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Comparative study of influence of Cu, CuO nanoparticles and Cu^{2+} on rainbow trout (*Oncorhynchus mykiss* W.) spermatozoa

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The same elements can yield disparate nanoproducts that may elicit different harmful effects in cells and organisms. This study aimed to compare the effects of copper (Cu NPs) and copper oxide (CuO NPs) nanoparticles and Cu^{2+} (from $CuSO_4$) on the physico-biochemical variables of rainbow trout spermatozoa. The cell death assay, along with the activation of caspases 8 and 9, the level of reactive oxygen species (ROS), and the percentage of cells exhibiting a high mitochondrial membrane potential (MMP) were quantified over 24-hour incubation. Interestingly, during exposure, all copper products induced cell apoptosis. However, Cu NPs had a stronger effect than CuO NPs, while the impact of the Cu in ionic form was found to be between the other two compounds. The extrinsic and intrinsic apoptotic pathways were activated, as evidenced by the activation of caspases 8 and 9. Initially, caspase activation increased without a corresponding decrease in MMPs but prolonged exposure resulted in a significant decrease in MMP levels. In all treated cells, the ROS levels increased over time. Further studies are needed to confirm the lower CuO NPs' toxicity compared to Cu NPs because their effect on cells also depends on many other parameters such as size or shape.

Keywords Nanotoxicology, Nanocopper, Fish spermatozoa, ROS, apoptosis, MMP

In recent years, there has been a notable advancement in the field of nanotechnology, resulting in the incorporation of nanoparticles into the environment. Among these developments, copper based nanoparticles e.g. Cu NPs or CuO NPs, have become widely used due to its antimicrobial activity[1–4] and prospective applications in the biomedical field, including drug delivery[5,6] and diagnostics[7], as well as in agricultural and other commercial applications[8–11]. However, the widespread use of nanoparticles (NPs) has raised concerns about their potential impact on the environment[12–15], particularly in the aquatic environment, where waste ultimately accumulates[16]. Pollutants may exert an adverse impact on aquatic species by impeding their growth and development[17,18], as well as the reproductive cell and the effectiveness of reproduction which may ultimately threaten their existence[19,20].

Fish spermatozoa have been used as biomarkers to determine the effects of contaminants in aquatic environments[21–23]. However, current state of the art related to the reproductive biology of aquatic organisms is limited to the effects of metal ions, such as copper[19,24,25], zinc[26,27], and heavy metal derivatives[28,29] on sperm motility, viability, and overall fertilisation potential. Recently, attention has shifted to studying the effects of nanoparticles on fish spermatozoa. Copper contamination of water has been found to primarily reduces sperm viability, which can negatively affect reproduction[19,30,31]. The harmful effects of copper on aquatic organisms such as fish[19,25,31–33], daphnia[34–37] and algae[38–41] have been demonstrated. The median lethal concentration (LC_{50}) for Cu and Cu NPs in catfish was found to be 8.0-10.5 and 3.9-5.0 mg L^{-1}, respectively, following an exposure period of between 24 and 96 h[42]. Organisms differ in their susceptibility to the adverse effects of pollution[25,37,43–45]. The

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toxicological impact of NPs is influenced by a number of factors, including concentration, exposure duration, particle size, surface area, dissolution, agglomeration and the characteristics of aquatic ecosystem. Our previous study[46] showed that the effect of copper nanoparticles on the kinetics of rainbow trout spermatozoa is product-dependent. Smaller Cu nanoparticles have also been shown to be more toxic to zebrafish embryos than larger particles[47]. Furthermore, different kind of nanoparticles based on the same elements have different effects on spermatozoa. In our previous study[31], the effects of copper based nanoparticles (that is, Cu NPs and CuO NPs) on changes in sperm motility, analysed by the CASA (computer-assisted sperm analysis), were investigated and compared with effect of the ionic form of copper (from $CuSO_4$). The findings demonstrated that Cu NPs and ionic Cu were more deleterious to spermatozoa kinematics than CuO NPs[31]. In the aforementioned study, incubation was conducted at various concentrations, including 50 mg Cu L^{-1}, which is identical to the concentration used in the current study.

The effects of copper nanoparticles on cellular mechanisms have been investigated by several authors. Available evidence indicates that nanoparticles containing copper have the capacity to disrupt enzymatic activity[30,48], induce oxidative stress[1,42,49,50], cause cell membrane disruption[51], and lead to mitochondrial dysfunction[52]. A substantial body of evidence from numerous studies has consistently demonstrated that CuO NPs induce cytotoxicity in a range of cell types, including Chinook salmon cells, human lung epithelial cells, human airway smooth muscle cells and airway epithelial cells, primarily by generating oxidative stress[53–56]. Furthermore, studies have indicated that CuO NPs induce mitochondria-mediated apoptosis in human hepatocarcinoma cells[57]. As proposed by Thit et al.[58], the toxicity mechanism involves the uptake of CuO NPs by cells via endocytosis, resulting in the generation of Reactive Oxygen Species (ROS). Subsequently, ROS causes DNA damage, initiating a signalling pathway that ultimately results in apoptosis or cell death[58]. Additionally, a study observed a significant increase in the expression of antioxidant enzymes in the epididymides of Chinese soft-shelled turtles after 24 h of incubation with Cu NPs and $CuSO_4$[30]. Several studies have indicated that the toxicity of copper nanoparticles can be attributed to a combination of two factors. First, copper nanoparticles can penetrate cells or migrate to various tissues and organs, thereby disrupting their metabolic activities[42]. Second, the toxicity of nanoparticles to organisms is linked to the release of soluble copper ions[11,59,60].

In our previous study on spermatozoa motility tracked by computer-assisted sperm analysis (CASA) we demonstrated that copper nanoparticles (Cu NPs) and ionic copper ($CuSO_4$) exert a more pronounced immobilisation effect than copper oxide nanoparticles (CuO NPs)[31].

This contribution compares their effects on the physico-biochemical variables of rainbow trout spermatozoa by means of cell death assay, along with the activation of caspases 8 and 9, the level of reactive oxygen species (ROS), and the percentage of cells exhibiting a high mitochondrial membrane potential (MMP) over 24-hour incubation.

Results

Cell death assay

After two hours of incubation of rainbow trout spermatozoa in solutions containing Cu NPs, CuO NPs and $CuSO_4$, a significant increase ($p < .05$) in the percentage of early apoptotic cells was found (1.82%, 1.56%, and 1.71%, respectively), compared to the negative control (0.14%). Changes in the percentage of viable cells, late apoptotic cells, and necrotic cells did not differ among the groups ($p > .05$) (Fig. 1).

