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Cytoprotective and genoprotective effects of taxifolin against oxidative damage in HTR-8/SVneo human trophoblast cells

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Abstract

An increase of reactive oxygen species in the placenta and oxidative disbalance has been recognized as a significant factor contributing to pregnancy complications. Dietary intake of food rich in antioxidants during pregnancy could exert a protective role in the prevention of adverse outcomes such as preeclampsia, miscarriage, and others. Flavonoid taxifolin has shown numerous health-promoting effects in a large number of studies conducted on animals, as well as various human cell types *in vitro*. However, its effects on human placental cells—trophoblasts—have yet to be determined. Therefore, cytoprotective and genoprotective effects of taxifolin on trophoblast cell line HTR-8/SVneo under induced oxidative stress were explored in this study. Cytotoxicity of a range of taxifolin concentrations (1–150 μM) was evaluated using the MTT and crystal violet assays. A model of oxidative stress was achieved by exposing HTR-8/SVneo cells to H_2O_2. To determine cytoprotective and antigenotoxic effects, the cells were pre-incubated with three concentrations of taxifolin (10, 50, and 100 μM) and then exposed to H_2O_2. Taxifolin in concentrations of 1, 5, 10, 25, 50, and 100 μM showed no cytotoxic effects on HTR-8/SVneo cells, but 150 μM of taxifolin caused a significant decrease in adherent cell number, as detected by crystal violet assay. Pretreatment with the chosen concentrations of taxifolin showed a significant cytoprotective effect on H_2O_2-induced cytotoxicity, as determined by the MTT assay. Furthermore, taxifolin showed a significant reduction in H_2O_2-induced DNA damage, measured by comet assay. This study showed protective effects of taxifolin on human trophoblast cells exposed to oxidative damage. Further studies are needed to explore the underlying mechanisms.

Keywords: taxifolin; human trophoblast cells; DNA damage; cytotoxicity; comet assay

Introduction

Oxidative stress has been defined as increased production of reactive oxygen species (ROS) which affects cellular metabolism and its regulation, by damaging main cellular constituents (1). ROS interact with macromolecules and may cause lipid peroxidation, protein, and DNA damage, which can lead to cellular malfunction and development of pathological processes (2). Considerable evidence has shown the role of oxidative stress in the pathogenesis of cardiovascular, neurological, respiratory diseases, and cancer, but also in pregnancy-related disorders (3,4).

For the establishment and maintenance of normal pregnancy, two initial steps are necessary: first, a successful implantation of the embryo into the uterine wall, and subsequently, invasion of extravillous trophoblast cells of the placenta into the uterine stroma and the walls of maternal spiral arteries (5). These processes require a highly controlled and synchronized communication and interaction of the maternal and fetal side through local uteroplacental mediators. A failure to accomplish these steps may lead to placental disfunction and early pregnancy failure. Approximately 8%–15% of clinically recognized, and about 30% of all pregnancies, result in a miscarriage—loss of pregnancy before 20 weeks of gestation (6). Impaired trophoblast invasion is among major causes of preeclampsia, a multisystemic pregnancy-related hypertensive disorder affecting 2%–8% of pregnant women (7). The placenta is a temporary, highly specialized organ crucial for proper fetal growth and development. It is responsible for the exchange of nutrients, gases, growth factors, hormones, and metabolic waste products between maternal and fetal circulation. ROS have a role in mediating not only pathophysiological but also physiological signal transduction of various pathways which regulate cell differentiation, proliferation, and apoptosis (8). In the early stages of pregnancy, ROS play an important role in the regulation of angiogenesis, proliferation, differentiation, and invasion of trophoblast (9). While it has been shown that low to moderate levels of ROS are necessary for normal fetal development (10–12), increased placental oxidative stress can cause irreversible changes to the tissue, and has been recognized as an important factor in various pregnancy complications, including spontaneous preterm birth, intrauterine growth restriction,

