INTERNATIONAL JOURNAL OF RADIATION BIOLOGY
2018, VOL. 94, NO. 9, 838–843
https://doi.org/10.1080/09553002.2018.1492757

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ORIGINAL ARTICLE

Radiosensitizer effect of usnic acid on *Biomphalaria glabrata* embryos

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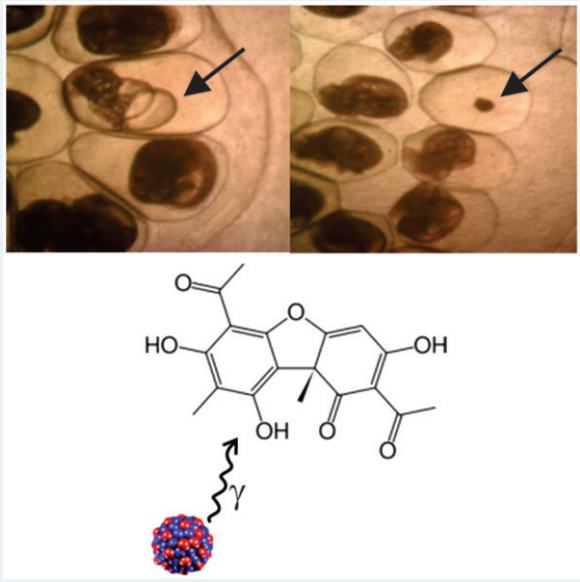
ABSTRACT

Purpose: Some phytochemicals have shown the potential of being radiomodifiers, especially phenolic compounds, such as lichenic secondary metabolites. To evaluate the phytochemical usnic acid as a radiomodifier, embryonic cells of molluscs have been used due to their ease of collection, high sensitivity to physical and chemical agents, well-known embryology and low cost for analysis.

Materials and methods: This study aimed to assess the radiosensitizing action of usnic acid on *Biomphalaria glabrata* embryos. Samples were irradiated with 4 Gy of gamma rays from a ^{60}Co source (dose rate 2.906 Gy/h). An acute toxicity test was performed using *B. glabrata* embryos in the blastula stage, in order to determine the toxicity of usnic acid and to establish the lethal Concentration for 50% (LC_{50}). Subsequently, the radiomodifing capacity of usnic acid was estimated using assays with *B. glabrata* embryos.

Results: Irradiation increased the number of non-viable embryos compared to unirradiated controls. Additionally, it was observed that embryos exposed to a non-toxic concentration of usnic acid (0.6 μg/mL) before irradiation showed a further enhancement in non-viable embryos when compared with exposure to ionizing radiation alone.

Conclusion: The results presented here indicate that usnic acid makes cells more sensitive to the damaging effects of radiation.



Abbreviations: ^{60}Co: Cobalt-60; DNA: deoxyribonucleic acid; Gy: gray; LC_{50}: lethal concentration for 50%; ROS: reactive oxygen species; UA: usnic acid

ARTICLE HISTORY
Received 14 December 2017
Revised 13 June 2018
Accepted 20 June 2018

KEYWORDS
Biomphalaria glabrata; radiation; radiosensitization; usnic acid

Introduction

Radiosensitizers are substances that make the target cells more susceptible to the biological effects of ionizing radiation (Nambiar et al. 2011). These molecules amplify the damage caused by the interaction of radiation with DNA and other cellular structures, regardless of whether the compounds cause cell damage individually (Verma 2016). Their main application occurs in the field of oncology, where they

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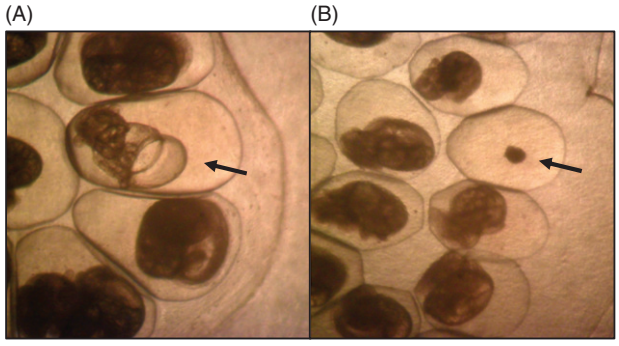

Figure 1. Spawns in veliger stage of embryonic development of *Biomphalaria. glabrata*. Viable and non-viable embryos. Indicated by black arrow: A) malformed and B) dead embryo.

are administered to enhance the effect of radiotherapy, sensitizing tumor cells and making them more susceptible to radiation damage (Javvadi et al. 2008).

