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# The impact of concentration and administration time on the radiomodulating properties of undecylprodigiosin *in vitro*

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Undecylprodigiosin pigment (UPP) is reported to display cytotoxic activity towards various types of tumours. Nevertheless, its efficacy in modifying the cellular response to ionising radiation is still unknown. In this study, the radiomodulating effects of UPP were investigated. The effects of UPP were assessed *in vitro* by treating cultures of human peripheral blood with UPP and ionising radiation using two treatment regimens, the UPP pre-irradiation treatment and UPP post-irradiation treatment. The activity of UPP was investigated evaluating its effects on the radiation-induced micronuclei formation, cell proliferation, and induction of apoptosis. The redox modulating effects of UPP were examined measuring the catalase activity and the level of malondialdehyde, as a measure of oxidative stress. The results showed that UPP effects on cellular response to ionising radiation depend on its concentration and the timing of its administration. At low concentration, the UPP displayed radioprotective effects in γ-irradiated human lymphocytes while at higher concentrations, it acted as a radiosensitiser enhancing either mitotic catastrophe or apoptosis depending on the treatment regimen. The UPP modified redox processes in cells, particularly when it was employed prior to γ-irradiation. Our data highlight the importance of further research of the potential of UPP to sensitize tumour cells to radiation therapy by inhibiting pathways that lead to treatment resistance.

KEY WORDS: *apoptosis; mitotic catastrophe; oxidative stress; radiosensitisation; undecylprodigiosin*

The main therapeutic modalities to treat cancer are surgery, radiotherapy, and chemotherapy. Radiotherapy is one of the most effective forms of cancer treatment. However, ionising radiation (IR) used for the elimination of malignant tumours induces persistent DNA double-strand breaks (DSBs) leading to the permanent cell growth arrest, apoptosis, necrosis, or mitotic catastrophe (1). The major difficulties encountered during the treatment of cancer are tumour resistance to therapy and therapy-associated normal tissue toxicity (2). Alternative approaches to enhancing the efficacy of radiotherapy and minimising its harmful side effects focus on a combined treatment with anticancer drug and radiation (2). The potential radiotherapeutic agents are commonly categorised as (i) radioprotectors, which reduce normal tissue toxicity, thus, minimising the side effects of radiotherapy, and (ii) radiosensitisers, which enhance the radiosensitivity of tumour cells, thereby minimising the radiation dosage and damage to surrounding normal tissues (3, 4). Radiosensitisation of tumour cells by different agents is mainly achieved by enhancement of radiation-induced DNA damage in tumour cells compared to surrounding tissues, inhibition of DNA synthesis and cell growth, and enhancement of radiation-induced apoptosis (2, 5). Another approach is to modulate reduction/oxidation reactions within tumour cells (4).

In the last few decades, a wide variety of naturally occurring compounds have been tested in order to identify effective radiosensitisers that could enhance the sensitivity of cancer to the effects of IR and improve the cancer patients' survival rate (6-8). Plant-derived anticancer drugs continue to play a significant role in the development of new therapeutics aimed at modifying the radiobiological response of cells. Plant compounds such as etoposide, pactitaxel, and *Vinca* alkaloids have been recognised as radiosensitisers with low toxicity and high effectiveness whose synergism with radiation therapy increases the likelihood of cancer treatments (9). However, besides plants, microorganisms represent a profuse source of diverse bioactive metabolites with anticancer and possible radiomodulating activities.

Prodigiosins (PGs), produced as secondary metabolites by many terrestrial and marine bacterial strains such as the species of *Serratia* (10) and *Streptomyces* (11, 12), are a family of natural red pigments that are reported to possess antimicrobial, immunosuppressive, and cytotoxic activities (13-15). It has been reported that PGs mainly target cancer cells (hematopoietic, gastrointestinal, breast, and lung

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cancer) with little or no effects on normal cells (16-18). Numerous studies indicated that PGs displayed selective cytotoxicity against cancer cells, anti-metastatic activity, and p53-independent proapoptotic effects, which points to their potency as anticancer agents (16, 19).

