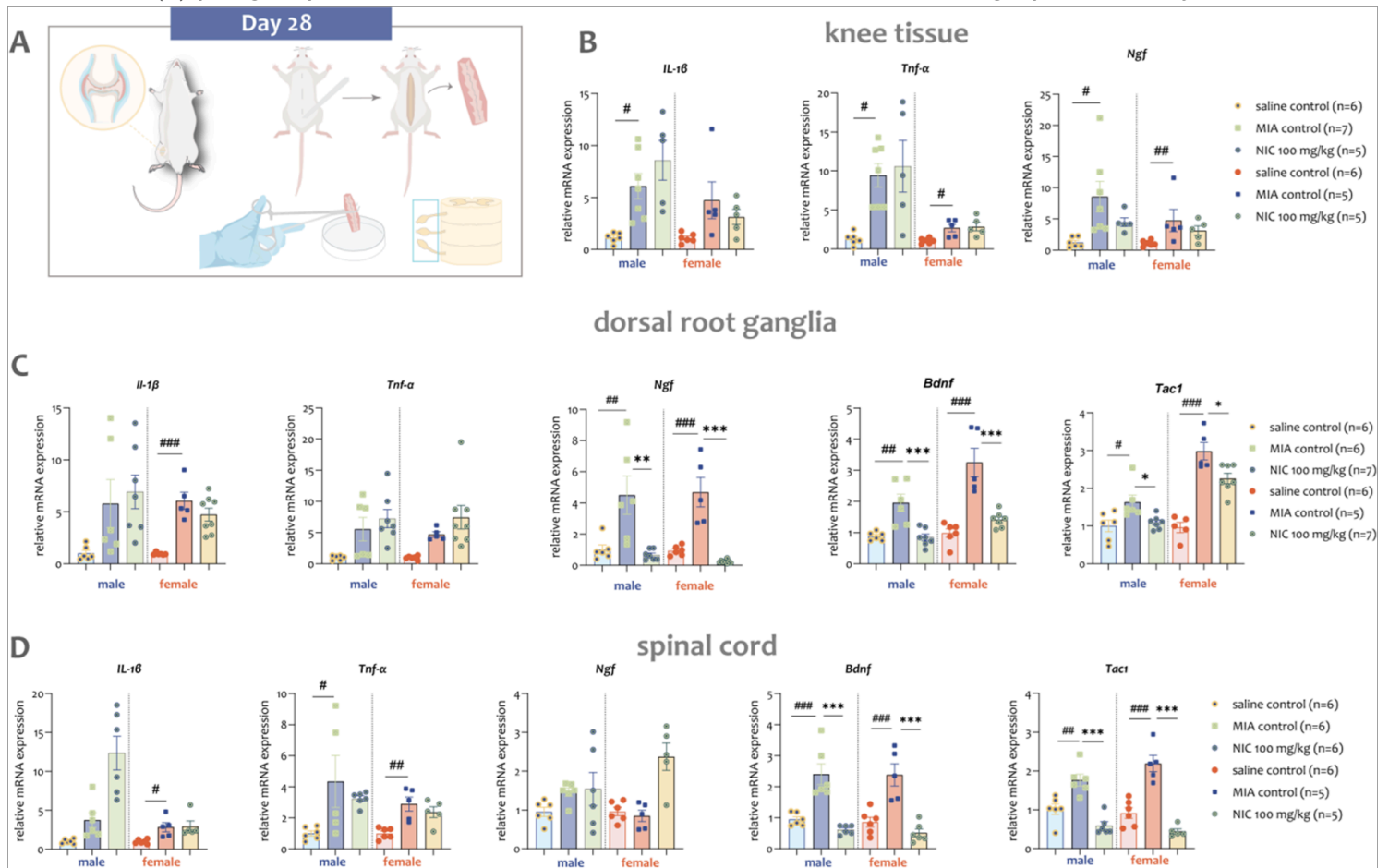


Figure 3

Effects of nicotinamide (NIC) on the mRNA expression of pain-related biomarkers in knee tissue, dorsal root ganglia (DRG) and spinal cord from male and female rats with OA induced by monoiodoacetate (MIA). (A) Schematic illustration of tissue isolation. At 28 days following MIA/saline injection, knee tissues, DRG, and spinal cord were collected to assess inflammatory biomarker mRNA expression via quantitative real-time PCR. (B) The effects of NIC on the mRNA expression of *IL-1 β* , *Tnf- α* and *Ngf* in knee tissue. (C) The effects of NIC on the mRNA expression of *IL-1 β* , *Tnf- α* , *Ngf*, *Bdnf* and *Tac1* in DRG. (D) The effects of NIC on the mRNA expression of *IL-1 β* , *Tnf- α* , *Ngf*, *Bdnf* and *Tac1* in the spinal cord. All results were normalized relative to the Gapdh housekeeping gene. To determine statistical significance, comparisons were made against the saline control group (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$) and the MIA control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) using one-way ANOVA followed by Tukey's *post hoc* test. Each panel legend specifies the number of animals (n) per group.