These treatments also entail a risk of inducing deleterious side effects to the patients. It is, therefore, necessary to undertake studies of natural radiosensitizers, which could maintain the efficacy of the treatment with less side effects of ionizing radiation exposure, due to the possibility of reduction of the total absorbed dose. The currently used synthetic drugs have limitations due to their toxicity such as myelosuppression, hypotension, bradycardia, neuropathy, and hepatic insufficiency (Levin et al. 2000). These findings reinforce the need to study alternative substances of natural origin with radiosensitizing properties.

Among the radiosensitizers of natural origin already known are phenolic compounds and antioxidants, such as curcumin (Goel and Aggarwal 2010), found in saffron, resveratrol (Araldi et al. 2018), polyphenol (Lagerweij et al. 2016), a genotype-abundant flavonoid found in leguminous plants and quercetin (Lin et al. 2012). derived from fruits, vegetables, grains, seeds and spices.

Lichens, symbiotic associations between fungi and algae, are the source of a variety of bioactive compounds, mainly derived from their secondary metabolism and having applications in medicine, the textile industry, cosmetics and food (Kosanić et al. 2012; Manojlovic et al. 2012; Paudel et al. 2012). Usnic acid, dibenzofuran found in several species of lichens, is one of the most studied lichen metabolites and one of the few commercially available today. A number of biological activities are attributed to this metabolite, and its analgesic, antiviral, antiparasitic, antimicrobial, anti-inflammatory, antiproliferative, antitumor and antioxidant effects are reported (Shang et al. 2014; Su et al. 2014). Such characteristics make usnic acid a promising candidate for bioassays that can verify its radiosensitizing activity.

With the advancement of molecular biology and experimental embryology, evidence has confirmed the correlation between tumourigenesis and early embryonic development, so the early embryo shares many characteristics with the development of cancer, both biologically and molecularly (Williams et al. 1993; Monk 1990; Ma et al. 2010). Embryos of *Biomphalaria* sp. are considered good biological models, having a short embryoic period, simple and low cost maintenance and translucent spawnings which makes observation easy. The genus has a wide geographic distribution in tropical countries and has proved to be a useful model organism for bioassays of ecotoxicity, radioprotection and immunological tests (Oliveira-Filho et al. 2009; Siqueira et al. 2014; Sullivan and Belloir 2014). Therefore, the objective of this study is to evaluate the possible radiosensitizing effect of usnic acid using the simple biological model of *Biomphalaria glabrata* embryos.

Material and methods

Experimental animals

Pigmented adult snails of *B. glabrata* were used, measuring 10–14 mm diameter, from São Lourenço da Mata, Pernambuco and maintained for successive generations in the Radiobiology Laboratory of the Department of Biophysics and Radiobiology of Federal University of Pernambuco. The snails were kept in plastic tanks of approximately 20 L filtered and dechlorinated water, pH 7 and a temperature of about 25 ± 3 °C. The snails were fed daily with fresh organic lettuce (*Lactuca sativa*). The animals deposited their spawn onto colorless polyethylene strips (5x5 cm) that were floated on the water surface and examined under a stereomicroscope (Tecnival SQZ-SD4, São Paulo, Brazil) for individualization and identification of embryonic stage. After identification, embryos in the blastula stage were collected and divided into groups of 100 ± 3 specimens.

Determination of the lethal concentration for 50% of embryos

The toxicity of usnic acid (Sigma-Aldrich, Saint Louis, USA) in *B. glabrata* embryos was assessed in order to determine the lethal concentration for 50% (LC_{50}) as well as the concentration which showed the lowest lethality for embryos.

The assay was performed according to the method described by Oliveira-Filho and Paumgartten (2000). To perform the toxicity test, the embryos in blastula stage were placed in 24-well polystyrene plates (Prolab, São Paulo, Brazil) and exposed for 24 h to concentrations of non-irradiated and irradiated usnic acid (aqueous solution): 0.15, 0.3, 0.6, 1.2, 1.5, 2, 2.5, 5 and 10 µg/mL. For each concentration, approximately 100 embryos were used in triplicate.

After the exposure, the embryos were washed with tap water for total removal of usnic acid and then were maintained with filtered and dechlorinated water and examined under a stereomicroscope for eight consecutive days. The viability analysis was performed according to the method described by Okazaki et al. (1996). The embryos which had a normal development (during the experimental period) were considered viable and those which demonstrated malformations during development or were obviously dead were considered non-viable (Figure 1). The control group consisted of embryos maintained with filtered and dechlorinated water in the same conditions as the experimental embryos. The results were expressed as the mean percentage of non-viable embryos ± standard error of the mean (non-viable embryos %).

