

Article

Investigation of Potential Effects of Ibuprofen on the Storage Cells and Anhydrobiosis Capacity of the Tardigrade *Paramacrobiotus experimentalis*

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Abstract: The surge in pharmaceutical consumption, particularly non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, has raised concerns about their presence in aquatic ecosystems. This study investigated the potential ecological impact of ibuprofen, focusing on the ultrastructure of storage cells in the tardigrade *Paramacrobiotus experimentalis*, renowned for its resilience to environmental stressors. Individuals were exposed to three ibuprofen concentrations (0.1 µg/L, 16.8 µg/L, and 1 mg/L) over 7 and 28 days. Storage cells were examined using light microscopy, transmission electron microscopy, and confocal microscopy. This study also explored ibuprofen's impact on the process of anhydrobiosis. In the short-term experiment, no ultrastructural changes in tardigrade storage cells were observed across ibuprofen concentrations. However, in the long-term incubation, autophagic structures in storage cell cytoplasm were identified, indicating potential adaptive responses. Individual mitochondria exhibited degeneration, and the rough endoplasmic reticulum displayed slight swelling. No evidence of increased oxidative stress or nuclear DNA fragmentation was observed in any research group. This study elucidates the complex responses of tardigrade storage cells to ibuprofen exposure. The findings emphasize the importance of understanding pharmaceutical impacts on aquatic organisms, highlighting the resilience of tardigrades to specific environmental stressors.

Keywords: tardigrades; anhydrobiosis; ibuprofen



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1. Introduction

Consumption of pharmaceuticals increases every year as they are widely used to prevent or treat human and animal diseases [1]. They can enter the aquatic environment as metabolites or in unchanged form. As these compounds contaminate aquatic habitats (through improper disposal or ineffective methods in wastewater treatment plants), they can have devastating effects on the environment and aquatic life [2,3]. One of the main groups of substances that pose a threat to aquatic environments is non-steroidal anti-inflammatory drugs [2,4]. Ibuprofen, acetylsalicylic acid (aspirin), diclofenac, and naproxen are reported to be contaminants in aquatic ecosystems. These drugs are widely used for their analgesic, antipyretic, and anti-inflammatory properties in both human and veterinary medicine. The presence of these substances in aquatic environments can have significant ecological and environmental implications. They can persist in aquatic systems for extended periods. Additionally, some of these compounds can bioaccumulate in the tissues of aquatic organisms. Bioaccumulation occurs when organisms at lower trophic levels consume contaminated water or food, and the accumulated substances become more concentrated

as they move up the food chain [1,5,6]. One of the most widely used NSAIDs is ibuprofen, with a history dating back to its synthesis in the 1960s. It was developed as a response to the need for an analgesic and anti-inflammatory medication that could be as effective as aspirin but with fewer intense side effects. Ibuprofen is commonly used for the treatment of acute and chronic pain, as well as various rheumatic diseases. It is effective in reducing pain, inflammation, and fever [7,8]. Ibuprofen exerts its anti-inflammatory and analgesic effects by inhibiting cyclooxygenase (COX) enzymes. Specifically, it inhibits both COX-1 and COX-2, which are involved in the synthesis of prostaglandins. Prostaglandins play a crucial role in inflammation, pain, and fever. By inhibiting their production, ibuprofen reduces these symptoms [8–10]. Tardigrades, commonly known as water bears, are small invertebrates known for their exceptional resilience to a wide range of environmental stressors, including extreme temperatures, lack of water, and radiation. Their ability to withstand these harsh conditions is attributed to a process called cryptobiosis [11]. The field of tardigrade research has gained increasing attention due to their remarkable survival mechanisms and their potential applications in various scientific and industrial fields. Studying the ultrastructure of tardigrades when exposed to pharmaceuticals or other stressors can provide insights into the mechanisms by which these organisms cope with adverse conditions. This study investigated the effect of short- and long-term exposure to ibuprofen on the ultrastructure of the storage cells of *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa, Roszkowska 2020 (Tardigrada, Eutardigrada). Storage cells are found either free floating in the body cavity fluid or attached to the basement membranes of internal organs. Their primary function is to accumulate reserve materials, which can be used during times of stress or unfavorable environmental conditions [11–15].

We also investigated the effect of ibuprofen on the process of anhydrobiosis of *Pam. experimentalis*. This adaptation allows certain organisms, such as tardigrades and nematodes, to survive near-complete dehydration by suspending their metabolic processes. During anhydrobiosis, these organisms lose most of their body water. When conditions become favorable again, these organisms can rehydrate and resume their normal life processes [16–20].

2. Materials and Methods

2.1. Materials

Research was conducted using *Paramacrobiotus experimentalis* (Tardigrada, Eutardigrada, Macrobiotidae), a predatory and gonochoric species. They lay ornamented eggs freely in their environment. The bodies of these animals are transparent or white [21]. They were collected in 2013 from moss samples in eastern Madagascar, specifically in the Toamasina and Antananarivo provinces. The specimens were generously provided by Professor Łukasz Kaczmarek from Adam Mickiewicz University in Poznań, Poland. To create suitable conditions, the animals were cultured in plastic Petri dishes with a textured bottom. They were placed in a mixture of Żywiec Zdrój mineral water and distilled water in a 1:3 ratio. The temperature was maintained at 19 °C, and they were subjected to a 12 h light and 12 h dark cycle. As for their diet, they were nourished with rotifers (*Lecane inermis*), with the addition of algae (*Chlorella* sp. and *Chlorococcum* sp.) to their culture.

2.2. Methods

2.2.1. Experiment

The specimens were divided into ten groups, consisting of four control groups and six experimental groups (see Table 1). Each group of 50 animals was cultured in 12-well plates with a textured bottom. The total amount of solution added to one well was 3000 µL. The PeC control group was cultured in the culture medium, as previously described in Section 2.1. The control groups PeC0, PeC7, and PeC28 were also cultured in the culture medium, but with the addition of 1 mL of ethanol per liter of the medium. This step was necessary because, in the experimental groups, ibuprofen was dissolved in 1 mL of ethanol before being introduced to the medium. It is worth noting that ibuprofen is more soluble in ethanol than in water. These control groups were instrumental in eliminating any varia-

tions caused by the presence of ethanol in the culture medium and those triggered by the aging of the animals. The experimental groups consisted of specimens cultured in 12-well plates with a culture medium enriched with ibuprofen. Three concentrations of ibuprofen were utilized in our research: two environmental concentrations, i.e., 0.1 µg/L [22] and 16.8 µg/L [23], and a higher concentration (1 mg/L). For each concentration, the experiments were conducted at two different exposure times: 7 days and 28 days (see Table 1). Importantly, neither the ibuprofen concentrations nor the exposure durations increased the mortality of the animals.

Table 1. Control and experimental groups.

PeC	specimens cultured in the culture medium
PeC0	specimens cultured in the culture medium with the addition of 1 mL of ethanol per liter of medium
PeC7	specimens cultured 7 days in the culture medium with the addition of 1 mL of ethanol per liter of medium
PeC28	specimens cultured 28 days in the culture medium with the addition of 1 mL of ethanol per liter of medium
Ib1a	specimens cultured in ibuprofen at a concentration of 0.1 µg/L for 7 days
Ib1b	specimens cultured in ibuprofen at a concentration of 16.8 µg/L for 7 days
Ib1c	specimens cultured in ibuprofen at a concentration of 1 mg/L for 7 days
Ib2a	specimens cultured in ibuprofen at a concentration of 0.1 µg/L for 28 days
Ib2b	specimens cultured in ibuprofen at a concentration of 16.8 µg/L for 28 days
Ib2c	specimens cultured in ibuprofen at a concentration of 1 mg/L for 28 days

2.2.2. Light and Transmission Electron Microscopy

The animals (Table 2) were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for a period of seven days at 4 °C. Following this, they were rinsed three times, each time for 30 min in a 0.1 M phosphate buffer at room temperature (RT) and postfixed in 2% osmium tetroxide in a 0.1 M phosphate buffer for 2 h. The specimens were then rinsed again in a 0.1 M phosphate buffer three times, 10 min each. Next, they underwent dehydration in a graded series of ethanol (30, 50, 70, 90, 96, and 100% for 10 min, 2 × 10 min, 15 min, 15 min, 15 min, and 4 × 15 min, respectively). After dehydration, the material was incubated in a solution of 100% ethanol and acetone (1:1) for 15 min and washed twice in acetone, 10 min each. After that, the material was embedded in epoxy resin (Poly/Bed 812 Embedding Media/DMP-30 Kit, Polysciences, Inc., Warrington, PA, USA). Semi-thin sections (800 nm) were cut on a Leica UCT25 ultramicrotome, stained with 1% methylene blue in 1% borax, and analyzed using a light microscope, while ultra-thin sections (70 nm) were cut on a Leica EM UC7 ultramicrotome, stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Hitachi H500 at 75 kV).

Table 2. The number of adult specimens of *Pam. experimentalis* for each method.