Recent studies put a PG family member, undecylprodigiosin pigment (UPP), in the focus of anticancer investigation based on its proapoptotic potency demonstrated in various types of tumours (20-23). It has been suggested that UPP inhibits cell proliferation by inducing G2/M phase arrest and apoptosis of cancer cells. The tumoricidal action of UPP alone and UPP conjugated to gold nanoparticles was observed in melanoma, lung, breast, and colon cancer cells, enabling the platform for further development of efficient anticancer drugs suitable for clinical application (22). On the other side, it has been shown that UPP provides protection against oxidative stress by delaying the lipid peroxidation process (24) and displaying the antioxidant properties and mitochondrial function improvement (25). Similar findings were obtained in our previous investigation showing that treatment of human lymphocytes with low UPP concentrations (0.1 μg $mL^{-1}$) significantly reduced DNA damages and maintained the redox homeostasis of cells, indicating its cytoprotective nature. At moderate concentrations (1 μg $mL^{-1}$), UPP displayed effects similar to that of the untreated control, while the highest applied concentration (5 μg $mL^{-1}$) was genotoxic (unpublished data). The protective effects of UPP against UV irradiation were described previously (26).

As far as we know, the effect of UPP as a modifier of cellular response to ionising radiation is still unknown. Therefore, in this study, we investigated potential radioprotective/radiosensitising effects of UPP in γ-irradiated human peripheral blood cells using different combined treatment regimens with UPP and IR. The influence of UPP on radiation-induced apoptosis, micronuclei (MNi) induction, and oxidative stress alterations was investigated.

## MATERIALS AND METHODS

### *Undecylprodigiosin pigment (UPP) preparation*

Undecylprodigiosin (UP) pigment was synthesised in a shake flask culture of *Streptomyces* sp. JS520 using mannitol-soy-yeast medium supplemented with methyl-oleate (0.2 %, v/v) for six days at 30 °C and recovered and purified by column chromatography. The quality of the purified UP was analysed by liquid chromatography coupled to mass spectroscopy (LC-MS) as described previously (24).

### *Cell cultures and treatments*

Blood sample was obtained from a healthy, non-smoking, 30-year-old male volunteer donor in accordance with current Health and Ethical Regulations in Serbia (27). Aliquots of heparinised whole blood (0.5 mL) were first added to culture tubes containing 4.5 mL of RPMI-1640 medium supplemented with 15 % calf serum (Invitrogen-Gibco, Paisley, UK) and were then treated with an increasing concentration of UPP (final concentrations 0.1 μg $mL^{-1}$, 1 μg $mL^{-1}$, and 5 μg $mL^{-1}$).

The UPP effects were studied employing the experimental set up used in the study of Shukla et al. (28). For each UPP concentration, two treatment regimens were applied: pre-treatment - UPP was added to cell cultures and then exposed to γ-irradiation, and post-treatment - cell cultures were treated with γ-irradiation and then exposed to UPP treatment. γ-irradiated cultures not treated with UPP served as control.

For pre-treatment, UPP was added to cell cultures and incubated at 37 °C for one hour. Cultures were subsequently exposed to 2 Gy of $^{60}Co$ γ-radiation, at a dose rate of 0.45 Gy $min^{-1}$. After irradiation, UPP was washed off by centrifugation and cultures were reconstituted by adding 4.5 mL of RPMI-1640 medium supplemented with 15 % calf serum and 2 % phytohemagglutinin (Invitrogen-Gibco, Paisley, UK). They were then incubated at 37 °C for 72 h.

For post-treatment, cell cultures were exposed to γ-irradiation as described above. Immediately after irradiation, phytohemagglutinin and UPP were added to the cultures, which were then incubated for 72 h.

The adequate number of blood cultures was established to enable examinations of the MNi frequency, cytokinesis-block proliferation index (CBPI), catalase activity (CAT), and malondialdehyde (MDA) level.

For the apoptosis (AP) assay, blood aliquots of 0.5 mL were incubated in a RPMI-1640 medium (Invitrogen-Gibco, Paisley, UK) supplemented with 15 % calf serum (Invitrogen-Gibco, Paisley, UK) for 24 h. The treatments with UPP and γ-irradiation were performed as described above.