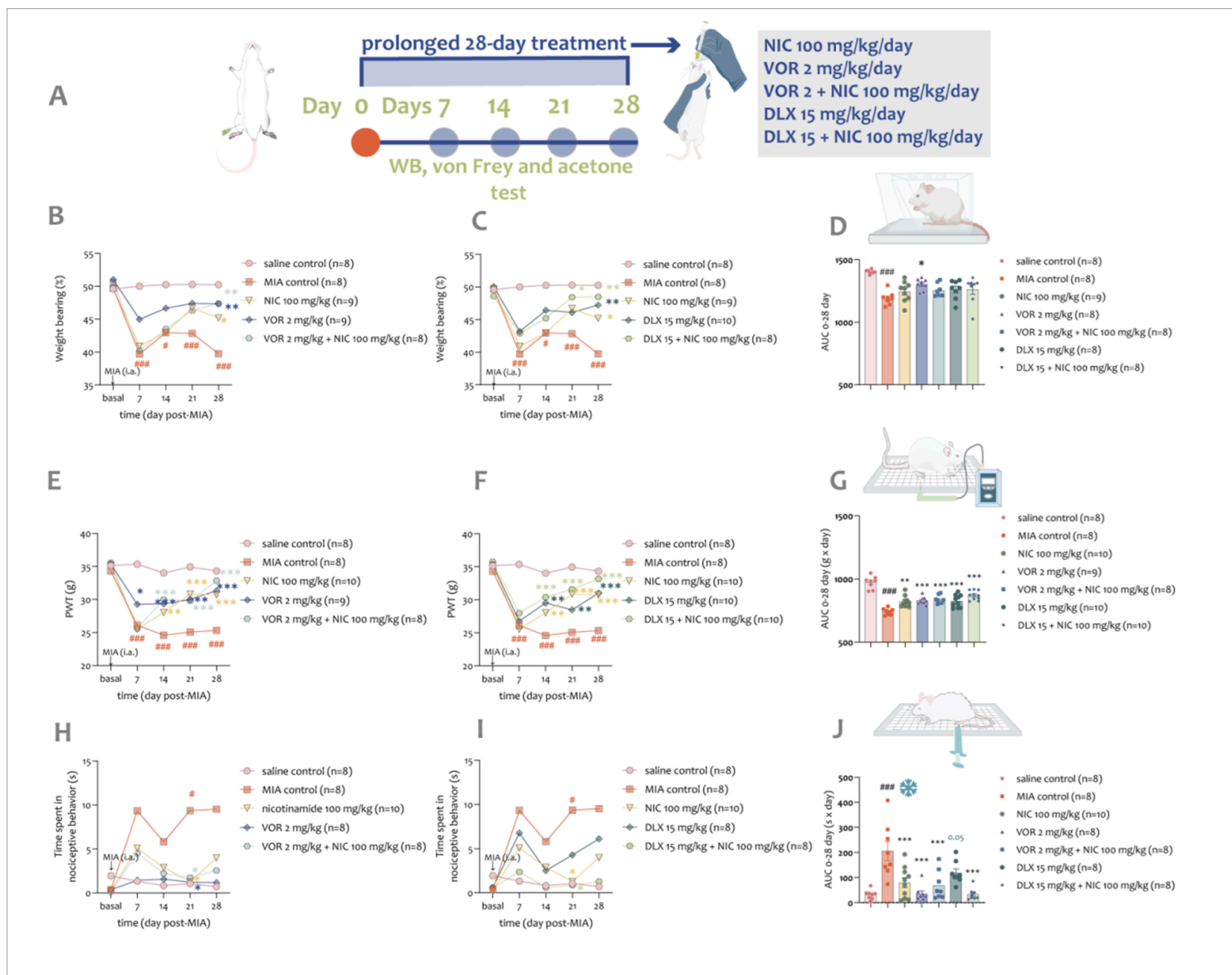


Figure 4

Effect of nicotinamide (NIC) in combination with vortioxetine (VOR) and duloxetine (DLX) on pain-related behavior in female rats with monoiodoacetate (MIA)-induced OA. (A) Schematic illustration of experimental timeline and treatment conditions. Oral administration of NIC and VOR+NIC/DLX+NIC began on the day of MIA intra-articular (i.a.) injection and continued for 28 days. (B, C and D) Effects of the combination of VOR+NIC (B) and DLX+NIC (C) in weight-bearing test in female rats. (D) Area under the curve (AUC) of the weight-bearing distribution reported in panel B and C reflects data collected over the 28-day experiment. (E, F and G) Effects of the combination of VOR+NIC (E) and DLX+NIC (F) in the von Frey test in female rats. (G) AUC of the paw-withdrawal threshold (PWT) reported in panels E and F reflects data collected over the 28-day experiment. (H, I and J) Effects of the combination of VOR+NIC (H) and DLX+NIC (I) in the acetone test in female rats. (J) AUC of the time spent in nociceptive behavior reported in panel H and I reflects data collected over the 28-day experiment. To determine statistical significance, comparisons were made against the saline control group (# $P < 0.05$, ## $P < 0.001$) and the MIA control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Two-way repeated measures ANOVA was used for

time course data, and one-way ANOVA for AUC data, followed by Tukey's *post-hoc* test. Each panel legend specifies the number of animals (n) per group. Standard errors are omitted from the time-course graph for clarity.