After a 12 h incubation of spermatozoa with copper products, the percentage of early-apoptotic cells remained significantly higher in solutions containing Cu NPs, CuO NPs and $CuSO_4$ (3.32, 2.17, 3.27%, respectively) compared to the control (0.46%) (Fig. 2). Additionally, a significant increase ($p < .05$) in the percentage of late apoptotic cells compared to the control occurred after incubation with Cu NPs and $CuSO_4$ (4.80% in the control, 32.77% in Cu NPs, and 30.77% in $CuSO_4$, respectively). The percentage of necrotic cells was similar in all the groups. At the same time, there was a significant decrease in the percentage of live cells with Cu NPs (68.23%) compared to the control (93.57%). In the case of CuO NPs and $CuSO_4$, the percentage decrease was not significant (84.40% and 85.22%, respectively) (Fig. 2).

Following a 24 h incubation period in copper-containing compounds, the number of late apoptotic cells increased significantly ($p < .05$) from 3.86% in the control group to 36.43% in Cu NPs, 31.55% in $CuSO_4$ and 23.95% in CuO NPs. The effect of copper compounds did not result in alterations in the proportion of necrotic and early apoptotic cells compared to the control (Fig. 3). The percentage of viable cells significantly decreased in Cu NPs and $CuSO_4$ (from 89.46% in the control to 63.96% and 68.83%, respectively), whereas no significant change was observed in the case of CuO NPs (79.69%). The toxic effect exerted by Cu NPs was found to be greater than that exerted by CuO NPs ($p < .05$) while for $CuSO_4$ exhibited a similar effect on both NPs (Fig. 3).

Caspase 8 and 9 activations

After 2 h of incubation, the relative level of caspase 8 activation in rainbow trout sperm was higher in all copper-containing compounds than in the negative control (Fig. 4). After 12 and 24 h of incubation, the level of caspase 8 activation fluctuated slightly, remaining similar or higher in the copper treatment compared to the control, with a range of 1.3 to 2.1 times that of the control (Fig. 4).

After 2 h of incubation, the relative levels of caspase 9 were similar (CuO NPs and $CuSO_4$, $p > .05$) or significantly lower (Cu NPs, $p < .05$) compared to the control (0.69-fold control) (Fig. 5). After 12 h of incubation in solutions containing copper, the level of caspase 9 activation increased significantly. The level was found to be similar and was approximately 1.80 times higher than that in the control group for all copper solutions. After 24 h of incubation, the greatest increase in caspase 9 activation levels was observed in sperm incubated with nanoparticles (3.19-fold control in Cu NPs and 2.98-fold control in CuO NPs). In $CuSO_4$ the level of caspase 9 activation was also significantly higher than that in the control (2.27-fold control), but lower than that in solutions containing nanoparticles (Fig. 5).

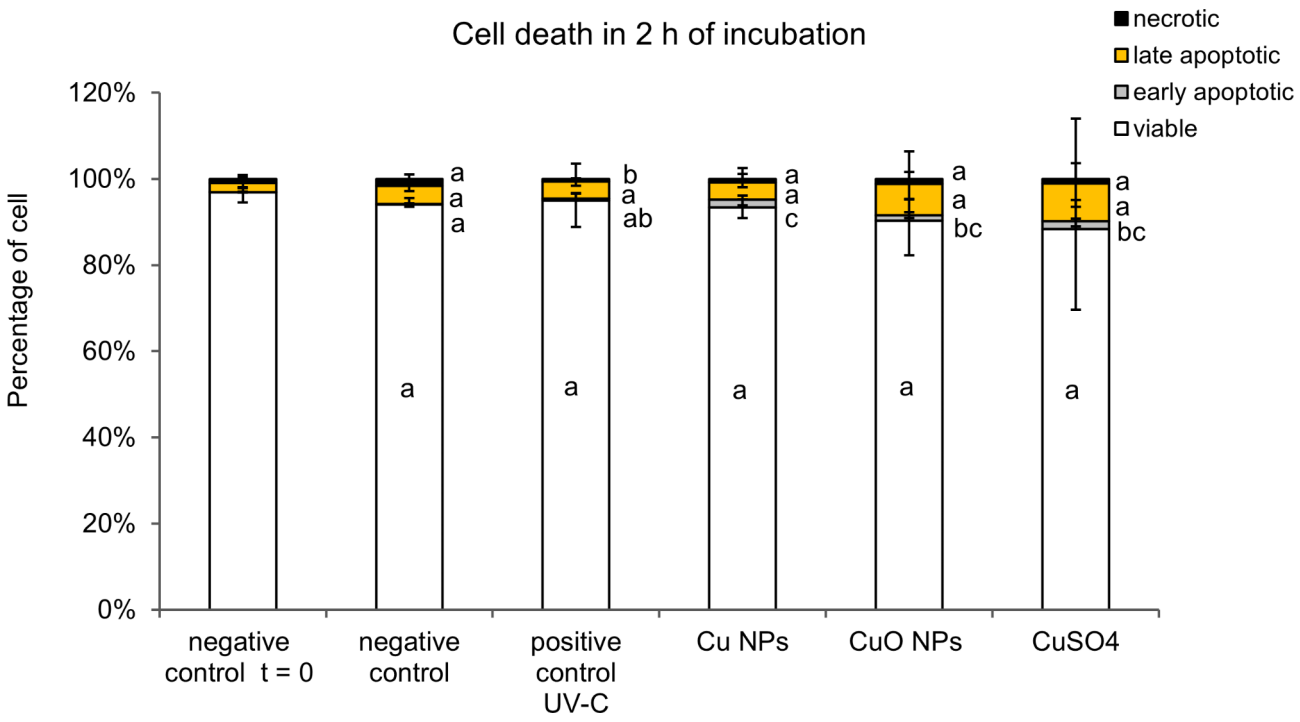

Fig. 1. The influence of two hours incubation in copper compounds Cu NPs, CuO NPs and $CuSO_4$, at a concentration of 50 mg Cu L^{-1} on cell death. Different superscripts indicate a significant difference in a given stage of cell death among the experimental groups. Median and interquartile range ($n = 17$, Kruskal-Wallis test, $p < .05$).