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preeclampsia, pregnancy loss and stillbirth (13). It has been shown that increased oxidative stress and disrupted placental antioxidative defense mechanisms could affect the success of the trophoblast invasion and transformation of spiral arteries, potentially causing preeclampsia (14), or leading to early pregnancy loss (15,16). Flavonoids are plant secondary metabolites with polyphenolic structure, widely found in fruits and vegetables (17). In the last decades, they have attracted large scientific interest due to their various health-promoting effects. They regulate redox homeostasis, mitochondrial function, and inflammatory response and activate survival genes and signaling pathways (18). Among flavonoids, taxifolin (dihydroquercetin) has lately attracted significant attention. Taxifolin (3,3',4',5,7-pentahydroxyflavan-4-one) is commonly found in olive oil, grape, citrus fruits, as well as certain conifers, including *Taxus chinensis*, *Larix siberica*, and *Cedrus deodara* (19). It is commercially available in the form of various extracts from milk thistle seeds, maritime pine tree bark. Numerous studies have shown that taxifolin exhibits various pharmacological effects, including antioxidative, anti-inflammatory, antimicrobial, anticancer, hepatoprotective, and cardioprotective activities (20–24).

Antioxidative properties of taxifolin have been determined in nontrophoblast cells. Xie and collaborators (25) demonstrated that taxifolin inhibits intracellular generation of ROS and increases cell viability of human retinal pigment epithelial cells treated with H_2O_2. Zhanatev *et al*. (26) evaluated genotoxic properties of taxifolin in mice, using comet assay and chromosome aberrations counting in blood, bone marrow, liver, and rectal cells and detected no DNA damage. Since numerous pregnancy disorders have been linked to placental oxidative stress, inhibition of oxidative damage and apoptosis could be efficient in preventing these adverse outcomes. Therefore, this study aimed to evaluate cytoprotective, as well as genoprotective, effect of taxifolin on the human first-trimester trophoblast cell line, HTR-8/SVneo in a model of H_2O_2-induced oxidative stress.

Materials and methods

Cell line

The HTR-8/SVneo trophoblast cell line was established from the human first-trimester placenta explant cultures immortalized by SV40 large T antigen (kindly provided by Dr Charles H. Graham, Queen's, Kingston, Canada). The cells were cultured in a humidified incubator with 5% CO_2 at 37°C in RPMI 1640 (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal calf serum (Pan Biotech, Germany) and 1% antibiotic-antimycotic solution (Capricorn Scientific GmbH, Germany) (complete RPMI medium).

Taxifolin preparations

A stock solution of taxifolin (Cas No. 480-18-2, Sigma–Aldrich, St. Louis, MO, USA, 100 mM in DMSO) was diluted in a complete RPMI medium to achieve the appropriate concentrations for the experiments. Seven different preparations of taxifolin were made: 1, 5, 10, 25, 50, 100 and 150 µM. All suspensions were vortexed before treatment.

Cytotoxicity of taxifolin

Cytotoxicity of taxifolin in a range of concentrations (1–150 µM) was evaluated using MTT (27) and crystal violet cell viability assays, as described previously (28). After reaching 70% confluence, the cells were harvested from flasks using 0.25% trypsin–EDTA solution (Capricorn Scientific, Ebsdorfergrund, Germany) and seeded in 96-well plates (2×10^4 cells/well) in 100 µl of the complete medium. The cells were allowed to adhere and were incubated for 24 h at 37°C in a humidified incubator with 5% CO_2 prior to treatment. After the incubation period, the medium was removed, and fresh control medium (0.1% DMSO in complete RPMI medium) or the preparations of taxifolin were added to a total culture volume of 100 µl/well. The cells were incubated at 37°C for 24 h.

Afterwards, for the MTT assay, 10 µl of MTT reagent (thiazolyl blue tetrazolium bromide, 1 mg/ml) was added to each well. The cells were then incubated for 2 h in the dark at 37°C, after which the formed formazan crystals were solubilized by adding 100 µl sodium dodecyl sulphate (10% SDS, 0.1 N HCl) to each well. The plates were kept at 37°C for 24 h and shaken before measuring, to ensure complete solubilization of the crystals. Finally, absorbance was read at 570 nm using a microplate reader (BioTek ELx800, VT, USA).