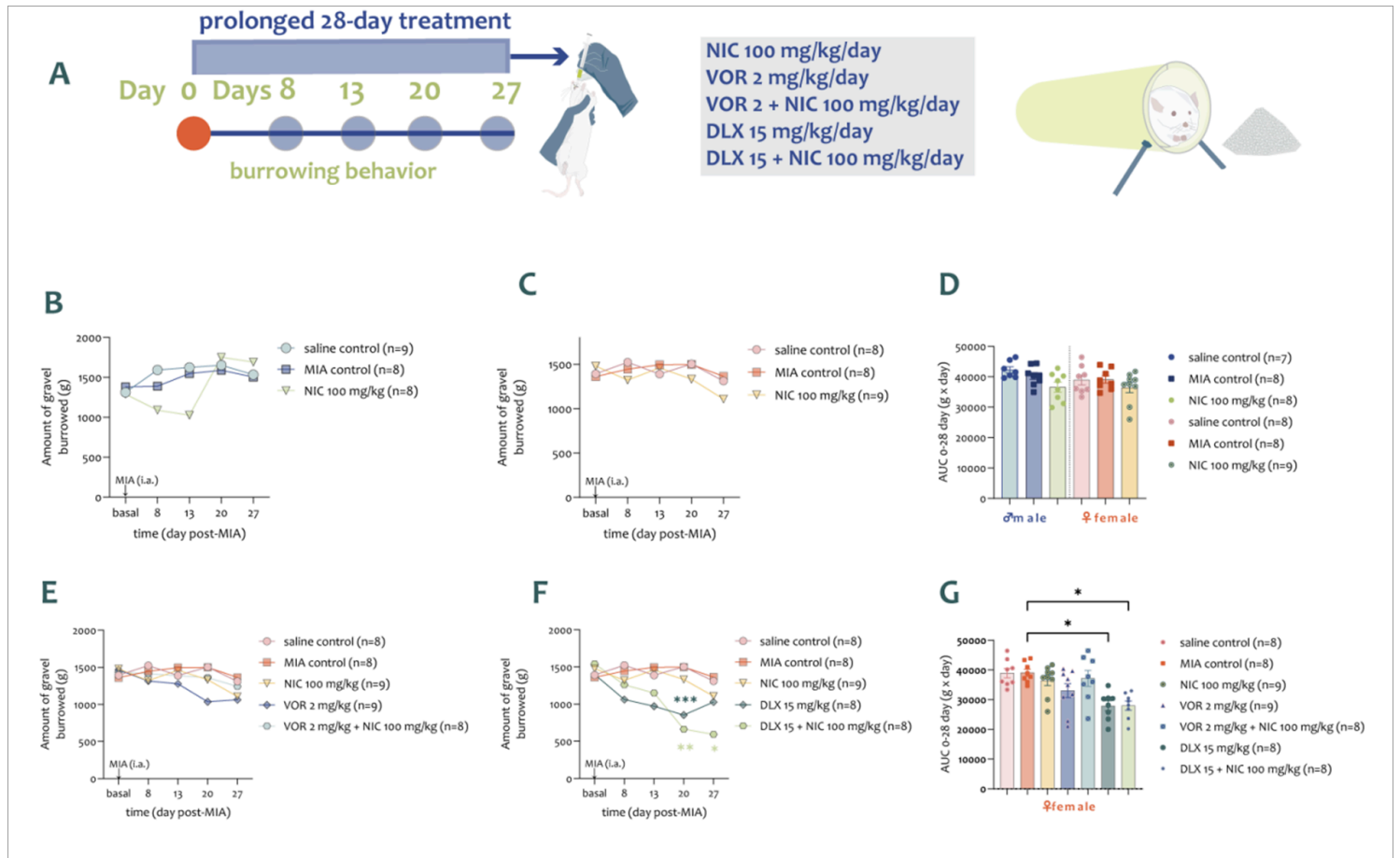


Figure 5

Effects of nicotinamide (NIC) and its combination with vortioxetine (VOR) and duloxetine (DLX) on burrowing activity of rats with OA induced by monoiodoacetate (MIA). (A) Schematic illustration of experimental timeline and treatment conditions. Oral administration of NIC and VOR+NIC/DLX+NIC began on the day of MIA intra-articular (i.a.) injection and continued for 28 days. (B, C and D) Effects of NIC in male (B) and female (C) rats on burrowing behavior. (D) Area under the curve (AUC) of the amount of gravel burrowed reported in panels B and C reflects data collected over the 28-day experiment. (E, F and G) Effects of the combination of VOR+NIC (E) and DLX+NIC (F) on burrowing behavior in female rats. (G) AUC of the amount of gravel burrowed reported in panel E and F reflects data collected over the 28-day experiment. To determine statistical significance, comparisons were made against the MIA control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Two-way repeated measures ANOVA was used for time course data, and one-way ANOVA for AUC data, followed by Tukey's *post-hoc* test. Each panel legend specifies the number of animals (n) per group. Standard errors are omitted from the time-course graph for clarity.