For each analysis performed, three independent experiments were carried out. The obtained data were pooled and results were expressed as the mean and standard deviation (SD) of the mean.

### *Micronucleus assay*

Radiosensitivity was assessed using the cytokinesis block method of Fenech (29). For MN preparation, Cytochalasin B (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 4 μg $mL^{-1}$ was added to each culture 44 h after incubation in order to inhibit cytokinesis. Lymphocyte cultures were incubated for further 28 h. Cells were collected by centrifugation and treated with a hypotonic solution (0.56 % KCl+0.90 % NaCl, mixed in equal volumes) at 37 °C. Cell suspension was fixed in

methanol/acetic acid (3:1), washed three times with a fixative, and dropped onto clean slides. Slides were air-dried and stained in alkaline Giemsa. For each sample, at least 1000 binucleated (BN) cells were scored and MNi were recorded using an Optech microscope (Munich, Germany) with 400x or 1000x magnification.

*Cytokinesis-block proliferation index*

The ability of cells to proliferate *in vitro* was evaluated by counting the number of cells with one to four nuclei on the same slides. The results of these analyses are presented as a cytokinesis-block proliferation index. CBPI was calculated according to the method of Surrales at al. (30) as follows: CBPI =[MI + 2MII + 3(MIII +MIV)]/N, where MI-MIV represents the number of cells with one to four nuclei, respectively, and N is the number of cells scored.

*Apoptosis of leukocytes*

After 24 h of incubation, cells were gently washed with physiological saline (0.9 % NaCl) at 37 °C, and fixed in methanol/acetic acid (3:1). Afterwards, the pellet was fixed in 96 % ethanol and stored at +4 °C. Samples were incubated at room temperature for 10-15 min in incubation phosphate buffer. Propidium iodide (PI, Sigma-Aldrich) and Ribonuclease A (RNase A, Sigma-Aldrich) were added 5 min prior to the flow cytometry analysis.

Apoptosis was assessed by flow cytometric (Partec, Germany) identification of cells displaying apoptosis-associated DNA condensation. DNA content was assessed by measuring the UV fluorescence of propidium iodide stained DNA. Apoptotic population was calculated using the Flow Max software (Partec, Germany).

*Blood culture preparation*

After 72 h of incubation, blood cultures were separated on a Lymphoprep (Lymphocyte separation medium, PAA Laboratories GmbH, Pasching, Austria); lymphocytes were collected by centrifugation, washed in a physiological saline, and frozen at -20 ºC for the TBA assay, while erythrocytes were haemolysed in ice cold deionised water and frozen at -20 ºC for subsequent analyses of the catalase activity.

*Assay of the catalase activity*

The catalase activity was measured using the method of Aebi (31) with minor modifications by following the catalytic reduction of hydrogen peroxide. The decomposition of the substrate $H_2O_2$ was measured using a Perkin Elmer Lambda 25 Spectrophotometer (Perkin Elmer Instruments, Norwalk, CT, USA) at 240 nm. The activity was expressed as K – rate constant of the first-order reaction per minute per mg of Hb. Haemoglobin concentration was determined using the Drabkin's method.

*Thiobarbituric acid (TBA) assay*

A thawed lymphocyte suspension was treated with thiobarbituric acid, and used to determine malondialdehyde levels, spectrophotometrically at 532 nm (32). Values were expressed as nmol TBA-reactive substance (MDA equivalent)/mg protein, using a standard curve of 1,1,3,3-tetramethoxypropane. Protein concentration was determined according to the method of Lowry et al. (33).

*Statistics*

Statistical analysis was carried out using the Statistica 8 and OriginPro 8.5.1 software packages for Microsoft Windows. Statistical analysis was done using the Mann-Whitney U test. *P* values of less than 0.05 were considered to be significant.

## RESULTS

The radiomodulating effects of UPP were assessed *in vitro* by treating human peripheral blood cultures with UPP and IR using two treatment regimens: UPP was added to the cultures one hour prior to γ-irradiation as pre-treatment and immediately after γ-irradiation as post-treatment. The activity of UPP was investigated evaluating its effects on the radiation-induced MNi formation, cell proliferation, and induction of apoptosis. The redox modulating effects of UPP were examined measuring the catalase activity and the level of malondialdehyde, the lipid peroxidation product, as markers of oxidative stress.