	Light and Transmission Electron Microscopy	Confocal Microscopy			Induction and Recovery from Anhydrobiosis
		TUNEL	LysoTracker Red	DHE	
PeC	20	10	10	10	24
PeC0	20	10	10	10	24
PeC7	20	10	10	10	24
PeC28	20	10	10	10	24
Ib1a	20	10	10	10	24
Ib1b	20	10	10	10	24
Ib1c	20	10	10	10	24
Ib2a	20	10	10	10	24
Ib2b	20	10	10	10	24
Ib2c	20	10	10	10	24

2.2.3. Confocal Microscopy

TUNEL (Terminal Deoxynucleotidyl Transferase)—Detection of DNA Fragmentation during Apoptosis

The non-fixed specimens (Table 2) were submerged in a 0.1% sodium citrate solution that had been freshly prepared for a duration of 2 min while placed on ice. After the sodium citrate incubation, the specimens were carefully washed three times for 5 min each with TBS (Tris-buffered saline). Following the TBS washing steps, the specimens were subjected to a staining process using a TUNEL reaction mixture. Specifically, the In situ Cell Death Detection Kit TMR red from Roche Applied Science was utilized for this purpose. The staining occurred in darkness at 37 °C for 1 h. After this hour of staining, the specimens were once again washed with TBS, the three washes being repeated for 5 min each. Subsequently, the specimens were stained with a solution containing 1 µg/mL of DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) in TBS. This staining lasted for 15 min at RT and was conducted in darkness. The DAPI-stained specimens were washed with TBS three more times, for 5 min each. To prepare them for microscopy, the specimens were mounted on microscopic slides and enclosed in a Vectashield medium. The final step involved the analysis of the specimens using an Olympus FluoView FV 1000 confocal microscope with a 60 x UPLSAPO lens. Excitation at 405 nm for DAPI dye and 570 nm for TMR red dye was provided by a diode laser 3000.

LysoTracker Red—Detection of Acidic Organelles

The non-fixed specimens (Table 2) were placed in a dark environment and immersed in a solution of 2.5 mM LysoTracker Red DND-99 (Molecular Probes, L 7528, Thermo Fisher Scientific, Waltham, MA, USA) for 15 min. This incubation took place in TBS at a pH of 7.4 and RT. After the LysoTracker Red incubation, the specimens were rinsed with TBS, the three washes being repeated for 5 min each. Then, the material was stained with a solution containing 1 µg/mL of DAPI in TBS. This staining process was carried out for 15 min. Following the DAPI staining, the specimens were washed in TBS, the process being repeated three times for 5 min each. To prepare them for microscopy, the specimens were mounted on microscopic slides and enclosed in a Vectashield medium. The final step involved analysis of the specimens using an Olympus FluoView FV 1000 confocal microscope with a 60 UPLSAPO lens. Excitation at 405 nm (DAPI) and 570 nm (LysoTracker Red) was provided by a diode laser 3000.

Dihydroethidium (DHE) Analyzing Superoxide Levels (Differentiation of ROS+ and ROS− Cells)

The non-fixed specimens (Table 2) were treated with TBS containing 0.0025% Triton X100 at RT. Following this, the specimens were stained with a solution of 30 µM DHE (Chemodex), which was prepared from a 30 mM stock solution of DHE in DMSO. The staining process lasted for 15 min at RT in darkness. After the DHE staining, the specimens were washed with TBS, this step being repeated three times for 5 min each. Subsequently, the specimens were stained with a solution containing 1 µg/mL of DAPI in TBS. This staining procedure also took 15 min at RT in darkness. Following the DAPI staining, the specimens were washed with TBS, this step being repeated three times for 5 min each. To prepare them for microscopy, the specimens were mounted on microscopic slides and enclosed in a Vectashield medium. The final step involved the analysis of the specimens using an Olympus FluoView FV 1000 confocal microscope with a 60× UPLSAPO lens. Excitation at 405 nm (DAPI) and 561 nm (DHE) was provided by a diode laser 3000.

Induction and Recovery from Anhydrobiosis

Specimens of *Pam. experimentalis* (Table 2) were transferred with a small amount of medium (450 µL; a mixture of Żywiec Zdrój mineral water and distilled water; ratio 1:3) to plastic Petri dishes lined with laboratory filter paper. They were stored for 3 days at 19 °C in a 12 h light–12 h dark cycle as the individuals were allowed to dry slowly. Next, 3 mL

of the culture medium was added to each Petri dish. Samples were transferred using an automatic pipette to a 24-well plate. The individuals were rehydrated, and their return to activity was observed after 24 h, 48 h, and 72 h. They were incubated for 3 days at 19 °C in a 12 h light–12 h dark cycle.

Statistical Analysis

The assessment of active individuals after rehydration at the 72 h mark was conducted using the ANOVA test. All statistical analyses were performed using Statistica version 13.0.

3. Results

3.1. Ultrastructural Changes in Storage Cells of *Pam. experimentalis* Exposed to Ibuprofen

3.1.1. Control Groups (PeC, PeC0, PeC7, PeC28)

Ultrastructural analysis of the control groups cultured in the medium containing ethanol (PeC0, PeC7, PeC28) and without ethanol (PeC) showed no differences. No differences due to aging were observed in the control groups (PeC0, PeC7, PeC28). Therefore, all control groups will be described together. The body cavity of tardigrades was filled with numerous storage cells (Figure 1A). The storage cells of tardigrades from control groups had an amoeboid shape (Figure 1B–D). These cells had a large, centrally located nucleus containing a heterogeneous nucleolus with a spongy structure (Figure 1B,D). Heterochromatin clumps surrounded the nucleolus. Numerous ribosomes and cell organelles including mitochondria with distinct cristae, cisterns of rough endoplasmic reticulum, and Golgi complexes were present in the cell cytoplasm (Figure 1B–D). Large numbers of spheres of reserve material were present in storage cells (Figure 1B–D). These spheres had various sizes and electron densities, which may reflect the different types of content.

3.1.2. Experimental Groups after 7 Days of Exposure to Ibuprofen: Ib1a (Ibuprofen Concentration 0.1 µg/L), Ib1b (Ibuprofen Concentration 16.8 µg/L), Ib1c (Ibuprofen Concentration 1 mg/L)

The shape of the storage cells of experimental group Ib1a (Figure 2A), Ib1b (Figure 2B), and Ib1c (Figure 2C,D) did not differ from the shape of cells in the control groups. The cell nuclei (Figure 2A,C,D) with heterogeneous nucleoli (Figure 2A,D) were positioned centrally within the cell. Compared to the control groups, no changes were observed in the ultrastructure of cell organelles (Figure 2A–D).

3.1.3. Experimental Group Ib2a (Ibuprofen Concentration 0.1 µg/L, 28 Days)

The storage cells had an amoeboid shape. The cell nucleus with nucleolus was observed in the central part of the cell (Figure 3A,B). Mitochondria, cisterns of rough endoplasmic reticulum, Golgi complexes, and spheres of reserve material were present in the cell cytoplasm. An accumulation of electron-dense material was observed in the mitochondrial matrix of some mitochondria (Figure 3A,B). A few autophagic structures appeared in the cytoplasm (Figure 3B).

3.1.4. Experimental Group Ib2b (Ibuprofen Concentration 16.8 µg/L, 28 Days)

The shape of the storage cells did not differ from the shape of cells in the control groups. Storage cells showed a similar ultrastructure to cells from the Ib2a experimental group. The only difference was the increased number of autophagic structures in the cell cytoplasm (Figure 3C,D).

3.1.5. Experimental Group Ib2c (Ibuprofen Concentration 1 mg/L, 28 Days)

As in the control animals, the storage cells had an amoeboid shape. The cell nucleus was observed in the central part of the cell (Figure 3E,F). Some of the mitochondria had degenerated. The degenerated mitochondria were bloated, had lost their cristae, and their matrix had become electron-lucent (Figure 3E). The cisternae of the rough endoplasmic reticulum were also slightly bloated. Autophagic structures were observed in the cytoplasm

of cells (Figure 3E,F). Moreover, the cytoplasm itself showed a heterogeneous structure, with some electron-lucent regions (Figure 3E,F).