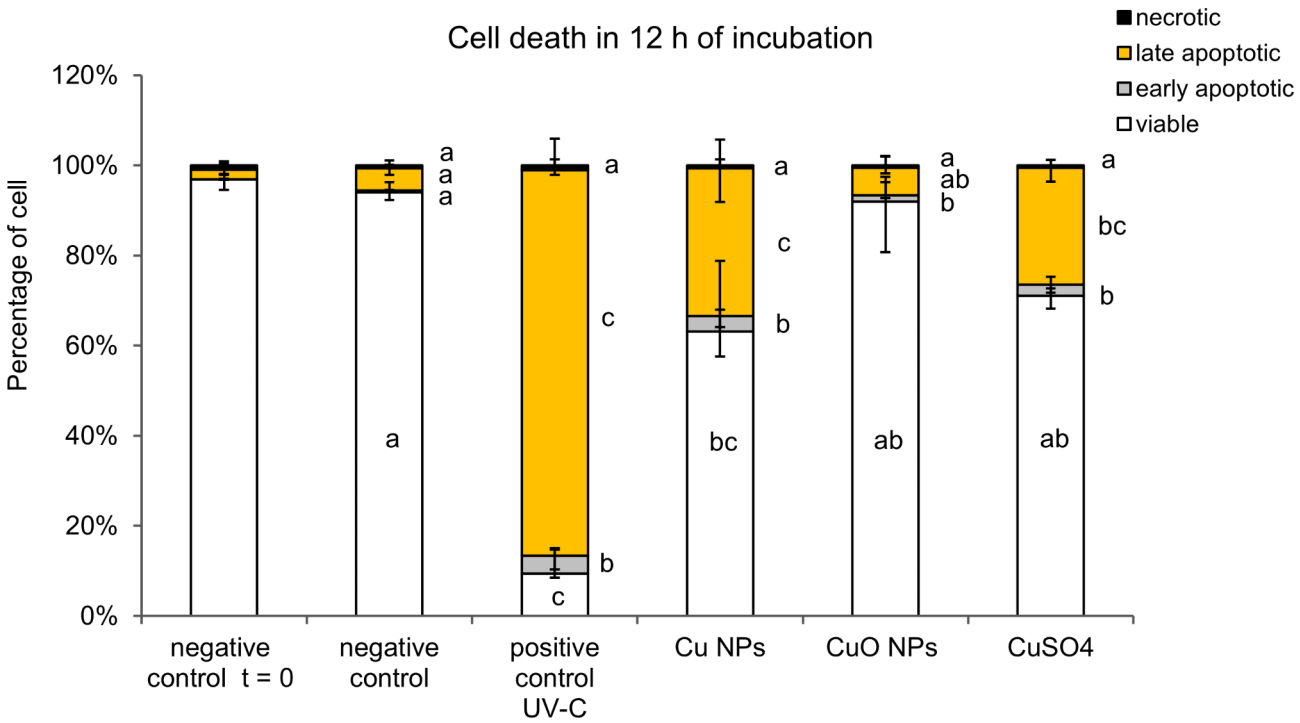

Fig. 2. The influence of 12 h incubation with copper compounds Cu NPs, CuO NPs and $CuSO_4$, at a concentration of 50 mg Cu L^{-1} on cell death. Different superscripts indicate a significant difference in a given stage of dying cells among experimental groups. Median and interquartile range ($n = 17$, Kruskal-Wallis test, $p < .05$).

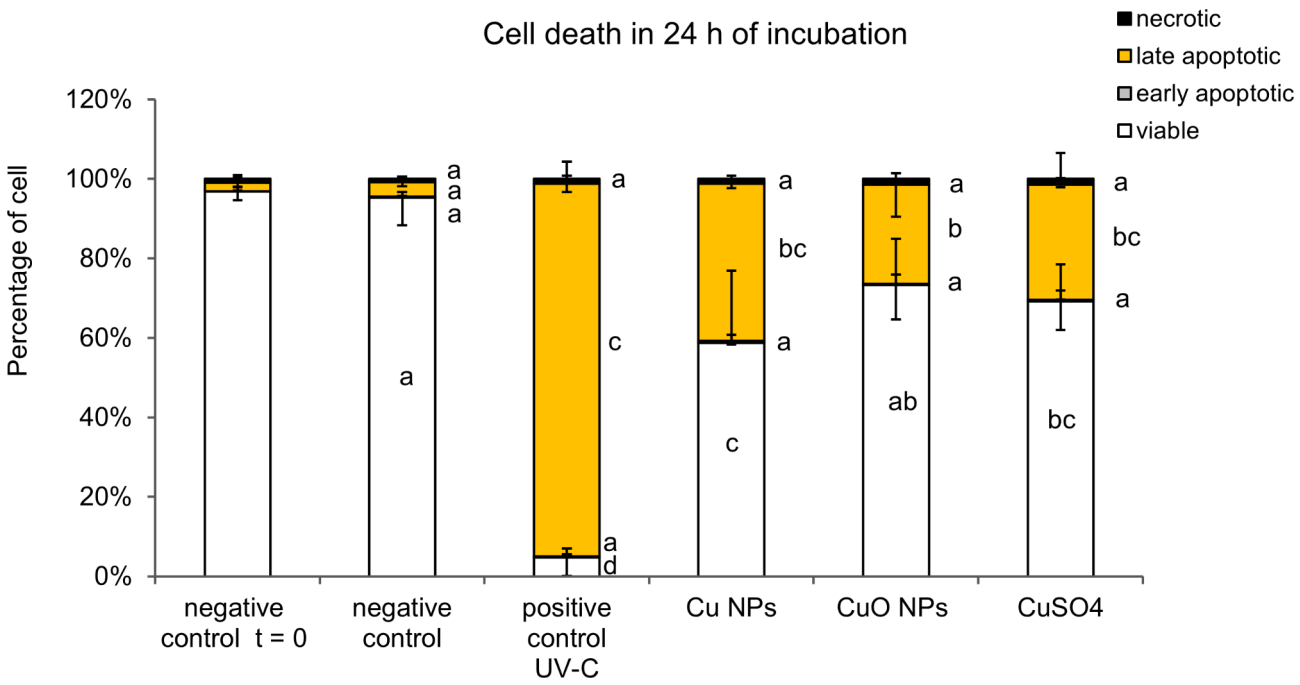

Fig. 3. The influence of 24 h incubation in copper compounds Cu NPs, CuO NPs and $CuSO_4$, at a concentration of 50 mg Cu L^{-1} on cell death. Different superscripts indicate a significant difference in a given stage of dying cells among experimental groups. Median and interquartile range ($n = 17$, Kruskal-Wallis test, $p < .05$).