For determination of adherent cell number, the crystal violet assay was performed. Following the incubation period with control or taxifolin preparations, the cells were rinsed with phosphate-buffered saline (PBS), dried, and fixed with ice-cold acetone–methanol (1:1). After the fixative was removed, and the cells were completely dried, 50 µl of 0.05% crystal violet dye in 25% methanol was added to each well. After 5 min incubation period, the excess dye was removed by immersing the plates in distilled water and drying at room temperature. The incorporated dye was dissolved in 0.1 M sodium citrate in 50% ethanol at 100 µl/well. Optical density was read at 540 nm using a microplate reader (BioTek ELx800, VT, USA).

Both MTT and crystal violet assays were performed in triplicate, and the entire experiment was repeated three times. The results are presented as a percentage of control values obtained for untreated cells.

Cytoprotective effect of taxifolin

Cytoprotective effect of taxifolin against H_2O_2-induced damage in HTR-8/SVneo cells was evaluated using the MTT assay. The cells were seeded in 96-well plates (2×10^4 cells/well) in 100 µl of the complete medium and allowed to grow overnight. The next day, the cells were treated with 10, 50, and 100 µM taxifolin or complete medium (0.1% DMSO in complete RPMI medium) for 24 h. Following the treatment, the cells were rinsed with PBS and incubated with 200 µM H_2O_2 in serum-free RPMI medium for 2 h to induce oxidative damage. The control cells were incubated with serum-free medium alone. The 200 µM H_2O_2 concentration was chosen as the 50% inhibitory concentration with a significant cytotoxic effect, compared with untreated trophoblast cells. After the treatment, a fresh serum-free medium was added, and the MTT assay was carried out as described above. The experiment was conducted in triplicate and repeated three times. The results are presented as a percentage of control values obtained for untreated cells.

Antigenotoxic effects of taxifolin

Antigenotoxic effect of taxifolin was evaluated using the alkaline comet assay. The cells were pretreated with 10, 50, and 100 µM taxifolin in the complete medium, and oxidative

damage was induced using 30 μM H_2O_2 in serum-free medium, as described in the text above. This peroxide concentration was chosen because it produced significant DNA damage, without causing total destruction. After the H_2O_2 treatment, the medium was removed and cells harvested using 0.25% trypsin–EDTA solution. The cells were then centrifuged at 300 × g for 5 min, the supernatant was discarded, and the cells were resuspended in a culture medium to obtain the single-cell suspension.

The alkaline comet assay was performed in accordance with MIRCA guidelines (29). The single-cell suspension was mixed with low-melting-point agarose (Sigma–Aldrich, St. Louis, MO, USA) in PBS to a final concentration of 0.7% and spread onto slides precoated with 1% normal melting point agarose (Sigma–Aldrich, St. Louis, MO, USA). The slides were placed in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris with freshly added 1% Triton X-100 and 10% DMSO) for 1 h at 4°C. After the lysis, the samples were placed in alkaline electrophoresis buffer (10 M NaOH, 200 mM EDTA) for 30 min to allow DNA unwinding, and electrophoresis was carried out at 25 V and 300 mA for 30 min. Finally, the slides were stained with ethidium bromide (20 μg/ml) and analyzed under Olympus BX 50 microscope (Olympus Optical Co., GmbH, Hamburg, Germany), equipped with mercury lamp HBO (50 W, 516-560 nm, Zeiss) pigment lens at a magnification of 100×. Comets were visually scored and classified into one of five classes as described previously by Collins *et al.* (30). Two replicate slides were prepared for each treatment, and the analysis was performed on 100 randomly selected cells per slide. Each comet class was given a value of 0, 1, 2, 3, or 4 (from undamaged, 0, to totally damaged, 4), and DNA damage was expressed as arbitrary units using the following equation: (percentage of cells in class 0 × 0) + (percentage of cells in class 1 × 1) + (percentage of cells in class 2 × 2) + (percentage of cells in class 3 × 3) + (percentage of cells in class 4 × 4). The entire experiment was carried out three times.