*Combined treatment with IR and UPP – the UPP pre-treatment regimen*

As shown in Table 1, the UPP pre-treatment, at low concentration, induced a significant reduction of the MNi frequency (by 13 %) compared to control (irradiated sample not treated with UPP) ($p<0.05$). At higher applied UPP concentrations, no considerable differences in the MNi frequency compared to control were observed. The proliferation potential of cells was almost unchanged up to the highest applied concentration when significant suppression of cell proliferation was found ($p<0.05$). As shown in Figure 1, the percentage of apoptotic cells was significantly higher (by approximately 50 %) than that in control ($p<0.01$), irrespective of the UPP concentration.

Considering the redox modulating activities of UPP, in cell cultures that were pre-treated with the lowest UPP concentration, a significant decrease in the MDA level (by 32 %) was observed ($p<0.05$), while the subsequent increase in UPP concentrations enhanced MDA level ($p<0.05$), potentiating hence the effects of IR (Figure 2a). As shown in Figure 2b, the catalase activity slightly increased in a dose-dependent manner reaching significant enhancement (by 36 %) at the highest UPP concentration applied ($p<0.05$).

**Table 1** *Frequency of micronuclei and proliferation potential of cells (mean±SD) in human lymphocytes treated with UPP and γ-irradiation (UPP pre-irradiation treatment and UPP post-irradiation treatment)*

| | | Frequency of micronuclei (MN) | Proliferation potential (CBPI) |
|---|---|---|---|
| Unirradiated Control | | 10.56±1.22 | 1.82±0.02 |
| γ-irradiated Control | | 215.87±8.46 | 1.51±0.04 |
| | UPP concentration | | |
| UPP pre-irradiation treatment | 0.1 μg ml$^{-1}$ | 189.04±9.41* | 1.53±0.01 |
| | 1 μg ml$^{-1}$ | 203.33±4.55 | 1.48±0.02 |
| | 5 μg ml$^{-1}$ | 215.43±8.38 | 1.40±0.03* |
| UPP post-irradiation treatment | 0.1 μg ml$^{-1}$ | 228.30±9.30 | 1.48±0.01 |
| | 1 μg ml$^{-1}$ | 231.27±6.81* | 1.41±0.02* |
| | 5 μg ml$^{-1}$ | 247.52±4.30* | 1.38±0.02* |

**Comparison with γ-irradiated control, p<0.05*

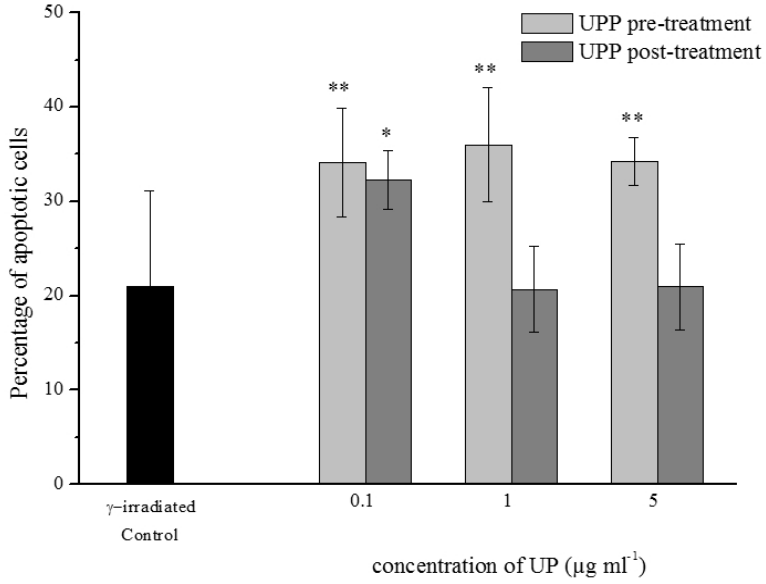


**Figure 1** *Apoptosis of leukocytes in a combined treatment with IR and UPP (mean± SD); Comparison with γ-irradiated control, *p<0.05, **p<0.01*