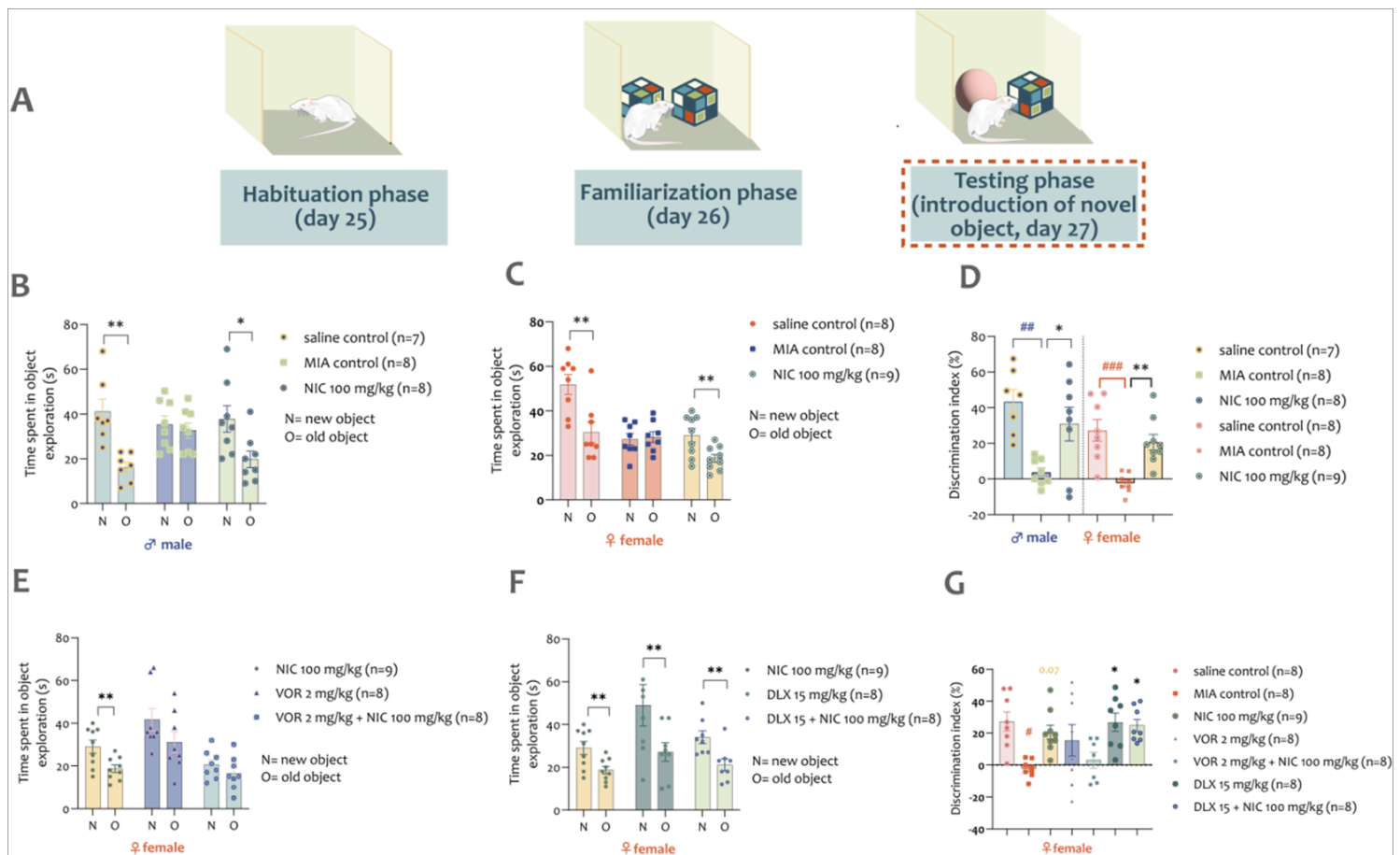


Figure 6

Effects of nicotine (NIC) and its combination with vortioxetine (VOR) and duloxetine (DLX) on cognitive ability in the novel object recognition test (NORT) of rats with OA induced by monoiodoacetate (MIA). (A) Schematic illustration of experimental timeline. (B, C and D) Effects of NIC in the NORT are expressed as the duration (in seconds) of exploration of the novel (N) and old (O) objects in male (B) and female (C) rats and discrimination indexes (D) obtained 27 days post-MIA injection. (E, F and G) Effects of the combination of VOR+NIC (E) and DLX+NIC (F) in the NORT are expressed as the duration (in seconds) of exploration of the novel (N) and old (O) objects (panels E and F) in female rats and discrimination indexes (panel G) obtained 27 days post-MIA injection. Duration of exploration of the N and O objects was analyzed using paired *t*-test for N vs. O object comparison (**P* < 0.05, ***P* < 0.01) and one-way ANOVA, followed by Tukey's *post-hoc* test for discrimination index data (##*P* < 0.01, ###*P* < 0.001 compared to the saline control group; **P* < 0.05, ***P* < 0.01 compared to the MIA control group). Each panel legend specifies the number of animals (n) per group.