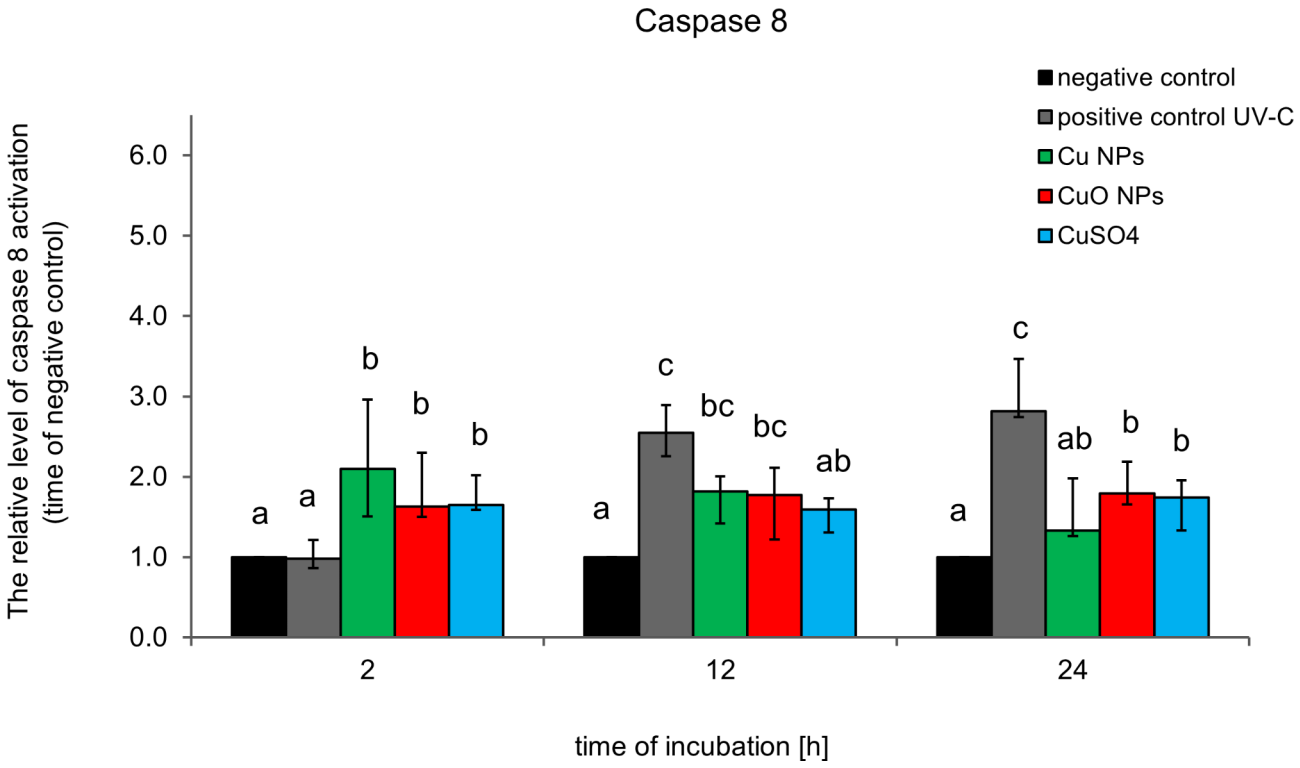

Fig. 4. The relative level of caspase 8 activation following incubation with copper-containing compounds at a concentration of 50 mg Cu L^{-1} including Cu NPs, CuO NPs, and $CuSO_4$, was determined as a fold change compared to the negative control. Different superscripts indicate significant differences among experimental groups at a given incubation time. Median and interquartile range ($n = 9$, Kruskal-Wallis test, $p < .05$).

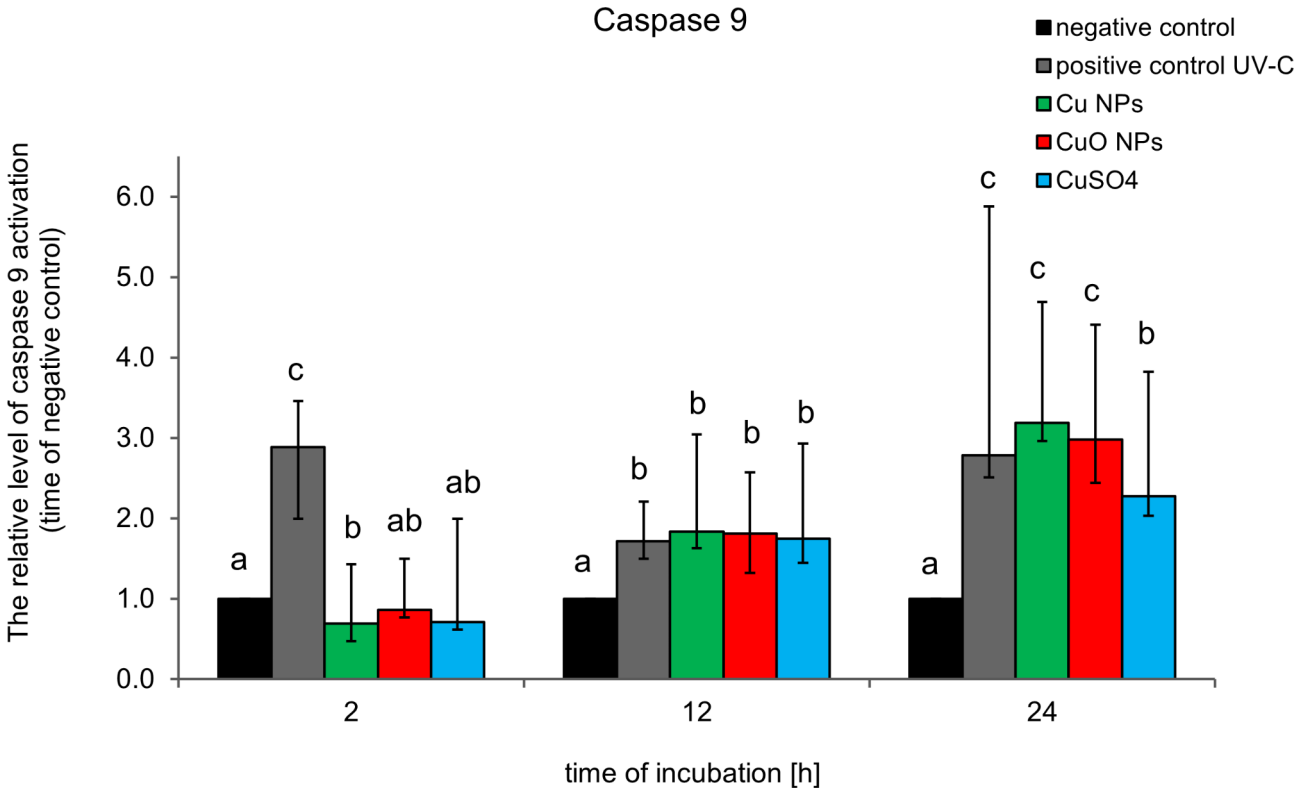

Fig. 5. The relative level of caspase 9 activation following incubation with copper-containing compounds at a concentration of 50 mg Cu L^{-1} including Cu NPs, CuO NPs, and $CuSO_4$, was determined as a fold-change compared to the control. Different superscripts indicate a significant difference among experimental groups at a given incubation time. Median and interquartile range ($n = 21$, Kruskal-Wallis test, $p < .05$).