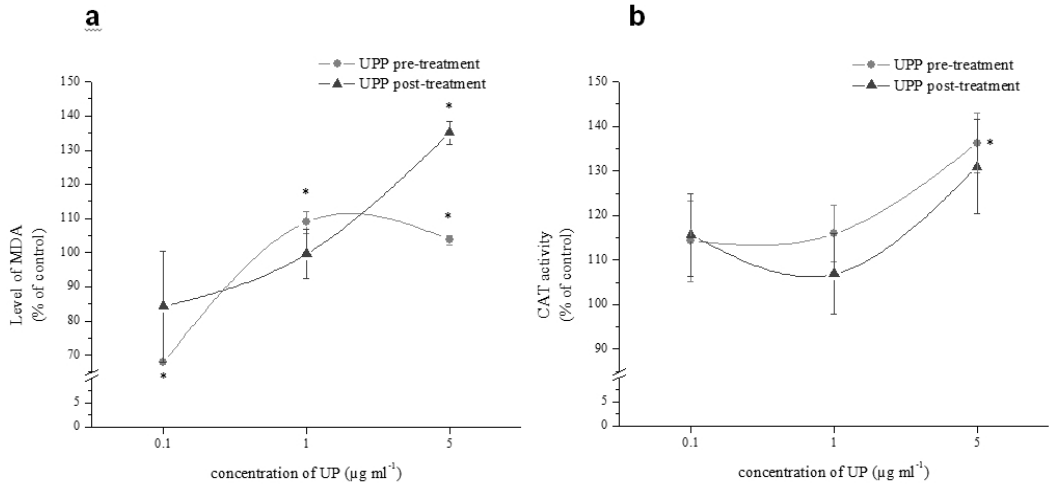


**Figure 2** *(a) Level of malondialdehyde and (b) Catalase activity in cell cultures treated with UPP and γ-irradiation (UPP pre-irradiation treatment and UPP post-irradiation treatment), expressed as a percentage of γ-irradiated control set to 100 % (mean±SD); Comparison with γ-irradiated control, *p<0.05*

*Combined treatment with IR and UPP – the UPP post-treatment regimen*

The UPP post-treatment resulted in significant enhancement of radiation-induced MN (by 15 %) and reduction of cell proliferation in a concentration dependent manner ($p<0.05$) (Table 1).

The percentage of apoptotic cells was significantly enhanced (by approximately 50 %) compared to control only at the lowest applied UPP concentration ($p<0.05$), while a further increase in UPP concentrations decreased apoptosis to control level (Figure 1).

As shown in Figure 2a, the UPP post-treatment caused no significant changes in the MDA level except for the highest UPP concentration, which enhanced radiation-induced lipid peroxidation by 35 % ($p<0.05$).

The UPP post-treatment caused mild, insignificant enhancement of the catalase activity (Figure 2b). Schematic representation of the treatment of human peripheral blood lymphocytes with UPP and γ-irradiation is given in Figure 3.

## DISCUSSION

Different classes of natural compounds have been investigated for their potencies to augment the therapeutic index of radiotherapy (1). Although PGs have been found to display anticancer activities, their efficacy in modifying cellular response to IR has not been investigated so far. In this study, we examined the influence of UPP on different end-points of radiation damage. Results of the study clearly indicate that UPP radiomodulating effects depend on its concentration and treatment regimens.

IR is known to cause direct DNA damage and indirect DNA damage is caused by free radicals derived from the ionisation or excitation of the water component of cells (34). Reactive oxygen species (ROS) generated upon irradiation induce DNA strand breaks and structural and functional alterations of biomolecules (35). Some ROS i.e. $HO_2^-$ and $H_2O_2$ can pass through cell membranes, which results in important changes in cellular functions (36).