Statistical analysis

All results are expressed as the mean and standard error of the mean (mean ± SEM). One-way analysis of variance (ANOVA) with Tukey and Games–Howell post hoc tests was used to determine statistical significance. The correlation between DNA damage and taxifolin concentration was also examined. Statistical analyses were performed using Statistical Package for the Social Science (SPSS) software version 23 and value $P < 0.05$ was considered significant.

Results

Cytotoxicity of taxifolin

Viability of HTR-8/SVneo cells following 24-h incubation period with 1, 5, 10, 25, 50, 100, and 150 μM concentrations of taxifolin is shown in Fig. 1. No significant change in the number of viable cells for all the studied concentrations, when compared with the control, was observed in the MTT assay (Fig. 1a).

Results of the crystal violet assay are shown in Fig. 1b. No change in the number of adherent cells after the exposure to taxifolin in the concentrations of 1, 5, 10, 25, 50, and 100 μM was detected. However, taxifolin in the concentration of 150 μM caused a significant decrease in the number of adherent cells (82% vs. 100% control).

Cytoprotective effects of taxifolin

For further evaluation of cytoprotective effect of taxifolin against H_2O_2-induced oxidative damage, from subcytotoxic concentrations that showed no decrease in cell viability in the previous MTT assay, concentrations of 10, 50, and 100 μM were selected. Exposure to 200 μM H_2O_2 for 2 h reduced the viability of HTR-8/SVneo cell to 51%, compared with the control, untreated cells. Pretreatment of HTR-8/SVneo cells with 10, 50, and 100 μM taxifolin led to a significant rise in the percentage of viable cells (69%, 69%, and 67%, respectively), indicating a cytoprotective effect (Fig. 2).

Genotoxic effects and antigenotoxic potential of taxifolin

Genotoxic effects and antigenotoxic potential of taxifolin in the concentrations of 10, 50, and 100 μM were evaluated using the comet assay. The results are presented in Fig. 3. Taxifolin exposure did not increase DNA damage, compared to control, untreated cells, and no genotoxic effect was observed for any of the studied concentrations. Antigenotoxic effect of taxifolin was evaluated on HTR-8/SVneo cells treated with H_2O_2. Hydrogen peroxide exposure led to a significant increase in DNA damage, compared to control, untreated cells (222 vs. 13 DNA damage, expressed in arbitrary units). Pretreatment with taxifolin led to a significant attenuation of H_2O_2-induced DNA damage for all three studied concentrations (163, 88, and 47 DNA damage, expressed in arbitrary units for 10, 50, and 100 μM taxifolin, respectively). A very strong negative correlation (Pearson correlation coefficient $r = -0.949$) between DNA damage and studied taxifolin concentrations was detected ($P < 0.001$). Taxifolin in concentrations of 10, 50, and 100 μM exhibited a genoprotective effect against H_2O_2-induced damage. The detected effect was concentration-dependent. Figure 4 illustrates DNA damage in control HTR-8/SVneo cells (Fig. 4a), cells exposed to H_2O_2 (Fig. 4b) and, cells pretreated with 100 μM taxifolin prior to peroxide exposure (Fig. 4c).

Discussion

Increased oxidative stress has been implicated in the pathophysiology of many pregnancy complications including miscarriage, preeclampsia, fetal growth restriction, and preterm labor (31). Dietary intake of food rich in antioxidants has been proposed as a mechanism of prevention, but a thorough understanding of their mechanism of action is required to confirm their benefits in pregnancy. To the authors' best knowledge, no previous study explored the effects of taxifolin on H_2O_2-induced cytotoxicity and DNA damage in HTR-8/SVneo cells. Therefore, this study aimed to determine whether taxifolin protects trophoblast cells from oxidative damage.