Reactive oxygen species (ROS)

After 2 h of incubation, the ROS level was found to be comparable between the Cu NPs and CuO NPs solutions, while exhibiting a lower level in $CuSO_4$ compared to the control (Fig. 6). A significant increase in ROS levels (1.22-fold control) compared to the control was observed after 12 h incubation of sperm in CuO NPs. After 24 h, increased ROS levels were observed for all the tested copper compounds. In Cu NPs and CuO NPs, ROS levels increased approximately 2.05 times compared to the control, whereas in $CuSO_4$, they increased by 2.86-times (Fig. 6).

Mitochondrial membrane potential (MMP)

After 2 h of incubation in solutions containing copper compounds, the percentage of cells with a high mitochondrial membrane potential remained unchanged in comparison to the control (Fig. 7). After 12 h, a significant ($p < .05$) increase in the percentage of cells with high MMP was observed in spermatozoa incubated with CuO NPs (98.75%) and $CuSO_4$ (97.59%) compared with the control (87.23%). No significant changes were observed in spermatozoa incubated with Cu NPs ($p > .05$). However, after 24 h of incubation in solutions containing copper compounds, a decrease in the percentage of cells with a high MMP was observed. A significant decrease was observed for Cu NPs (64.99%) and $CuSO_4$ (74.37%) compared with the control (93.29%). Conversely, the decrease in the percentage of cells with high MMP levels incubated with CuO NPs (79.25%) was not significant ($p > .05$) when compared to the control. The percentage were similar in the three-copper solution (Fig. 7).

Discussion

The increasing production of nanocopper products has highlighted the necessity to gain a deeper understanding of the effects of nanoparticles on aquatic organisms and the pathways through which they exert their effects. However, data concerning the influence of Cu NPs on fish (and particularly on spermatozoa) are limited, and their results are not homogeneous[45,61–63].

A previous study[31] and the present study demonstrated that copper nanoparticles (Cu NPs) are more toxic to rainbow trout spermatozoa than copper oxide nanoparticles (CuO NPs). The initial study indicated that there was a higher degree of spermatozoa immobilisation, whereas a subsequent study demonstrated a lower proportion of viable cells. In the aforementioned studies, the effects of the Cu NPs were comparable to those of the ionic form of Cu. Similarly, Al-Bairuty et al.[64] observed that the toxicity of Cu NPs and $CuSO_4$ to juvenile rainbow trout is comparable. Administration of both substances resulted in the development of pathological conditions in the target organs. In a separate study of this species, a notable distinction was observed in the

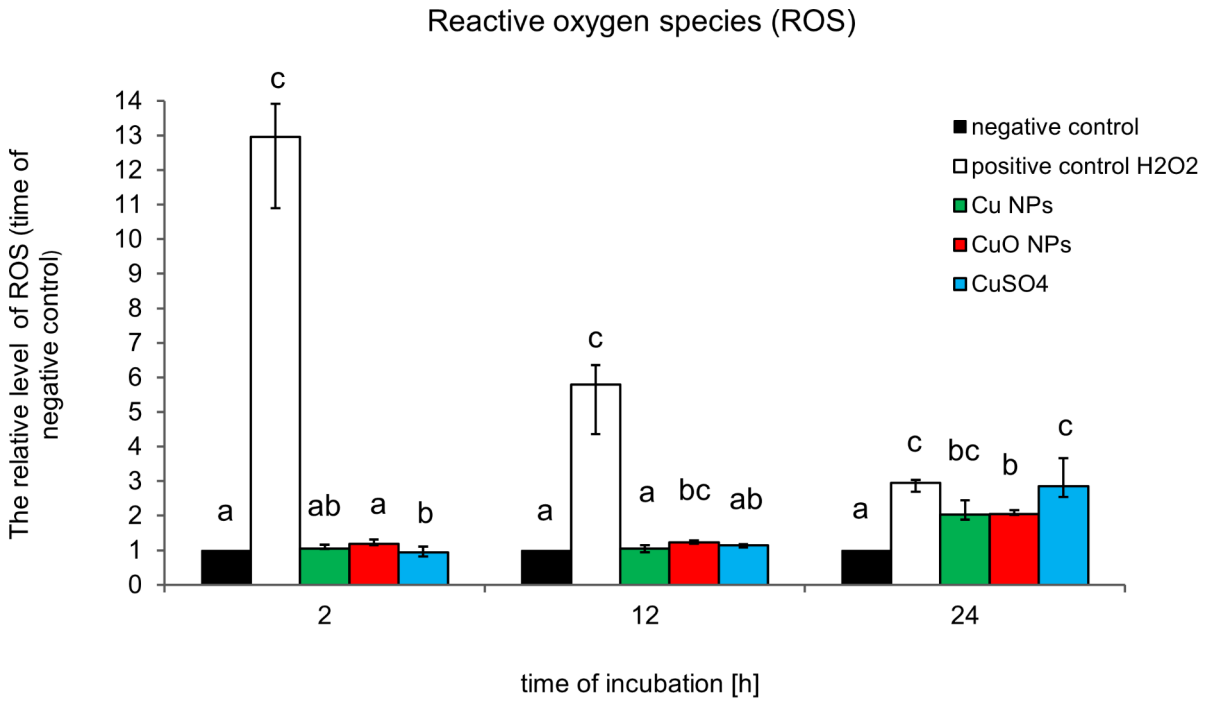

Fig. 6. The reactive level of Reactive Oxygen Species (ROS) in rainbow trout spermatozoa cells was followed by incubation in solutions containing copper compounds Cu NPs, CuO NPs, and $CuSO_4$ at a concentration of 50 mg Cu L^{-1}. Different superscripts indicate a significant difference among experimental groups at a given incubation time. Median and interquartile range ($n = 12$, Kruskal-Wallis test, $p < .05$).