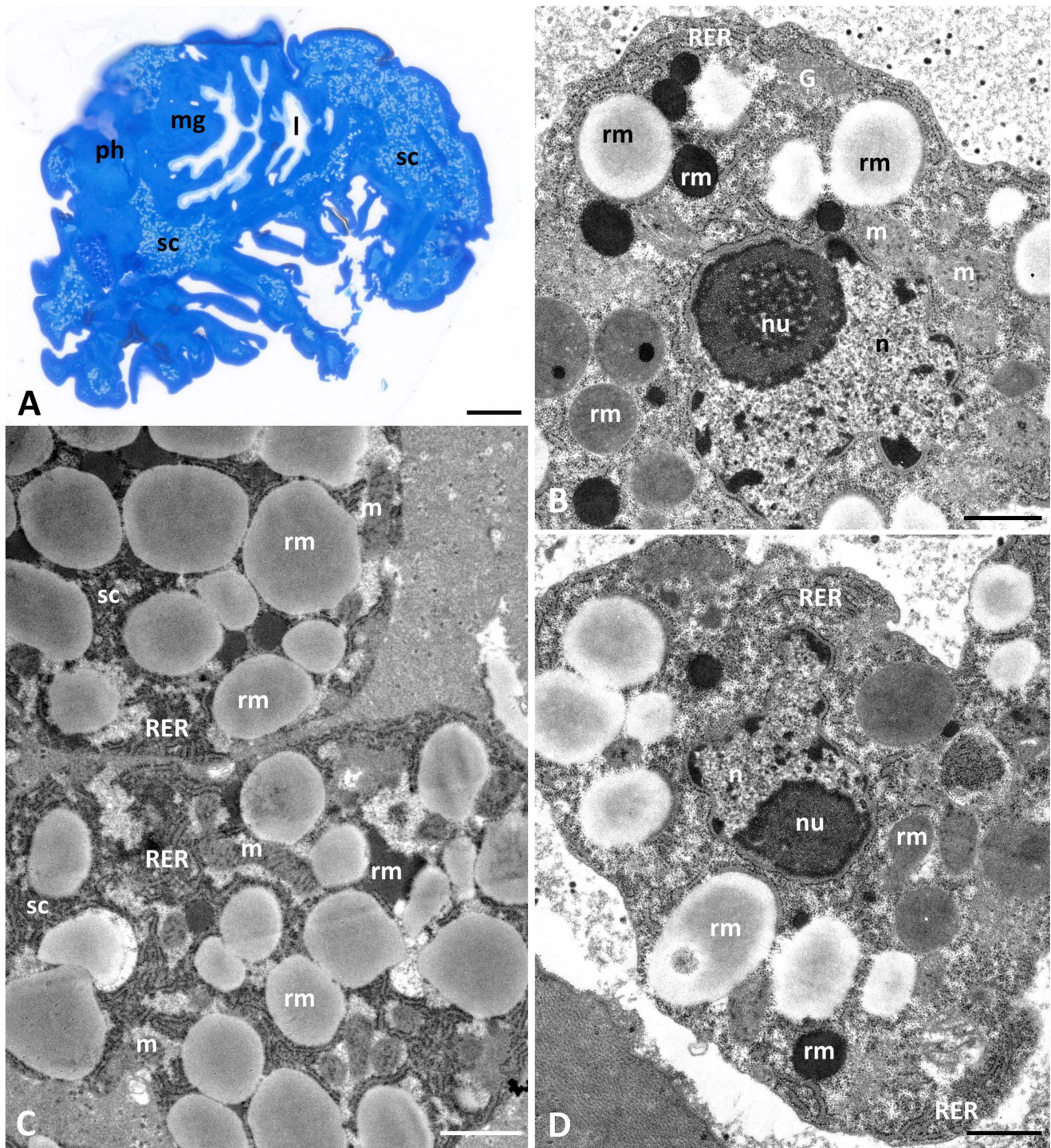


Figure 1. Storage cells of *Pam. experimentalis* in control groups—PeC, PeC0, PeC7, PeC28. (A) Localization of storage cells in the animal body, methylene blue staining, LM, scale = 20 μ m; (B–D) Ultrastructure of storage cells in the control groups, TEM, scale = 1 μ m. Abbreviations: storage cells (sc), Golgi complexes (G), midgut lumen (l), midgut (mg), mitochondrion (m), nucleus (n), nucleolus (nu), pharynx (ph), rough endoplasmic reticulum (RER), spheres of reserve material (rm).

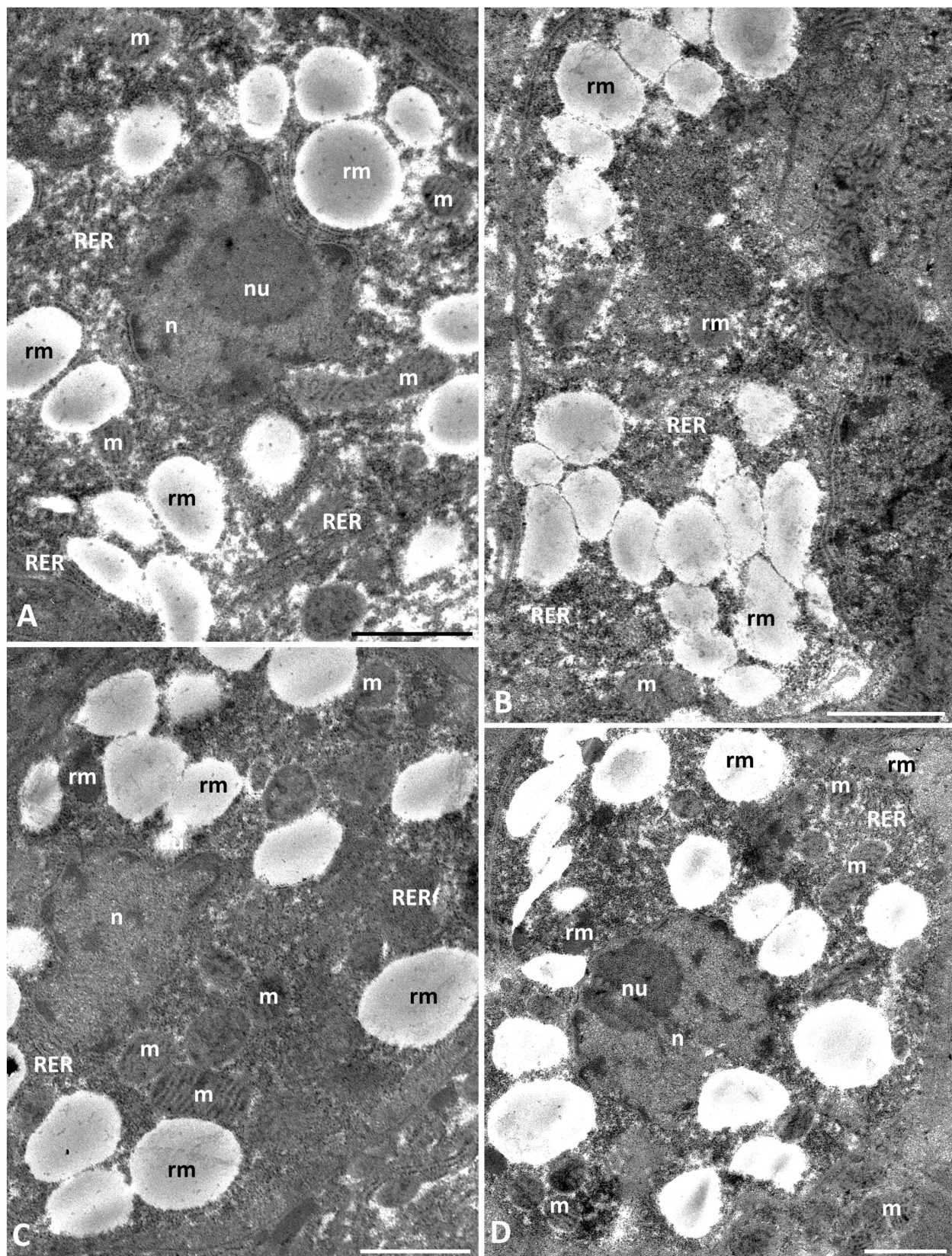


Figure 2. Ultrastructure of storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; (A) concentration of ibuprofen 0.1 µg/L—Ib1a, (B) 16.8 µg/L—Ib1b, and (C,D) 1 mg/L—Ib1c, TEM. (A) scale = 1 µm. Abbreviations: mitochondrion (m), nucleus (n), nucleolus (nu), rough endoplasmic reticulum (RER), spheres of reserve material (rm).