Protective effects of taxifolin have been evaluated in a large number of studies on various cells *in vitro*. Massunari *et al.* (32) showed that taxifolin in concentrations of 5 and 10 μM is not cytotoxic to Saos-2 osteoblast-like cells, while Chen *et al.* (22) reported protective effects of 200 μM taxifolin against TNF-α/ActD-induced apoptosis in HepG2 cells. Similarly, the study by Shu *et al.* (23), which evaluated protective effects of taxifolin in H9c2 cardiomyocytes (in a series of concentrations from 2.5 to 80 μM), showed no cytotoxic effects. Therefore, the range of concentrations from 1 to 150 μM was chosen for this study.

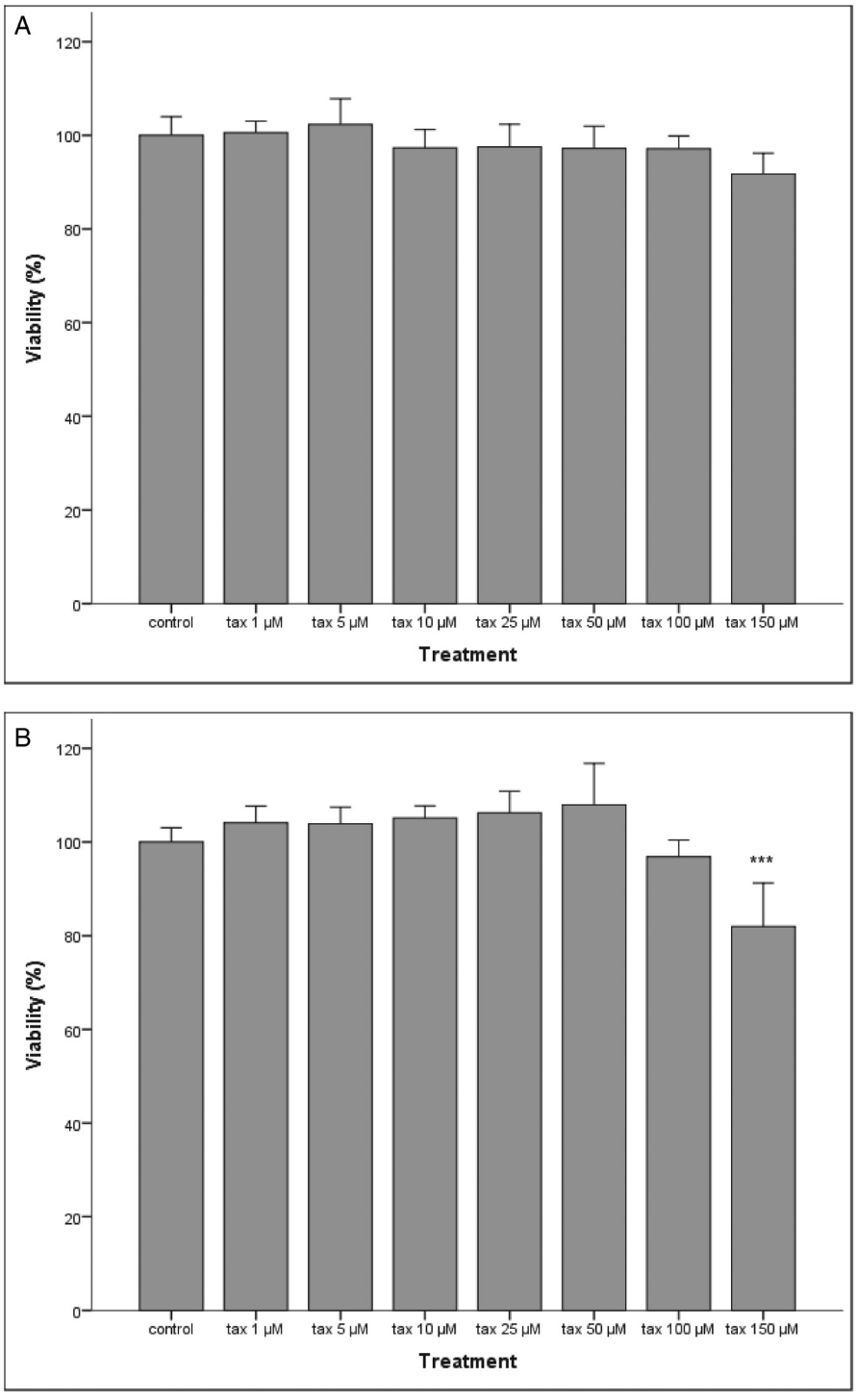

Figure 1. (a) Cytotoxicity of taxifolin evaluated using the MTT assay. Values are shown as mean ± SEM. (b) Effects of taxifolin on adherent cell number evaluated using the crystal violet assay. Values are shown as mean ± SEM. Tax, taxifolin, ***$P < 0.001$ compared with the cells treated with medium only.