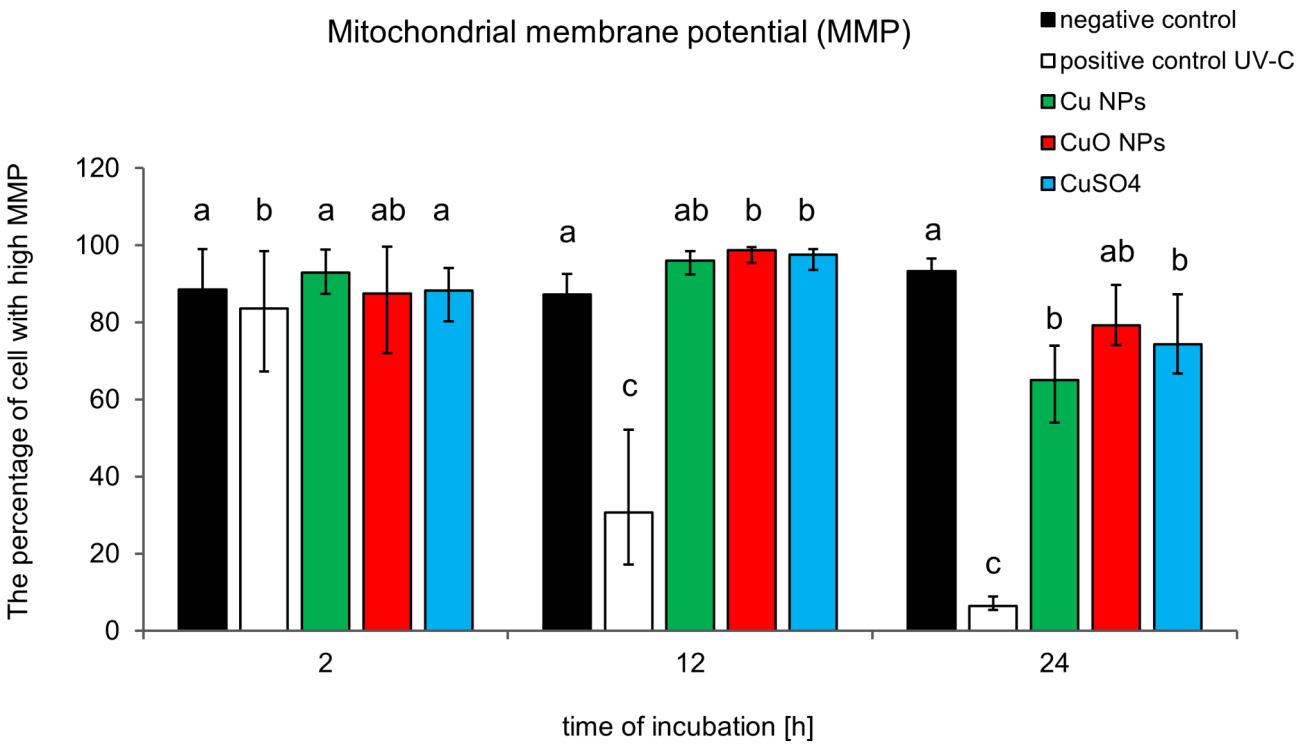

Fig. 7. Changes in the percentage of rainbow trout spermatozoa with high mitochondrial membrane potential (MMP) following incubation in solutions containing copper compounds Cu NPs, CuO NPs, and $CuSO_4$ at a concentration of 50 mg Cu L^{-1}. Different superscripts indicate a significant difference among experimental groups at a given incubation time. Median and interquartile range ($n = 17$, Kruskal-Wallis test, $p < .05$).

extent of brain damage, whereby the damage caused by Cu NPs was more pronounced than that induced by the same concentrations of $CuSO_4$[65]. In their investigation of the impact of Cu NPs and $CuSO_4$, Hoseini et al.[61] observed that the ionic form induced a more pronounced harmful effect and necrosis in common carp (*Cyprinus carpio*) liver and kidney cells compared to the nano form[61]. Similarly, a more harmful effect was observed in the ionic form by Yang et al.[30], who investigated the effects on Chinese soft-shelled turtle (*Pelodiscus sinensis*) spermatozoa and Kowalska-Góralska et al.[63], examined the effects on the eggs of rainbow trout (*Oncorhynchus mykiss* W.).

The preceding CASA study indicated that CuO NPs exhibit a diminished level of toxicity in comparison to $CuSO_4$[31]. This could be attributed to the fact that the initial studies encompassed a longer exposure period of up to 96 h, whereas the current study was conducted for a duration of 24 h. The reduced toxicity of CuO NPs compared to the ionic form of copper (derived from $CuCl_2$) was also demonstrated by Mansano et al.[66], who evaluated toxicity in fish gill cells. The study demonstrated that copper ions caused greater increases in the proportion of early apoptotic and late apoptotic/necrotic cells than CuO NPs[66].

Analysis of caspase activation within the cell enables the identification of the pathway that directs the cell towards programmed cell death. In the present study, the activation levels of caspase 8 were measured for the extracellular pathway (extrinsic) and caspase 9 for the mitochondrial (intrinsic) pathway of cell death. The level of caspase 8 activition under the influence of the tested copper compounds was observed to be higher from as early as two hours of incubation, indicating that the cell death signal originates externally through death receptors. The studies revealed no significant differences in caspase 8 activity between copper nanoproducts and the ionic form. Conversely, caspase 9 activation was observed to increase with incubation duration. Following a 24-hour incubation period, a significant elevation in caspase 9 activation was observed in spermatozoa, particularly in the presence of nanoproducts. This indicated that the studied nanoproducts induced apoptosis by intensifying the activation of the intrinsic pathway in response to their presence. This finding is in accordance with the results of a study conducted by Sarkar et al.[50], who observed the activation of caspase 3 and 8 in kidney tissue under Cu NPs exposure. The same type of nanocopper induced the activity of caspase 3 and caspase 9 in the liver of juvenile fish *Takifugu fasciatus*[67]. Caspase 3 is an essential protein. Siddiqui et al.[57] observed a significant increase in caspase 3 activity in human hepatocarcinoma cells in the presence of Cu NPs.

Reactive oxygen species (ROS) play a crucial role in cellular signalling and the maintenance of cellular homeostasis. Excess of these substances can result in oxidative stress and subsequent damage to cells. The present study revealed a significant elevation in ROS generation in cells following a 24-hour incubation period with copper products. The findings of the present study are consistent with those previously reported by Yang et al.[30], who demonstrated that after 24 h, both Cu NPs and $CuSO_4$ induced a significant increase in ROS production in spermatozoa from Chinese soft-shelled turtles (*Pelodiscus sinensis*), where $CuSO_4$ markedly elevated ROS production compared to Cu NPs[30]. Exposure of fish gills of *H. eques* to Cu ions (from $CuCl_2$) also resulted in a higher increase in ROS production than that observed with CuO NPs[66]. Similarly, Sarkar et al.[50] observed that exposure to Cu NPs elevated ROS production.