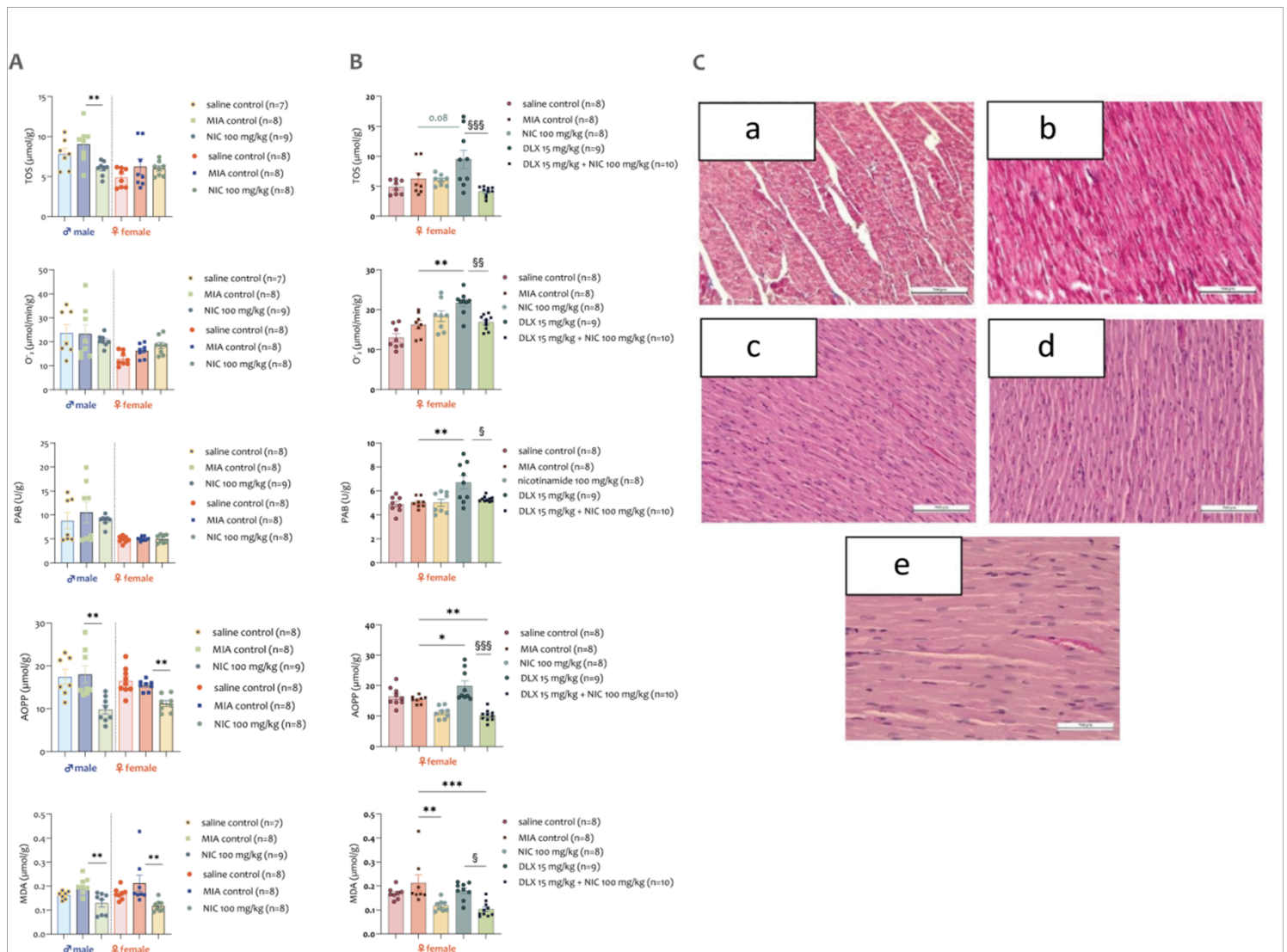


Figure 7

Effects of nicotine (NIC) and its combination with duloxetine (DLX) on oxidative stress markers and histology of cardiac muscle tissue in rats with OA induced by monoiodoacetate (MIA). After 28 days of NIC and DLX+NIC administration the levels of total pro-oxidant status (TOS), superoxide anion radicals ($O_2^{\cdot-}$), pro-oxidant antioxidant balance (PAB), advanced oxidation protein products (AOPP) and malondialdehyde (MDA) were measured. (A) Effects of NIC on cardiac oxidative stress markers in male and female rats. (B) Effects of the combination of DLX+NIC on cardiac oxidative stress markers in female rats. (C) Histology of the heart muscle tissue. (a) Representative image (Masson trichrome staining, 100x magnification) of heart tissue from male rat receiving DLX 15 mg/kg/day. (b) Representative image (Masson trichrome staining, 100x magnification) of heart tissue from female rat receiving DLX 15 mg/kg/day. (a) and (b): Cardiomyocytes with hypercontraction of sarcomeres, condensation of eosinophilic cytoplasm and intracellular edema, as well as perivascular edema. (c) Representative image (hematoxylin eosin staining, 40x magnification) of heart tissue from male rat receiving NIC 100 mg/kg/day. (d) Representative image (hematoxylin eosin staining, 40x magnification) of heart tissue from female rat receiving NIC 100 mg/kg/day. (e) Representative image (hematoxylin

eosin staining, 400x magnification) of heart tissue from female rat receiving DLX 15 mg/kg/day + NIC 100 mg/kg/day. To determine statistical significance for oxidative stress markers, comparisons were made against the MIA control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) or DLX 15 mg/kg/day group (§ $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$), using one-way ANOVA followed by Tukey's *post hoc* test. Each panel legend specifies the number of animals (n) per group.