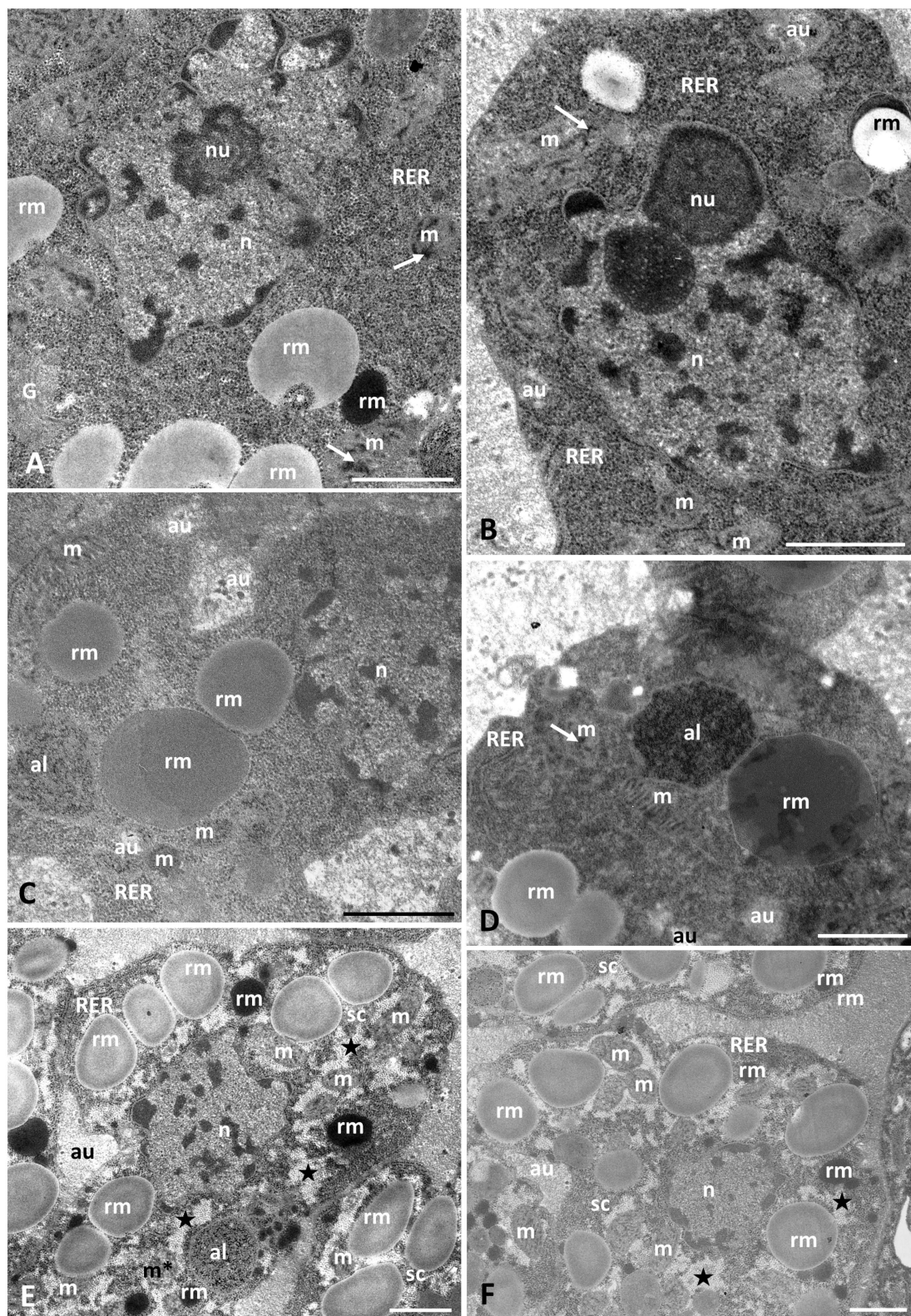


Figure 3. Ultrastructure of storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; (A,B) concentration of ibuprofen 0.1 µg/L—Ib1a, (C,D) 16.8 µg/L—Ib1b, and (E,F) 1 mg/L—Ib1c, TEM, scale = 1 µm. Abbreviations: storage cells (sc), mitochondrion (m), degenerating mitochondrion (m*), nucleus (n), nucleolus (nu), rough endoplasmic reticulum (RER), spheres of reserve material (rm), Golgi apparatus (G), autolysosome (al), autophagosome (au), accumulation of electron-dense material (arrow), electron-lucent regions of cytoplasm (star).

3.2. Confocal Microscopy Analysis

3.2.1. ROS+/ROS− Cells in Storage Cells of *Pam. experimentalis* Exposed to Ibuprofen

The use of dihydroethidium (DHE) revealed weak signals in storage cells of the control groups (Figure 4A,E), as well as in experimental groups Ib1a, Ib1b (low concentrations of ibuprofen, incubation 7 days) (Figure 4B,C), and Ib2a (Figure 4F). Slightly stronger signals were observed in storage cells from the experimental groups Ib1c (Figure 4D), Ib2b, and Ib2c (Figure 4G–H).

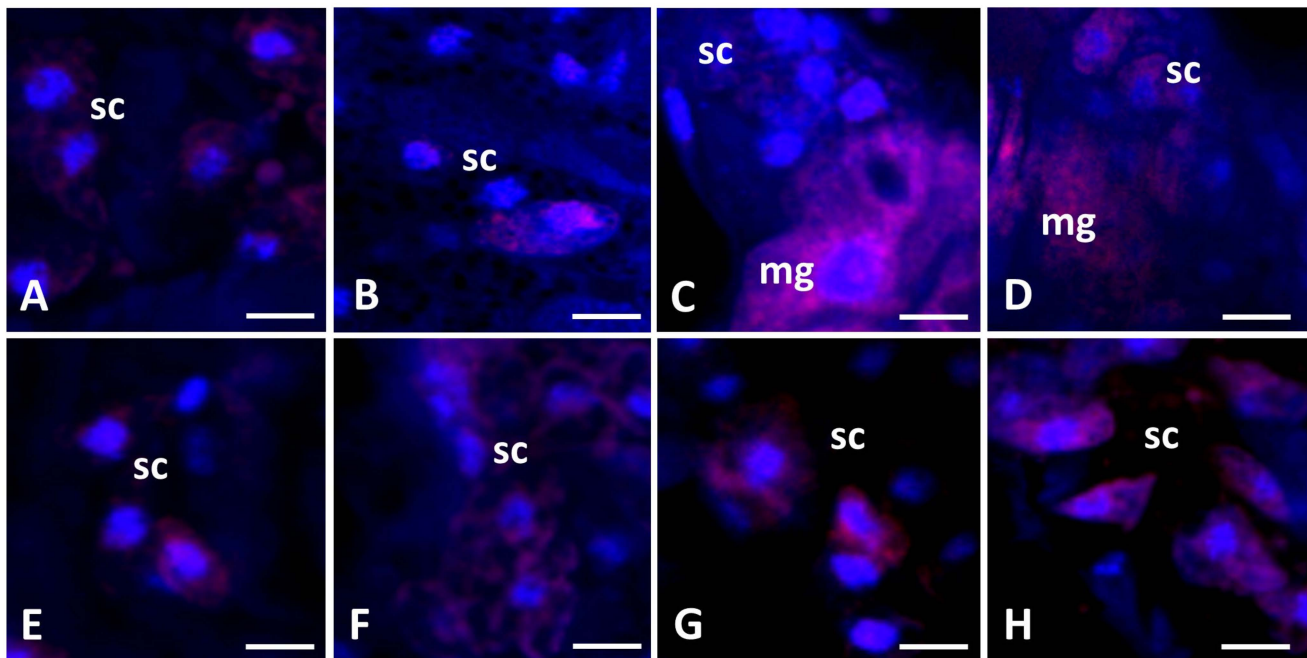


Figure 4. Storage cells of *Pam. experimentalis* stained with dihydroethidium (DHE) and DAPI, confocal microscopy. ROS-positive cells (red signals), nuclei (blue signals). (A,E) Storage cells of *Pam. experimentalis* in control groups; (A) PeC7 group; (E) PeC28 group; (B) storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; concentration of ibuprofen 0.1 $\mu\text{g/L}$ —Ib1a; (C) storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; concentration of ibuprofen 16.8 $\mu\text{g/L}$ —Ib1b; (D) storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; concentration of ibuprofen 1 mg/L—Ib1c; (F) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 0.1 $\mu\text{g/L}$ —Ib2a; (G) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 16.8 $\mu\text{g/L}$ —Ib2b; (H) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 1 mg/L—Ib2c, (A–H) scale = 4.71 μm . Abbreviations: midgut (mg), storage cells (sc).

3.2.2. Apoptosis in Storage Cells of *Pam. experimentalis* Exposed to Ibuprofen

Analysis using TUNEL showed no nuclear DNA fragmentation, which is a marker of cell apoptosis, in any of the control groups (Figure 5A,E). Also, no signals of DNA fragmentation were found in storage cells from the experimental groups (Figure 5B,C,F–H).

3.2.3. Autophagy in Storage Cells of *Pam. experimentalis* Exposed to Ibuprofen

Qualitative analysis using LysoTracker Red showed that signals from acidic organelles (autolysosomes, lysosomes) were comparable in control groups (Figure 6A,E) and in experimental groups (Ib1a, Ib1b, Ib1c) (Figure 6B–D) in which animals were treated with ibuprofen for seven days. After 28 days of treatment with ibuprofen (Ib2a, Ib2b, Ib2c), the emitted signals were much stronger (Figure 6F–H).