Table 1. Effect of usnic acid non-irradiated and irradiated with 4 Gy in embryos of *Biomphalaria glabrata*.[a]

	Concentration (μg/mL)	Non-viable embryos (%)
Control	0	1.8 ± 0.5
	0.15	1.9 ± 0.6
	0.3	2.7 ± 0.9
	0.6	4 ± 1.7
	1.2	19.3 ± 0.7*
Usnic acid	1.5	54.4 ± 3.4*
	2	98 ± 0.2*
	2.5	99 ± 0.1*
	5	99 ± 1*
	10	99.5 ± 0.5*
	0.15	1.4 ± 0.9
	0.3	3.1 ± 0.4
	0.6	3.6 ± 1.1
	1.2	23.7 ± 0.5*
Usnic acid irradiated	1.5	49.7 ± 4.8*
	2	97.3 ± 0.7*
	2.5	98.8 ± 0.5*
	5	99.2 ± 0.3*
	10	99.8 ± 0.2*

[a]The results are expressed as mean ± standard error mean. The significant differences were related between the groups by * vs. control ($p < .05$).

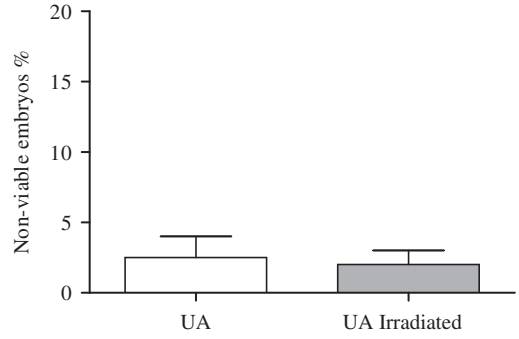

Figure 2. Embryotoxicity of usnic acid irradiated in *Biomphalaria glabrata*. The results are expressed as a mean and error bars indicate the standard deviation for $n = 30$ independent experiments. UA: embryos exposed to non-irradiated usnic acid; UA irradiated: embryos exposed to irradiated usnic acid.

Evaluation of radiosensitivity

The evaluation of the radiosensitizer capacity of usnic acid was performed with the concentration that showed the lowest toxicity (0.6 μg/mL), where the viability of the embryos was analyzed as described above. The embryos were divided into the following groups:

- Control (C): embryos maintained with filtered and dechlorinated water throughout the experimental period (eight days);
- Irradiated control (I): irradiated embryos (4 Gy) were maintained with filtered and dechlorinated water throughout the experiment;
- Usnic acid (UA): embryos exposed to usnic acid. After 24 h of exposure to the substance, the embryos were removed and kept in filtered and dechlorinated water until the end of the experiment;
- UA + bI: embryos exposed to usnic acid before being exposed to ionizing radiation. After 24 h of exposure to the substance, the embryos were removed and kept in filtered and dechlorinated water until the end of the experiment;
- UA + aI: embryos exposed to usnic acid after being exposed to ionizing radiation and after 24 h, the embryos were removed and kept in filtered and dechlorinated water until the end of the experiment.

The embryos were irradiated using a source of Gamma Cell ^{60}Co (Radionics Labs, dose rate = 2.906 Gy/h).

Statistical analysis

The results were expressed as mean ± standard error mean. The determination of the LC_{50} was analyzed by Probit regression using the software StatPlus® 2009 Professional. The difference between two groups was analyzed using a t-test, whereas the difference between three or more groups was analyzed using the analysis of variance (ANOVA) followed by Newman-Keuls test. The differences were considered significant when $p < .05$.

Results

Analysis of toxicity to embryos

The result of embryotoxicity of usnic acid is shown in the Table 1. The tested concentrations ranged from 0.15 to 10 μg/mL. The concentration of 0.6 μg/mL did not produce any change in the viability of the embryos, but for concentrations of 1.2, 1.5 and 2 μg/mL, there was an increase in the embryonic deterioration compared to the control group (19.3 ± 0.7, 54.4 ± 2.3 and 98 ± 0.2 vs. 1.8 ± 0.5). The concentration of 2 μg/mL showed a maximal response. In addition, there was no significant difference between the irradiated and non-irradiated groups. With these results, the LC_{50} of usnic acid non-irradiated was calculated, where its value was approximately 1.36 μg/mL, which is a concentration similar to that observed by Araújo et al. (2018). The LC_{50} of usnic acid irradiated was calculated, where its value was approximately 1.43 μg/mL. The highest concentration tested that did not show toxicity to embryos was 0.6 μg/mL. This concentration was selected to assess the capacity of usnic acid as a radiosensitizer.