Acting on cells before radiation exposure, UPP displayed significant proapoptotic effects enhancing the radiation-induced apoptosis independently of the concentration applied. This result is in accordance with the previously reported propensity of PGs to induce apoptosis. It has been shown that prodigiosins modulate the expression of Bcl-2 family proteins, IAP proteins, and death ligand/receptors involving both mitochondrial and death-receptor apoptotic pathways (19). The influence of UPP on radiation-induced MNi formation appeared to be concentration dependent. Specifically, the low UPP concentration attenuated the radiation-induced MNi frequency while the subsequent increase of UPP concentrations kept the level of MNi similar to that of control. On the other hand, the UPP post-treatment induced enhanced radiation-induced MNi and suppressed cell proliferation in a concentration dependant manner. A dose-dependent increase in the frequency of MNi in the lymphocytes treated with UPP was also observed in our previous study (unpublished data). These results suggest that UPP added to cell cultures after irradiation displayed cytostatic effects and promoted mitotic catastrophe, a type of cell death that results from abnormal mitosis and leads to the formation of cells with multiple MNi (37). In this treatment regimen, the observed decrease in radiation-induced apoptosis is compensated by an

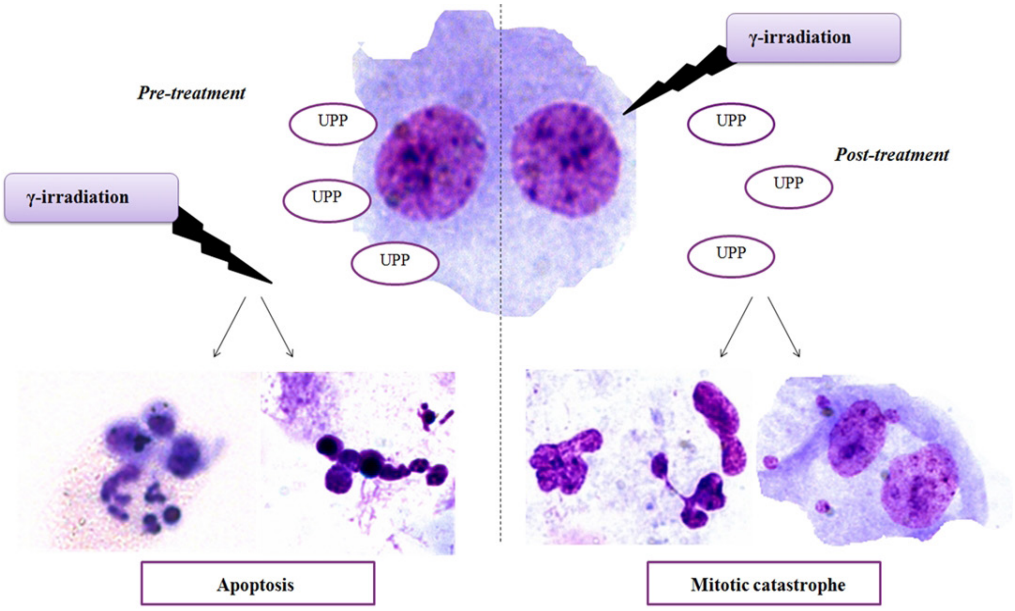


**Figure 3** *Schematic representation of the treatment of human peripheral blood lymphocytes with UPP and γ-irradiation. Left panel: Treatment with UPP prior to γ-irradiation enhanced radiation-induced apoptosis; Right panel: The UPP post- irradiation treatment enhanced radiation-induced mitotic catastrophe*

increase in the fractions of cells that die through the process of mitotic catastrophe. Mitotic catastrophe represents the main form of cell death induced by IR (38). Thus, in a post-treatment regimen, UPP contributed to the inappropriate entry of cells into mitosis possibly *via* suppression of multiple central checkpoint proteins or disturbance of the mitotic spindle formation.