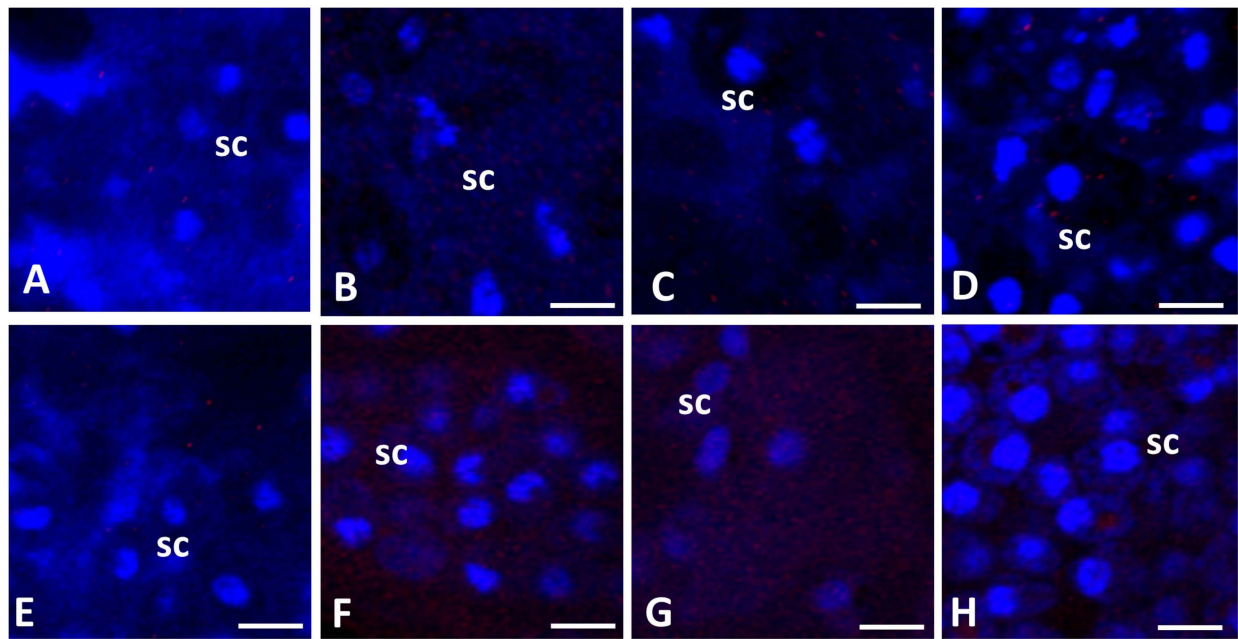


Figure 5. Storage cells of *Pam. experimentalis* stained with TUNEL assay and DAPI, confocal microscopy. Nuclei (blue signals), DNA fragmentation (red signals). (A,E) Storage cells of *Pam. experimentalis* in control groups; (A) PeC7 group; (E) PeC28 group; (B) storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; concentration of ibuprofen 0.1 $\mu\text{g/L}$ —Ib1a; (C) storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; concentration of ibuprofen 16.8 $\mu\text{g/L}$ —Ib1b; (D) storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; concentration of ibuprofen 1 mg/L—Ib1c; (F) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 0.1 $\mu\text{g/L}$ —Ib2a; (G) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 16.8 $\mu\text{g/L}$ —Ib2b; (H) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 1 mg/L—Ib2c, (A–H) scale = 4.71 μm . Abbreviations: storage cells (sc).

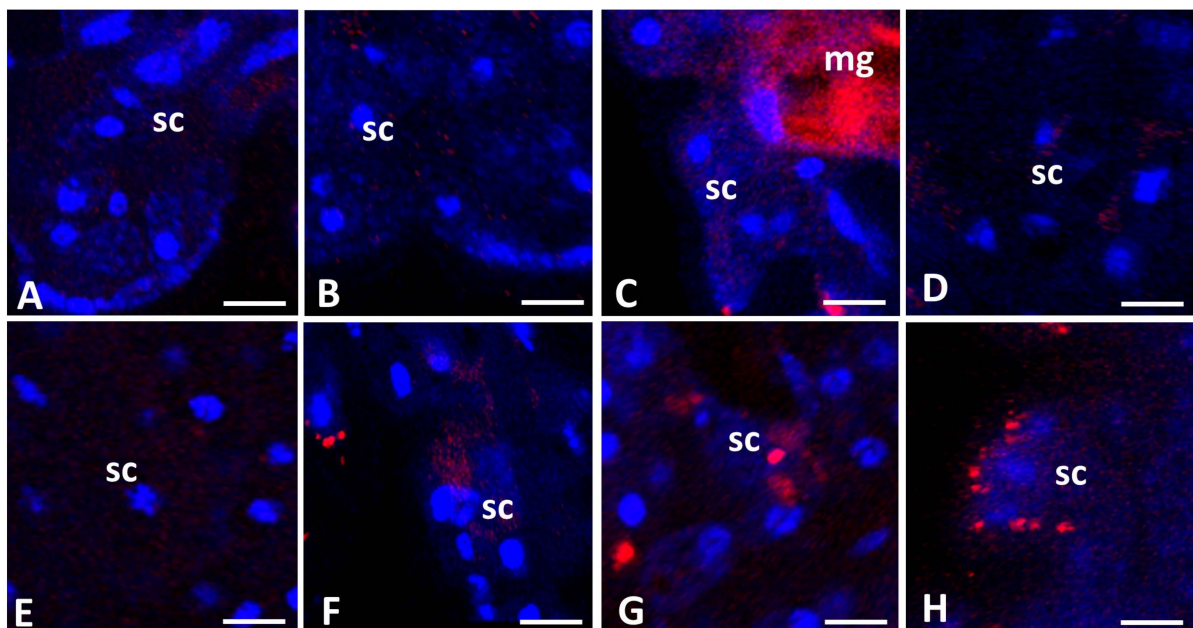


Figure 6. Storage cells of *Pam. experimentalis* stained with LysoTracker Red and DAPI, confocal microscopy. Nuclei (blue signals), acidic organelles (red signals). (A,E) Storage cells of *Pam. experimentalis* in control groups; (A) PeC7 group; (E) PeC28 group; (B) storage cells of *Pam. experimentalis*

treated with ibuprofen for 7 days; concentration of ibuprofen 0.1 µg/L—Ib1a; (C) storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; concentration of ibuprofen 16.8 µg/L—Ib1b; (D) storage cells of *Pam. experimentalis* treated with ibuprofen for 7 days; concentration of ibuprofen 1 mg/L—Ib1c; (F) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 0.1 µg/L—Ib2a; (G) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 16.8 µg/L—Ib2b; (H) storage cells of *Pam. experimentalis* treated with ibuprofen for 28 days; concentration of ibuprofen 1 mg/L—Ib2c, (A–H) scale = 4.71 µm. Abbreviations: storage cells (sc), midgut (mg).

3.3. Recovery from Anhydrobiosis Analysis

The analysis of the ability of individuals to recover from anhydrobiosis to have an active life did not show any significant difference between the experimental and control groups (Figure 7).

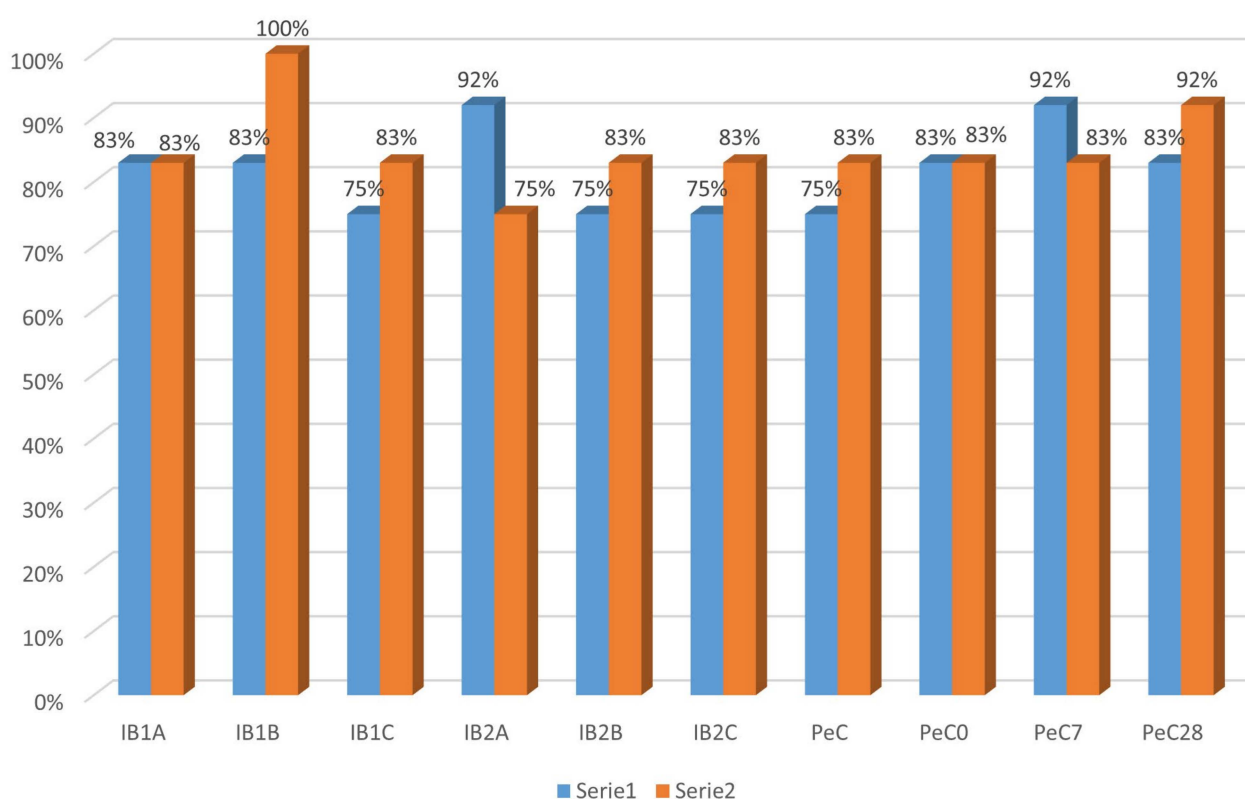


Figure 7. Graphical representation of anhydrobiosis survival rate of *Paramacrobiotus experimentalis* 72 h after rehydration in experimental control groups.