Stability of usnic acid after irradiation

An evaluation was performed after the irradiation of the substance at a dose of 4 Gy, to verify whether there was any change in the biological activity, as measured by the embryo toxicity test with irradiated and non-irradiated solutions of usnic acid at the selected concentration of 0.6 μg/mL (Figure 2). No difference was found in the numbers of non-viable embryos in the group exposed to the irradiated substance compared to the group with the non-irradiated substance.

Radiosensitizer activity

The results of the radiosensitizer capacity of usnic acid are present in Figure 3.

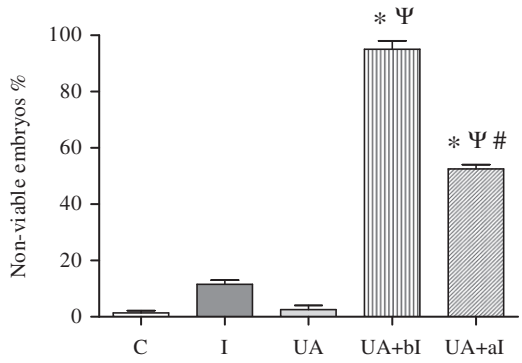

Figure 3. Effect radiosensitizer of usnic acid on embryos of *Biomphalaria glabrata*. C: control group; I: irradiated animals; UA: exposed to usnic acid; UA + bI: exposed to usnic acid before irradiation and UA + aI: animals exposed to usnic acid after irradiation. The results are expressed as a mean and error bars indicate the standard error mean for $n = 30$ independent experiments. The significant differences were related between the groups by # vs. UA + bI, Ψ vs. UA and * vs. I ($p < .05$).

The group I presented the highest number of non-viable embryos (11.5 ± 1.5) compared to group C (1.3 ± 0.9).

With regard to the usnic acid, the embryos exposed to the substance before irradiation (UA + bI) showed an increase in the number of non-viable embryos (95 ± 3) when compared to groups UA (2.5 ± 1.5) and I (11.5 ± 1.5).

Those embryos that were exposed to usnic acid after irradiation (group UA + aI) also showed an increase in the number of non-viable embryos (52.5 ± 1.5) in comparison to the groups UA (2.5 ± 1.5) and I (11.5 ± 1.5), however, when compared to animals that were exposed to group UA + bI (95 ± 3), the later presented a higher number of non-viable embryos.

Discussion

In recent years, lichens have been widely studied for their potential pharmacological properties. Their metabolites have also received special attention due to antibacterial (Segatore et al. 2012), antifungal (Shahi et al. 2011), antioxidant (Ranković et al., 2010) and anti-tumor properties (Einarsdóttir et al. 2010). In the field of radioprotection, great emphasis has been given to the use of natural products as radioprotectors or radiosensitizers as these may have advantages over synthetic compounds in, for example, producing fewer unwanted side effects.

The results of this study demonstrated an increase in mortality of embryos exposed to usnic acid in low concentrations, possibly due to the harmful effects caused by reactive oxygen species and mitochondrial dysfunction, both of which may be related to the induction of apoptotic mechanisms in embryonic cells. These effects were corroborated by studies in human liver cells (Chen et al. 2015) and rats (Han et al. 2004). In tests with neural cells exposed to usnic acid, there was evidence of a cytotoxic effect expressed as morphological changes and reduced viability which may lead to cell death possibly due to the increased production of reactive oxygen species (ROS; Rabelo et al. 2012). In other organisms similar toxicity was observed as demonstrated by Luz et al. (2015), who evaluated the action of usnic acid for promastigotes of *Leishmania infantum chagasi*. It was verified that the substance presented a leishmanicidal effect possibly related to morphological alterations in parasite cells. In addition, similar results were obtained in studies performed to verify the activity of usnic acid and its derivatives against the influenza A virus (H1N1)2009, where the lichen-derived substance showed antiviral activity (Sokolov et al. 2012).

The embryo toxicity test was first used in order to check whether 4 Gy irradiation might cause changes in the biological activity of usnic acid. No significant differences in the number of non-viable embryos were found, 4 Gy is a low dose in terms of changing chemical compounds (but not in terms of biological effects on DNA and other biomolecules) when compared with the doses used in the literature (Okazaki et al. 1996).