The effect of UPP on radiation-induced lipid peroxidation is mainly observed in the UPP pre-treatment; the experimental data showed that high concentration of UPP displayed pro-oxidant effects, while at low concentration UPP possessed antioxidant properties and acted as a scavenger of highly reactive free radicals generated upon irradiation. The attenuation of radiation-induced injury was also seen through the reduction of IR-induced MNi as described above. However, UPP at the same concentration proved to be ineffective as antioxidant if administered at the post-irradiation stage. Augmented levels of intrinsic oxidative stress have been found in a variety of tumours, possibly due to more active metabolism, mitochondrial mutation, cytokines, and inflammation (39). Therefore, modulating the redox status of cancer cells might be critical for their survival (40). In that way, UPP as the IR-induced oxidative stress enhancer may sensitise cancer cells to undergo apoptotic death, which was the scenario observed in cell cultures pre-treated with UPP. It can be assumed that UPP promotes the fixation of IR-induced free radicals and prevents the repair of cellular radiation damage. In addition, the highest UPP concentration added to cells prior to gamma irradiation induced a significant enhancement of the catalase activity. Knowing that many human cancers including melanoma, neuroblastoma, colon carcinoma, and ovarian carcinoma, constitutively generate a high amount of $H_2O_2$ (41), it can be assumed that UPP may induce cell cycle arrest and apoptosis of these cancer cells through the scavenging of $H_2O_2$ by overexpressed catalase. It is worth noting that in a post-treatment regimen, the UPP redox modulating activities were attenuated. Radiation-induced lipid peroxidation was potentiated by UPP only at the highest employed concentration.

## CONCLUSION

Taken together, the results of this study suggest that UPP effects on cellular response to IR vary from mild radioprotection to radiosensitisation depending on its concentration and the timing of its administration, i.e. pre- or post-irradiation stage. At higher concentrations, the UPP's genotoxic and pro-oxidant behaviour augmented radiation-induced injury and it acted as a radiosensitiser enhancing either mitotic catastrophe or apoptosis depending on the treatment regimen. These findings highlight the importance of further research of UPP effects in cancer cells in terms of its utilisation for sensitising tumour cells to radiation therapy. If proven to be inefficient in killing cancer cells, this kind of exposure and high concentrations of UPP should not be applied. When applied at pre-irradiation stage, UPP, at low concentration, displayed radioprotective effects in gamma-irradiated human lymphocytes reducing MNi formation and acting as an antioxidant, thus exhibiting potential for further research.

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**Utjecaj koncentracije i vremena administracije na radiomodulirajuća svojstva undecilprodigozina *in vitro***

Undecilprodigiozin pigment (UPP) pokazuje citotoksičnu aktivnost kod različitih tipova tumora. Međutim, njegova učinkovitost u modulaciji staničnog odgovora na ionizirajuće zračenje i dalje je nepoznata. U ovoj studiji ispitani su radiomodulirajući učinci UPP-a *in vitro* tretiranjem kultura ljudske periferne krvi s UPP-om i ionizirajućim zračenjem. Pri ispitivanju su se koristile dvije vrste tretmana: tretman s UPP-om prije ozračivanja (predtretman) i tretman s UPP-om poslije ozračivanja (posttretman). Djelotvornost UPP-a ispitivana je procjenom njegovih učinaka na zračenjem inducirano formiranje mikronukleusa, staničnu proliferaciju i apoptozu. Redoks-modulirajući učinci UPP-a ispitivani su mjerenjem aktivnosti katalaze i razine malondialdehida kao parametara oksidacijskoga stresa. Rezultati pokazuju da učinci UPP-a na stanični odgovor na ionizirajuće zračenje ovise o njegovoj koncentraciji i vrsti tretmana. Pri niskim koncentracijama UPP pokazuje radioprotekcijski učinak u ozračenim humanim limfocitima, a pri visokim koncentracijama djeluje kao radiosenzibilizator inducirajući ili mitotsku katastrofu ili apoptozu, ovisno o vrsti tretmana. UPP modificira redoks procese u stanici, osobito ako se primjenjuje prije zračenja. Naši rezultati upućuju na značaj daljnjeg ispitivanja UPP-a u cilju njegove primjene za senzibilizaciju tumorskih stanica u terapiji zračenjem inhibicijom puteva koji vode rezistenciji na tretman.

KLJUČNE RIJEČI: *apoptoza; ionizirajuće zračenje; mitotska katastrofa; oksidacijski stres; stanična proliferacija*