4. Discussion

The contamination of NSAIDs extends beyond surface waters to include groundwater and, infrequently, drinking water [6]. Ibuprofen has been identified in surface waters, groundwater, and tap water on a global scale, with a maximum measured environmental concentration of 303 µg/L [22].

Studies on the toxicity of ibuprofen towards water organisms have been conducted on various aquatic invertebrates, including *Daphnia magna* [24–27], *Hydra vulgaris* [28], and *Dreissena polymorpha* [29]. Additionally, the toxicity of ibuprofen has been explored in vertebrates such as the fishes *Rhamdia quelen* [30] and *Oryzias latipes* [31]. These investigations have mainly focused on the effects of ibuprofen on parameters such as mortality, growth, or reproduction. Notably, the existing literature lacks data on ultrastructural changes in invertebrate cells treated with ibuprofen. Interestingly, there are no data on the effect of

ibuprofen on tardigrades, organisms that are famous for their resistance to environmental stressors. The only data that can be found concern the effect of paracetamol on the storage cells of *Hypsibius exemplaris* [32].

Tardigrades are tiny invertebrate animals that need at least a thin layer of water to live actively [11]. They lack a conventionally structured respiratory system, leading to gas exchange happening through their body wall. The circulatory system's function is assumed by the fluid that fills the body cavity, accompanied by storage cells submerged within it, moving freely. The animal's motions induce the fluid and storage cells to move unrestrictedly, resulting in the occupation of available spaces around internal organs. Additionally, storage cells have the ability to affix themselves to the basement membranes of internal organs and the epidermis. Storage cells serve the role of gathering reserve materials, including lipids, peptides, and polysaccharides. These cells act as an energy reservoir essential for the animals to enter the state of cryptobiosis (also known as latent or hidden life) [13,14,33,34]. Another function is that in certain species, storage cells have the ability to synthesize vitellogenin precursors. These precursors are then conveyed to the ovary, where they accumulate in the oocyte [13,35]. In the context of the accumulation of reserve materials and the synthesis of vitellogenin precursors, storage cells perform a function similar to that of the fat body cells in arthropods. This comparison suggests that, similarly to the role of fat body cells in arthropods, storage cells contribute to the storage and synthesis of essential materials, playing a crucial part in the reproductive and physiological processes of the organism [36].

The present research findings indicated that ibuprofen induced alterations in the ultrastructure of storage cells in *Pam. experimentalis*. The extent of these changes varied and was influenced by both the concentration of ibuprofen and the duration of exposure to the stressor. This suggests a concentration- and time-dependent relationship between ibuprofen exposure and the observed ultrastructural modifications in the storage cells of *Pam. experimentalis*.

The short-term experiment (7 days) showed that the storage cells of tardigrades treated with ibuprofen at all studied concentrations showed no ultrastructural changes compared to the storage cells of animals from the control groups. However, long-term incubation with this pharmaceutical lasting 28 days showed the appearance of autophagic structures in the cytoplasm of storage cells. This was also confirmed by the experiment using LysoTracker Red. However, there was no increased level of oxidative stress or nuclear DNA fragmentation (which is a marker of cell apoptosis) in any of the research groups. Additionally, single mitochondria degenerated and the cisternae of the rough endoplasmic reticulum exhibited slight swelling.

The median lethal concentration (LC₅₀) of the tested species for 24 h exposure is 282 mg/L. Our previous study on *Pam. experimentalis* revealed that ibuprofen induced changes at the ultrastructural level in the digestive cells of the midgut [37]. The studies were conducted at identical concentrations and incubation times. Similarly to the storage cells of this species, short-term exposure of digestive cells to concentrations of 0.1 µg/L and 16.8 µg/L did not show significant changes in digestive cells compared to control groups. However, exposure to a concentration of 1 mg/L for the same duration revealed alterations in the mitochondria's ultrastructure and autophagy intensification, while no changes were observed in storage cells. Moreover, at all three tested concentrations (0.1 µg/L, 16.8 µg/L, 1 mg/L) during long-term incubation, similar changes to those presented in storage cells were observed, including degenerated mitochondria and the presence of autophagosomes and autolysosomes in the cytoplasm of digestive cells [37]. Interestingly, regenerative cells that have no contact with the midgut lumen did not show any changes regardless of the concentration of ibuprofen and the length of incubation. The lack of changes in regenerative cells and storage bodies after short-term exposure to ibuprofen and slight changes in storage bodies after long-term exposure to the stressor may indicate a protective role of the midgut. It probably acts as a barrier against the stressor, protecting its regenerative and body cavity cells (storage cells).

The presence of autophagosomes and autolysosomes serves as evidence for the heightened activity of autophagy. Autophagy is now recognized as a mechanism that safeguards cells from dying. This process is prevalent in eukaryotic cells and is employed to break down damaged or unnecessary intracellular proteins and organelles. It can also be triggered in response to various adverse factors, such as pathogen infection, oxidative stress, or exposure to toxins [20,32,38–42]. In instances of autophagy induced by starvation, reserve materials amass in the cytoplasm of the cells of the animal. This phenomenon can be triggered by environmental factors such as a scarcity of food, molting, or hibernation [34,43]. During these conditions, the autophagic process allows the organism to utilize accumulated reserve materials as a source of energy or essential nutrients, facilitating survival in the face of limited external resources. *Pam. experimentalis* treated with ibuprofen intensified the process of autophagy. It is suggested that autophagy plays a role in mitigating the toxic effects of this substance by eliminating damaged cell organelles. Similar observations of autophagy have been reported in the digestive cells of the midgut of *Grevenius granulifer* (formerly *Isohypsibius granulifer granulifer*—see Gąsiorek et al., 2019) infected with microsporidia [44]. In that instance, both pathogens and damaged organelles were effectively removed from the cell [38]. The appearance of autophagic structures and the consequent escalation of autophagy triggered by pharmaceuticals have been documented in the storage cells of the tardigrade *Hys. exemplaris* when exposed to paracetamol. The autophagic activity was found to be correlated with both the concentration of the drug and the duration of incubation [32]. The same process has been observed in the cytoplasm of trophocytes in *Grevenius granulifer*, specifically during late choriogenesis when they are being detached from the oocyte. In this scenario, the augmentation of autophagy has been noted to guide the cell toward the pathway of apoptosis [45].

The presence of excessive autophagosomes and autolysosomes in cells has been associated with the initiation of cell death through apoptosis [38,43]. This suggests that an accumulation of these autophagic structures can lead to the demise of the cells. Apoptosis may serve as a mechanism to maintain homeostasis in multicellular organisms, particularly when it is coordinated with the proliferation of new cells [46]. In this way, the removal of aged or damaged cells through apoptosis contributes to the overall balance and functionality of the organism. In the process of apoptosis, the cell shrinks, and structural alterations occur in organelles such as the endoplasmic reticulum, Golgi complexes, and mitochondria. Subsequently, apoptotic bodies are formed [45]. Typically, they contain cellular fragments and organelles. The coordination of processes of autophagy and apoptosis has been documented in various organisms, including ovarian nurse cells of *Drosophila virilis* [47] and the midgut epithelium, salivary glands, and gonads of *Lithobius forficatus* [20,40,41]. These studies highlight the widespread occurrence of interactions between autophagy and apoptosis in various tissues and organisms, underscoring the importance of these processes in cellular homeostasis and development.

These processes are influenced by various factors, including the presence of reactive oxygen species (ROS). They can affect apoptosis in both pathological and physiological conditions. Reactive oxygen species exert a damaging effect on cellular components, including DNA and proteins [48]. The role of ROS in apoptosis highlights their dual nature, acting as essential signaling molecules in physiological processes while also contributing to cellular damage under certain conditions. Our study showed that in *Pam. experimentalis*, the level of free radicals increased in storage cells treated with various concentrations of ibuprofen for 28 days and in cells treated with ibuprofen at a concentration of 1 mg/L for seven days. A similar situation was observed in the digestive cells of this species [37]. Research on *Neocaridina davidi* shrimp indicated that long-term starvation led to an increase in ROS concentration in both the intestine and liver. When re-feeding occurred, there was a reduction in ROS concentration [49]. The antimicrobial properties of reactive oxygen species find support in their production in the midgut of *Drosophila melanogaster* when the organism faces a bacterial challenge [50].

Cryptobiosis (also known as latent or hidden life) is a state of life entered by an organism in response to adverse environmental conditions [51]. Anhydrobiosis is a form of cryptobiosis induced by desiccation. In this state, organisms undergo extreme dehydration, exhibiting almost no signs of metabolic activity. However, they retain the capacity to revive and resume life once rehydration occurs [18,52]. Regarding tardigrades, their anhydrobiotic capabilities show considerable diversity among different species. A study showed that the carnivorous *Milnesium inceptum* has greater survivability in anhydrobiosis compared to the herbivorous *Ramazzottius subanomalous*. Interestingly, despite this difference in anhydrobiotic abilities, both species are frequently found coexisting in the same habitat [53]. *Pam. experimentalis* also has the ability to undergo anhydrobiosis [54].