ROS generation is associated with impairment in mitochondrial membrane permeability and the respiratory chain. The mitochondrial membrane potential (MMP) serves as a crucial indicator for evaluating the functional capacity of mitochondria, a pivotal organelle in cellular energy production. Modifications of this parameter are associated with a range of physiological and pathological conditions. It is widely acknowledged that during apoptosis, there is a decrease in the mitochondrial membrane potential (MMP) as a result of mitochondrial outer membrane permeabilisation (MOMP). The findings of our study support this hypothesis, as after 24 h of incubation of rainbow trout spermatozoa, a decrease in MMP was observed for both Cu NPs and $CuSO_4$. An anomalous result was observed after 12 h of incubation, whereby an increase in MMP was observed in the copper-containing solutions. This finding is inconsistent with the widely described mechanism of caspase 9 activation which requires earlier MOMP. It is plausible that a 12-hour incubation period with copper products may result in caspase 9 activation through a mechanism that does not involve MOMP. An alternative mechanism has also been described in other studies. Specifically, caspase 9 can be directly activated by caspase 8 without MOMP in the extrinsic apoptotic pathway in cancer HeLa cells[68] and murine cells[69,70]. This temporary increase in MMP may explain the higher spermatozoa movement speed following incubation with the same copper compounds at specific concentrations, as reported in our earlier study[31]. Furthermore, an increase in spermatozoa velocity under the influence of nanoproduct exposure under certain experimental conditions has also been documented by other researchers[48,71]. It may be related to the fact that several studies have shown that copper can improve (or inhibit) enzyme function and influence gene expression, affecting some vital functions[17,18,72] but further research is required in this case.

The toxicity of NPs depends on a multitude of factors, including their concentration, exposure time, chemical composition, particle size, surface area, dissolution, agglomeration, and other physical properties. Several studies have indicated that the toxicity of copper nanoparticles can be attributed mainly to penetration into cells or migration to various tissues and organs, thereby disrupting their metabolic activities and linked to the release of soluble copper ions[11,42,59,60]. Some studies have demonstrated that smaller nanoparticles have a greater capacity to exaggerate toxic effects than larger ones[46,47]. In the present study, it was observed that the average size of the Cu NPs was approximately twice smaller that of CuO NPs (Fig. S1). Additionally, its positive zeta potential was four times smaller in respect to CuO NPs (Table S1). It is plausible that these parameters contributed to enhanced cell penetration, resulting in a greater degree of toxicity. What is more, from optical study it was observed that the CuO nanoparticles released more Cu^{2+} to the artificial seminal plasma solution within 24 h incubation test (Fig. S3). This could indicate that the harmful effect of CuO NPs can be related to the released of Cu^{2+} instead of the solid CuO NPs. It also explains the reason why the toxicity of $CuSO_4$ is placed between Cu NPs and CuO NPs. Therefore, basing on our research one can conclude that copper based compounds increase their harmful effect on the studied cells in the following order: Cu NPs > Cu^{2+} > CuO NPs.

Conclusion

The study demonstrated that the diverse copper based nanoparticles and Cu^{2+} subjected to examination exhibited disparate toxic effects on rainbow trout spermatozoa. The Cu NPs were observed to induce cell death by apoptosis to a greater extent than the CuO NPs. The toxic effect of $CuSO_4$ was found to be situated between the other two compounds. The physico-biochemical variables were found to be similar, with the exception of caspase 9 activation, which was observed to be higher under the influence of the nanoparticles. Different effects exerted by NPs on spermatozoa, apart from their chemical properties, could have been influenced by their different size and zeta potential. The size and zeta potential of Cu NPs were found to be lower than those of CuO NPs. It may be hypothesised that the parameters of Cu NPs contribute to easier penetration into cells resulting in greater toxicity to cells. Nevertheless, further research is recommended to determine what property of the nanoparticles predominantly influences its impact on cells.

Methods

Material

During the artificial spawning season, employees of The Trout Breeding Center "Kuźniczka" in Wieleń (coordinates: 52°57′03.8232″N 16°13′52.0320″E) stripped sperm from rainbow trout (*Oncorhynchus mykiss* W.) into plastic containers. The containers were then placed on ice (2–4 °C) and transported to the laboratory, which took approximately 2 h. Samples with high milt quality (motility exceeding 80% and curvilinear velocity above 100 µm s^{-1}) were selected for experiments based on computer-assisted sperm analysis (CASA) by Microptic S.L. (Barcelona, Spain), the Sperm Class Analyzer (SCA) v. 4.0.0 (https://www.micropticsl.com/products/sperm-class-analyzer-casa-system/).

To select the best portion of milt for the study, spermatozoa activation was triggered with 30 mM NaCl buffered with 20 mM Tris, pH 9, at 8 °C with 200-fold dilution. The temperature of the solution was maintained using a cooling block (FINEPCR, Seoul, Korea) and microscope table cooling device (Semic Bioelektronika, Kraków, Poland). Half-second films (25 frames) were recorded at 10 s after activation. Activation and film recording was repeated three times. The criterion for defining motility was an average path velocity VAP > 20 µm s^{-1}. Spermatozoa concentration was assessed in a Bürker chamber counting by the SCA at a dilution of 3000x with 0.8% NaCl. The percentage of motile sperm in the samples selected for the study ranged from 80 to 90% and mean curvilinear velocity and linearity were above 100 µm s^{-1} and 80%, respectively. Spermatozoa concentration ranged from 11.1 to 17.3×10^9 mL^{-1}. The experimental procedures conducted on sperm do not require permission from the Ethics Committee for Animal Experimentation, according to Polish law.