However, when the radiomodifying activity was investigated, it was demonstrated that the exposure of *B. glabrata* embryos to usnic acid, both before and after irradiation, the animals became more susceptible to the damaging effects of ionizing radiation. It has been suggested that this enhanced toxicity could be related to the increase of ROS present in the extra and intracellular environment. Possibly, usnic acid, when utilized in combination with ionizing radiation, may inhibit or reduce the activity of antioxidant enzymes or the molecular mechanisms of DNA repair or even the proliferative state of the cells which make them more susceptible to suffer biological damage.

Until now no studies have been reported evaluating the radiosensitizing capacity of usnic acid. However, some studies have demonstrated an antiproliferative activity of this substance (Bessadottir et al. 2012; Singh et al. 2013), which are directly related to the characteristic of a radiomodifier, more specifically, a radiosensitizer (Grem 2000). Other studies performed with cell lines of breast carcinoma (T-47D cell line human) and human pancreatic cancer (Capan-2), have demonstrated that usnic acid showed antiproliferative activity at a concentration of 4 μg/mL against the T-47D cell line and 5 μg/mL against Capan-2 cells (Einarsdóttir et al. 2010).

El-Beltagi et al. (2011) assessed the interaction of gamma radiation on the production of secondary metabolites in rosemary (*Rosmarinus officinalis* L.). They found that ionizing radiation induced an increase in the concentrations of phenols, flavonoids, amino acids, proteins and sugars. Similar results were obtained by Hussain et al. (2013), who studied the effect of ionizing radiation on damask and observed the occurrence of increased levels of flavonoids, phenols and β-carotene. In studies using *Ginko biloba* exposed to ionizing radiation phenolic compounds present in the plant extract showed a significant increase in comparison to a control group, with a dose-dependent response (Pereira et al. 2015). These alterations in the structure of the molecule may occur, since radiation can break chemical bonds and thus modify the structural characteristic of a substance, therefore, it is capable of altering the activity of a molecule, such as, increasing or decreasing toxicity for some organisms (Siqueira et al. 2014). Thus, depending on the substance

exposed to radiation, substances can be formed with radiosensitizing or radioprotective properties.

The present work has shown the potential of usnic acid being used in combination with ionizing radiation to enhance the destruction of groups of cells. If this phenomenon were to be applied to radiotherapy lower radiation doses could be needed to achieve the same tumor control as with present treatment regimens and with consequently better sparing of healthy tissue. Similarly, tests (Katz et al. 2008) using soy isoflavone and genistein alone or in combination with gamma radiation have led to the improved inhibition of cell growth in the human salivary gland. Moreover it has also been shown that the use of certain substances in combination with radiotherapy can increase the effectiveness of treatment of head and neck cancer (Katz et al. 2008). In studies performed with curcumin, it has been shown that it exhibited a potentially radiosensitizing effect, observed by optimization of the radiotherapy, either by increasing the number of destroyed tumor cells or allowing a decrease of the dose needed to produce the same therapeutic effect, and further, to reduce and so reducing the potential side effects due to ionizing radiation (Javvadi et al. 2008). Parthenolide is another substance shown to have a radiosensitizing effect when, combined with the radiation-induced increase of ROS levels reducing thiol in cancerous prostate cells (Watson et al. 2009). Another substance that has radiosensitizer action against cancer cells was ferulic acid. When associated with ionizing radiation, it produced a decrease in the viability of cells and also a decrease of antioxidant capacity and an increase of intracellular ROS, lipid peroxidation and DNA damage in HeLa and ME-80 human cells lines (Karthikeyan et al. 2011). Similar effects were also observed in studies performed with ellagic acid, where tumor cells treated, in vitro, with the combination of the phenolic compound and exposure to ionizing radiation with 6 Gy exhibited high levels of ROS. Changes in the transmembrane potential of mitochondria, reduced levels of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, together with a decrease in cell viability 24 h after treatment, in vivo, were found when using ellagic acid together with ionizing radiation in fractionated doses of 2 Gy (Bhosle et al. 2005).

Conclusion

This study has demonstrated the radiosensitizing effect of usnic acid on *B. glabrata* embryos exposed to ionizing radiation. The mechanisms responsible for potentiation of the damage caused by the combination of radiation with usnic acid may involve the action of bioactive free radicals. Further studies are necessary, commencing perhaps with human cancer cell lines, to evaluate the applicability of the combination of this substance with radiation with a view to its potential applications in radiotherapy.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

We thank the Brazilian Fostering Agency CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), State Fostering Agency FACEPE (Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the financial support.

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