Storage cells play a crucial role in anhydrobiosis. These cells are instrumental in accumulating and storing substances that support the survival of an organism during the extreme conditions of dehydration. The stored materials provide essential resources for the organism to maintain metabolic functions and facilitate recovery upon rehydration, contributing to the overall success of anhydrobiosis. It was demonstrated that the DNA in the storage cells of the tardigrade *Milnesium tardigradum* was effectively protected during the transition from the active to the anhydrobiotic state. However, as the anhydrobiotic phase extended, an increase in DNA damage within these cells was observed [55]. In several species of tardigrades, a reduction in storage cell size after anhydrobiosis was found, suggesting that the energy demands of entering and exiting the dehydrated state utilized the stored material [14].

Research on the species *Pam. experimentalis* revealed that the recovery from anhydrobiosis is influenced by several factors. These include age, the combination of the number and duration of anhydrobiosis episodes, and the presence of other individuals [56]. The current study demonstrated that none of the tested concentrations of ibuprofen had a significant impact on the recovery from anhydrobiosis of *Pam. experimentalis*. This lack of impact was observed across both short and long incubation periods. The absence of changes in the ultrastructure of storage cells in short-term incubation, as well as slight changes in the long-term experiment, suggests a lack of significant damage leading to cell death. This observation hints at the potential protective role of structures such as the midgut and body wall, which may act as barriers against the stressor, in this case, ibuprofen.

Our research has raised further questions: (1) Are there ultrastructural changes in epidermal cells, or does the cuticle provide protection against the impact of ibuprofen? (2) Do gonadal somatic cells exhibit a similar response to ibuprofen as storage cells? Addressing these questions will contribute to a more comprehensive understanding of how ibuprofen affects the biology of *Pam. experimentalis*, shedding light on the different impacts on various cell types and the potential protective mechanisms in place.

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References

- Boxall, A.B.; Rudd, M.A.; Brooks, B.W.; Caldwell, D.J.; Choi, K.; Hickmann, S.; Innes, E.; Ostapych, K.; Staveley, J.P.; Verslycke, T.; et al. Pharmaceuticals and personal care products in the environment: What are the big questions? *Environ. Health Perspect.* **2012**, *120*, 1221–1229. [\[CrossRef\]](#)
- Fent, K.; Weston, A.A.; Caminada, D. Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.* **2006**, *76*, 122–159. [\[CrossRef\]](#) [\[PubMed\]](#)
- Szymonik, A.; Lach, J. Zagrożenie środowiska wodnego obecnością środków farmaceutycznych. *Inżynieria i Ochrona Środowiska* **2012**, *15*, 249–263.
- Nikolaou, A.; Meric, S.; Fatta, D. Occurrence patterns of pharmaceuticals in water and wastewater environments. *Anal. Bioanal. Chem.* **2007**, *387*, 1225–1234. [\[CrossRef\]](#) [\[PubMed\]](#)
- Harshkova, D.; Aksmann, A. Zanieczyszczenie środowiska niesteroidowymi lekami przeciwzapalnymi na przykładzie diklofenaku? Przyczyny, skutki, bioindykacja. *Kosmos* **2019**, *68*, 185–194. [\[CrossRef\]](#)
- Parolini, M. Toxicity of the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) acetylsalicylic acid, paracetamol, diclofenac, ibuprofen and naproxen towards freshwater invertebrates: A review. *Sci. Total Environ.* **2020**, *740*, 140043. [\[CrossRef\]](#)
- Karaźniewicz, M. Czterdzieści lat doświadczeń stosowania ibuprofenu w lecznictwie. *Nowiny Lekarskie* **2007**, *76*, 195–197.
- Bushra, R.; Aslam, N. An overview of clinical pharmacology of Ibuprofen. *Oman Med. J.* **2010**, *25*, 155. [\[CrossRef\]](#)
- Stanley-Samuelson, D.W. Physiological roles of prostaglandins and other eicosanoids in invertebrates. *Biol. Bull.* **1987**, *173*, 92–109. [\[CrossRef\]](#)
- Batucan, N.S.P.; Tremblay, L.A.; Northcott, G.L.; Matthaei, C.D. Medicating the environment? A critical review on the risks of carbamazepine, diclofenac and ibuprofen to aquatic organisms. *Environ. Adv.* **2022**, *7*, 100164. [\[CrossRef\]](#)
- Nelson, D.R.; Guidetti, R.; Rebecchi, L. Phylum Tardigrada. In *Thorpe and Covich's Freshwater Invertebrates*, 4th ed.; Thorpe, J.H., Rogers, D.C., Eds.; Elsevier: London, UK, 2015; Volume 1, pp. 347–380.
- Węglarska, B. Studies on the morphology of *Macrobiotus richtersi* Murray, 1911. *Mem. Ist. Ital. Idrobiol.* **1975**, *32*, 445–464.
- Poprawa, I. Ultrastructural changes of the storage cells during oogenesis in *Dactylobiotus dispar* (Murray, 1907) (Tardigrada: Eutardigrada). *Zool. Pol.* **2006**, *51*, 13–18.
- Reuner, A.; Hengherr, S.; Brümmer, F.; Schill, R.O. Comparative studies on storage cells in tardigrades during starvation and anhydrobiosis. *Curr. Zool.* **2010**, *56*, 259–263. [\[CrossRef\]](#)
- Czerneková, M.; Janelt, K.; Student, S.; Jönsson, K.I.; Poprawa, I. A comparative ultrastructure study of storage cells in the eutardigrade *Richtersius coronifer* in the hydrated state and after desiccation and heating stress. *PLoS ONE* **2018**, *13*, e0201430. [\[CrossRef\]](#)
- Crowe, J.H.; Madin, K.A. Anhydrobiosis in tardigrades and nematodes. *Trans. Am. Microsc. Soc.* **1974**, *93*, 513–524. [\[CrossRef\]](#)
- Hengherr, S.; Brümmer, F.; Schill, R.O. Anhydrobiosis in tardigrades and its effects on longevity traits. *J. Zool.* **2008**, *275*, 216–220. [\[CrossRef\]](#)
- Welnicz, W.; Grohme, M.A.; Kaczmarek, Ł.; Schill, R.O.; Frohme, M. Anhydrobiosis in tardigrades—The last decade. *J. Insect Physiol.* **2011**, *57*, 577–583. [\[CrossRef\]](#)
- Kaczmarek, Ł.; Roszkowska, M.; Fontaneto, D.; Jezierska, M.; Pietrzak, B.; Wiczorek, R.; Poprawa, I.; Kosicki, J.Z.; Karachitos, A.; Kmita, H. Staying young and fit? Ontogenetic and phylogenetic consequences of animal anhydrobiosis. *J. Zool.* **2019**, *309*, 1–11. [\[CrossRef\]](#)
- Poprawa, I.; Chajec, Ł.; Chachulska-Żymełka, A.; Wilczek, G.; Student, S.; Leśniewska, M.; Rost-Roszkowska, M. Ovaries and testes of *Lithobius forficatus* (Myriapoda, Chilopoda) react differently to the presence of cadmium in the environment. *Sci. Rep.* **2022**, *12*, 6705. [\[CrossRef\]](#)
- Kaczmarek, Ł.; Roszkowska, M.; Poprawa, I.; Janelt, K.; Kmita, H.; Gawlak, M.; Fiałkowska, E.; Mioduchowska, M. Integrative description of bisexual *Paramacrobiotus experimentalis* sp. nov. (Macrobiotidae) from republic of Madagascar (Africa) with microbiome analysis. *Mol. Phylogenet. Evol.* **2020**, *145*, 106730. [\[CrossRef\]](#)
- aus der Beek, T.; Weber, F.A.; Bergmann, A.; Hickmann, S.; Ebert, I.; Hein, A.; Küster, A. Pharmaceuticals in the environment—Global occurrences and perspectives. *Environ. Toxicol. Chem.* **2016**, *35*, 823–835. [\[CrossRef\]](#)
- Mrowiec, B. Farmaceutyki—zagrożenie środowiska wodnego. *Edukacja Biologiczna i Środowiskowa* **2015**, *4*, 25–33.
- Heckmann, L.H.; Connon, R.; Hutchinson, T.H.; Maund, S.J.; Sibly, R.M.; Callaghan, A. Expression of target and reference genes in *Daphnia magna* exposed to ibuprofen. *BMC Genom.* **2006**, *7*, 175. [\[CrossRef\]](#)
- Heckmann, L.H.; Callaghan, A.; Hooper, H.L.; Connon, R.; Hutchinson, T.H.; Maund, S.J.; Sibly, R.M. Chronic toxicity of ibuprofen to *Daphnia magna*: Effects on life history traits and population dynamics. *Toxicol. Lett.* **2007**, *172*, 137–145. [\[CrossRef\]](#)
- Hayashi, Y.; Heckmann, L.H.; Callaghan, A.; Sibly, R.M. Reproduction recovery of the crustacean *Daphnia magna* after chronic exposure to ibuprofen. *Ecotoxicology* **2008**, *17*, 246–251. [\[CrossRef\]](#)
- Gómez-Oliván, L.M.; Galar-Martínez, M.; García-Medina, S.; Valdés-Alanís, A.; Islas-Flores, H.; Neri-Cruz, N. Genotoxic response and oxidative stress induced by diclofenac, ibuprofen and naproxen in *Daphnia magna*. *Drug Chem. Toxicol.* **2014**, *37*, 391–399. [\[CrossRef\]](#)