Nanoproducts and experiment description

Cu and CuO nanoproducts and copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were purchased from Sigma Aldrich. Suspensions with a concentration of 50 mg Cu L^{-1} were prepared in artificial seminal plasma (ASP[31], pH 8.5). The solutions were sonicated in an ultrasonic bath for 30 min. The morphology and dimensions of nanoparticles were examined using transmission electron microscopy (TEM) at the Department of Physicochemistry of Nanomaterials, West Pomeranian University of Technology in Szczecin. The mean diameter of Cu NPs and CuO NPs was 14.3 nm and 24.8 nm, respectively. The morphology of the nanoproducts was presented in the Supplementary material (Fig. S1). XRD diffraction has been conducted to reveal the crystal phase in nanoproducts and copper sulfate. The characteristics are included in the Supplementary material (Fig. S2). Milt was diluted with copper solutions in a ratio of 1:10, respectively. The diluted milt was incubated in a flat-bottomed container, mixture height 5 mm, for 2, 12 and 24 h at 6 °C. Through optical spectroscopy, the qualitative assessment of ion dissolution from Cu and CuO nanoparticles has been conducted during the incubation process and included in the Supplementary material (Fig. S3). To assess the behaviour of the nanoparticles (agglomeration effect) dynamic light scattering has been conducted in the incubation conditions at 0, 2, 12, and 24 h (Supplementary material, Fig. S4). At the same time intervals, cell death ($n = 17$), mitochondrial membrane potential (MMP) ($n = 17$), internal reactive oxygen species (ROS) ($n = 12$), and caspase 8 ($n = 9$), and 9 ($n = 21$), activity were measured using cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA). Fluorochromes for flow cytometry analysis FITC Annexin V Apoptosis Detection Kit I and MitoScreen JC-1 Kit were purchased from Becton Dickinson, 2′,7′-dichlorofluorescein diacetate (2′,7′-DCFH-DA) was obtained from Merck, CaspGLOW™ Fluorescein Active Caspase-8 Staining Kit from Thermo Fisher Scientific and Caspase-9 FITC staining kit from Abcam.

Cell death assay

Cell death tests were performed with the use of FITC Annexin V Apoptosis Detection Kit I. The 50 µL of cells diluted in ASP to a concentration 10^7 were incubated with 2 µL Annexin V FITC and 2 µL of PI detection kit solutions for 15 min, at 6 °C in the dark. Additionally, the cells in a 2 mm layer in a Petri dish exposed in the experiment to UV-C irradiation were used as a positive control (UV-C 70 mJ cm^{-2}, 30 min, 4°C). After the indicated incubation time, 300 µL of ASP was added and flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) analysis was carried out. The green fluorescence (FL-1) corresponding to the FITC-Annexin V signal was recorded using a laser beam at 530 ± 15 nm (λex = 488 nm) and the red fluorescence (FL-2) corresponding to the PI signal was recorded using a laser beam at 585 ± 21 nm (λex = 488 nm). The measurements were made for 10×10^3 events per sample. The percentages of living, early apoptotic, late apoptotic, and necrotic cells, respectively were determined using BD CellQuest Pro (Becton Dickinson) software ver. 5.2.1 (BD) Bioscience, (Franklin Lakes, NJ, USA) (https://www.bdbiosciences.com/en-pl/products/instruments/flow-cytometers).

Caspase activity assays

The activation of caspase 8 and caspase 9 in the examined cells was measured with the use of FLICA assay commercial kits containing fluorochrome-labelled inhibitors of caspases. The 50 µL of cells diluted in ASP to a concentration of 10^7 were incubated with 50 µL of the inhibitor of the active form of caspase-8 (Ile-Glu-Thr-Asp-fluoromethylketone, IETD-FMK) or caspase-9 (Leu-Glu-His-Asp-fluoromethyl ketone, LEHD-FMK), respectively, conjugated with FITC for 30 min at 6 °C, then washed in ASP by mixing and centrifugating 1000 *g* for 6 min, at 4 °C. After adding 300 µL of ASP, the samples were analysed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA). Additionally, the cells in a 2 mm layer in a Petri dish exposed in the experiment to UV-C irradiation were used as a positive control (UV-C 70 mJ cm^{-2}, 30 min, 4ºC). The green fluorescence (FL-1) of 10×10^3 events was recorded using a laser beam at 530 ± 15 nm (λex = 488 nm). The data were analysed using the BD CellQuest Pro (Becton Dickinson) software ver. 5.2.1 (BD) Bioscience, (Franklin Lakes, NJ, USA) (https://www.bdbiosciences.com/en-pl/products/instruments/flow-cytometers).

Intracellular ROS level assay

2',7'-DCFH-DA easily diffuses through the cell membrane of cells and undergoes non-specific hydrolysis by esterases in the cytoplasm. The product of this reaction is oxidised by hydrogen peroxide (H_2O_2), hydroxyl radical (HO•) and peroxyl radicals (ROO•) to the fluorescent compound 2',7'-DCF. The method is commonly used to determine the overall level of ROS in the cell[73].

The 50 µL of cells diluted in ASP to a concentration 10^7 were stained with 4 µL of 0.5 mM 2′,7′-dichlorofluorescein diacetate (2′,7′-DCFH-DA) for 10 min, at 6 °C in the dark. Thereafter, 300 µL of ASP was added. Additionally, the cells exposed to H_2O_2 were used as a positive control (1.5% H_2O_2, 30 min, 4ºC). The samples were analysed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA). The green fluorescence (FL-1) of 10×10^3 events per sample was recorded using a laser beam at 530 ± 15 nm (λex = 488 nm). The data were analysed using BD CellQuest Pro (Becton Dickinson) software ver. 5.2.1 (BD) Bioscience, (Franklin Lakes, NJ, USA) (https://www.bdbiosciences.com/en-pl/products/instruments/flow-cytometers).

Mitochondrial membrane potential detection assay kit

Changes in mitochondrial membrane potential (MMP) were detected using the JC-1 MitoScreen Kit. The 50 µL of cells diluted in ASP to a concentration of 10^7 were stained with 1 µL of JC-1 dye (which was reconstituted according to the manufacturer's instructions to create a working solution) and incubated for 30 min at 6 °C in the dark. Additionally, the cells in a 2 mm layer in a Petri dish exposed in the experiment to UV-C irradiation were used as a positive control (UV-C 70 mJ cm^{-2}, 30 min, 4ºC). After the indicated incubation time, cells were analysed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA). The green fluorescence (FL-1) and red fluorescence (FL-2) of 10×10^3 events were recorded using a laser beam at 530 ± 15 nm (λex = 488 nm) and at 581 ± 21 nm (λex = 488 nm), respectively. The data were analysed using the BD CellQuest Pro (Becton Dickinson) software ver. 5.2.1 (BD) Bioscience, (Franklin Lakes, NJ, USA) (https://www.bdbiosciences.com/en-pl/products/instruments/flow-cytometers).

Statistical analysis

The normality of the data distribution was verified with the use of the Shapiro–Wilk test. Significant differences were analysed by ANOVA nonparametric Kruskal-Wallis test using Statistica software (version 13.1) (StatSoft, Kraków, Poland) (https://www.statsoft.pl/statistica_13/) and $p < .05$ was considered statistically significant. The data were presented as median and interquartile range.

Data availability

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Author contributions

Conceived and designed the experiments: MGM, AM, KD. Performed the experiments: MGM, AM, KD. Analysed the data: MGM, AM, KD. Contributed reagents/materials/analysis tools: AM, MKG, EM, KZ, MGM, KD. Writing — original draft preparation: MGM, KD. Supplementary material – writing: EM, KZ. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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