28. Pascoe, D.; Karntanut, W.; Müller, C.T. Do pharmaceuticals affect freshwater invertebrates? A study with the cnidarian *Hydra vulgaris*. *Chemosphere* **2003**, *51*, 521–528. [\[CrossRef\]](#)
29. Parolini, M.; Binelli, A.; Provini, A. Chronic effects induced by ibuprofen on the freshwater bivalve *Dreissena polymorpha*. *Ecotoxicol. Environ. Saf.* **2011**, *74*, 1586–1594. [\[CrossRef\]](#)
30. Mathias, F.T.; Fockink, D.H.; Disner, G.R.; Prodocimo, V.; Ribas, J.L.C.; Ramos, L.P.; Cestari, M.M.; de Assis, H.C.S. Effects of low concentrations of ibuprofen on freshwater fish *Rhamdia quelen*. *Environ. Toxicol. Pharmacol.* **2018**, *59*, 105–113. [\[CrossRef\]](#)
31. Flippin, J.L.; Huggett, D.; Foran, C.M. Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, *Oryzias latipes*. *Aquat. Toxicol.* **2007**, *81*, 73–78. [\[CrossRef\]](#)
32. Wieczorkiewicz, F.; Sojka, J.; Poprawa, I. Effect of paracetamol on the storage cells of *Hypsibius exemplaris*—Ultrastructural analysis. *Zool. J. Linn. Soc.* **2024**, *200*, 258–268. [\[CrossRef\]](#)
33. Jönsson, K.I.; Rebecchi, L. Experimentally induced anhydrobiosis in the tardigrade *Richtersius coronifer*: Phenotypic factors affecting survival. *J. Exp. Zool.* **2002**, *293*, 578–584. [\[CrossRef\]](#)
34. Hyra, M.; Rost-Roszkowska, M.M.; Student, S.; Włodarczyk, A.; Deperas, M.; Janelt, K.; Poprawa, I. Body cavity cells of Parachela during their active life. *Zool. J. Linn. Soc.* **2016**, *178*, 878–887. [\[CrossRef\]](#)
35. Hyra, M.; Poprawa, I.; Włodarczyk, A.; Student, S.; Sonakowska, L.; Kszuk-Jendrysik, M.; Rost-Roszkowska, M.M. Ultrastructural changes in the midgut epithelium of *Hypsibius dujardini* (Doyère, 1840) (Tardigrada, Eutardigrada, Hypsibiidae) in relation to oogenesis. *Zool. J. Linn. Soc.* **2016**, *178*, 897–906. [\[CrossRef\]](#)
36. Kamińska, K.; Lipovšek, S.; Kaszuba, F.; Rost-Roszkowska, M. Ultrastructure of the fat body in the soil centipedes *Lithobius forficatus* (Lithobiidae) and *Geophilus flavus* (Geophilidae) according to their seasonal rhythms. *Zool. Anz.* **2019**, *279*, 82–93. [\[CrossRef\]](#)
37. Miernik, A.; Student, S.; Fiałkowska, E.; Poprawa, I. Does the midgut of the tardigrade *Paramacrobrotus experimentalis* responds to the effects of ibuprofen. *Eur. Zool. J.* **2023**, under review.
38. Rost-Roszkowska, M.M.; Poprawa, I.; Kaczmarek, Ł. Autophagy as the cell survival in response to a microsporidian infection of the midgut epithelium of *Isohypsibius granulifer granulifer* (Eutardigrada: Hypsibiidae). *Acta Zool.* **2013**, *94*, 273–279. [\[CrossRef\]](#)
39. Yin, Z.; Pascual, C.; Klionsky, D.J. Autophagy: Machinery and regulation. *Microbial Cell.* **2016**, *3*, 588. [\[CrossRef\]](#)
40. Rost-Roszkowska, M.M.; Vilimova, J.; Sosinka, A.; Skudlik, J.; Franzetti, E. The role of autophagy in the midgut epithelium of *Eubbranchipus grubii* (Crustacea, Branchiopoda, Anostraca). *Arthropod Struct. Dev.* **2012**, *41*, 271–279. [\[CrossRef\]](#)
41. Włodarczyk, A.; Student, S.; Rost-Roszkowska, M. Autophagy and apoptosis in starved and refed *Neocaridina davidi* (Crustacea Malacostraca) midgut. *Can. J. Zool.* **2019**, *97*, 294–303. [\[CrossRef\]](#)
42. Ostróżka, A.; Tiffert, Z.; Wilczek, G.; Rost-Roszkowska, M. Can insecticide-free clean water regenerate the midgut epithelium of the freshwater shrimp after dimethoate treatment? *Micron* **2022**, *155*, 103162. [\[CrossRef\]](#)
43. Rost-Roszkowska, M.M.; Janelt, K.; Poprawa, I. The role of autophagy in the midgut epithelium of Parachela (Tardigrada). *Zoomorphology* **2018**, *137*, 501–509. [\[CrossRef\]](#)
44. Gąsiorek, P.; Stec, D.; Morek, W.; Michalczyk, Ł. An integrative redescription of *Hypsibius dujardini* (Doyère, 1840), the nominal taxon for Hypsibioidae (Tardigrada: Eutardigrada). *Zootaxa* **2018**, *4415*, 45–75. [\[CrossRef\]](#)
45. Poprawa, I.; Hyra, M.; Kszuk-Jendrysik, M.; Rost-Roszkowska, M.M. Ultrastructural changes and programmed cell death of trophocytes in the gonad of *Isohypsibius granulifer granulifer* Thulin, 1928 (Tardigrada, Eutardigrada, Isohypsibiidae). *Micron* **2015**, *70*, 26–33. [\[CrossRef\]](#)
46. Hettis, S.W. To die or not to die: An overview of apoptosis and its role in disease. *Jama* **1998**, *279*, 300–307. [\[CrossRef\]](#)
47. Velentzas, A.D.; Nezis, I.P.; Stravopodis, D.J.; Papassideri, I.S.; Margaritis, L.H. Apoptosis and autophagy function cooperatively for the efficacious execution of programmed nurse cell death during *Drosophila virilis* oogenesis. *Autophagy* **2007**, *3*, 130–132. [\[CrossRef\]](#)
48. Simon, H.U.; Haj-Yehia, A.; Levi-Schaffer, F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* **2000**, *5*, 415–418. [\[CrossRef\]](#)
49. Włodarczyk, A.; Wilczek, G.; Wilczek, P.; Student, S.; Ostróżka, A.; Tarnawska, M.; Rost-Roszkowska, M. Relationship between ROS production, MnSOD activation and periods of fasting and re-feeding in freshwater shrimp *Neocaridina davidi* (Crustacea, Malacostraca). *PeerJ* **2019**, *7*, e7399. [\[CrossRef\]](#)
50. Chaitanya, R.K.; Shashank, K.; Sridevi, P. Oxidative stress in invertebrate systems. In *Free Radicals and Diseases*; Intech: Manchester, UK, 2016; Volume 19.
51. Keilin, D. The Leeuwenhoek Lecture—the problem of anabiosis or latent life: History and current concept. *Proc. Royal Soc.* **1959**, *150*, 149–191.
52. Alpert, P. The limits and frontiers of desiccation-tolerant life. *Integr. Comp. Biol.* **2005**, *45*, 685–695. [\[CrossRef\]](#)
53. Roszkowska, M.; Gołdyn, B.; Wojciechowska, D.; Kosicki, J.Z.; Fiałkowska, E.; Kmita, H.; Kaczmarek, Ł. Tolerance to anhydrobiotic conditions among two coexisting tardigrade species differing in life strategies. *Zool. Stud.* **2021**, *60*, e74. [\[PubMed\]](#)
54. Roszkowska, M.; Gołdyn, B.; Wojciechowska, D.; Książkiewicz, Z.; Fiałkowska, E.; Pluskota, M.; Kmita, H.; Kaczmarek, Ł. How long can tardigrades survive in the anhydrobiotic state? A search for tardigrade anhydrobiosis patterns. *PLoS ONE* **2023**, *18*, e0270386. [\[CrossRef\]](#) [\[PubMed\]](#)

-
55. Neumann, S.; Reuner, A.; Brümmer, F.; Schill, R.O. DNA damage in storage cells of anhydrobiotic tardigrades. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2009**, *153*, 425–429. [[CrossRef](#)]
 56. Nagwani, A.K.; Melosik, I.; Kaczmarek, Ł.; Kmita, H. Recovery from anhydrobiosis in the tardigrade *Paramacrobiotus experimentalis*: Better to be young than old and in a group than alone. *bioRxiv* **2023**. [[CrossRef](#)]

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