Cytotoxicity of taxifolin was evaluated using the MTT cell viability assay and crystal violet adherent cell number assay. Both assays showed no significant decrease in cell viability/adherent cell number following 24-h incubation with 1, 5, 10, 25, 50, and 100 µM concentrations of taxifolin. A statistically significant decrease in the number of adherent HTR-8/SVneo cells was detected with the highest tested taxifolin concentration (150 µM), while the same concentration of taxifolin showed no significant decrease in the MTT assay. This discrepancy could be explained by the limitations of the two assays. The MTT assay measures cell metabolic activity, by measuring the reduction of the tetrazolium dye to purple, insoluble formazan crystals, thus reflecting the numbers of metabolically active cells or the increase in metabolic activity without the increase in the cell number. On the other hand, the crystal violet assay detects differences in the number of

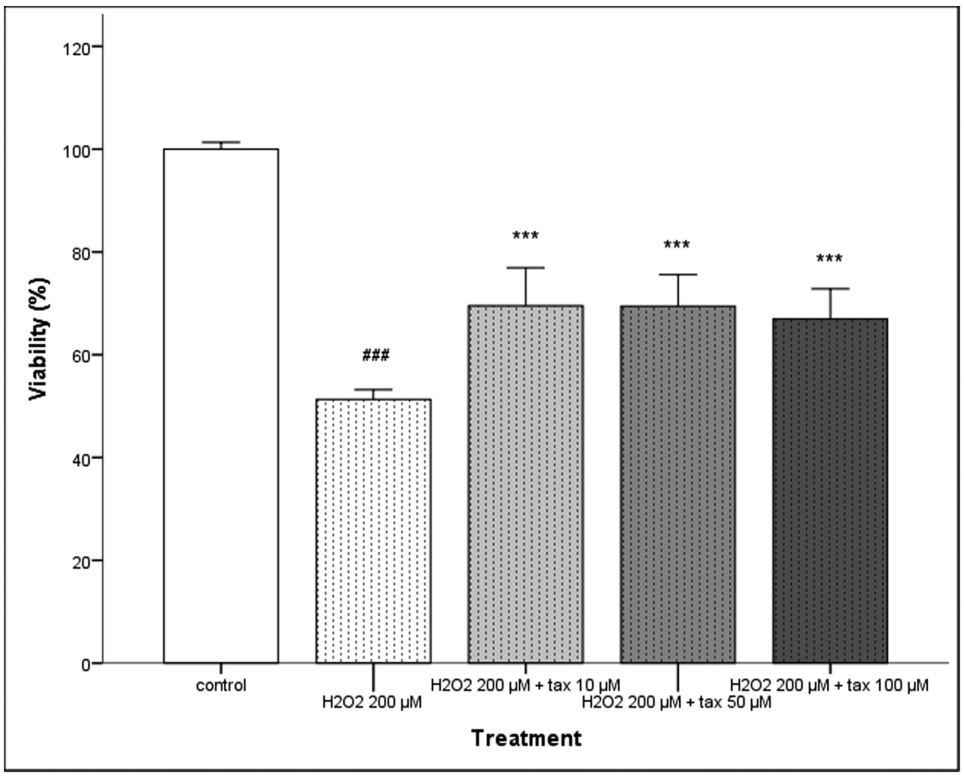

Figure 2. Cytoprotective effect of taxifolin on H_2O_2-induced damage. Values are shown as mean ± SEM. Tax, taxifolin, $^{###}P < 0.001$ compared with the cells treated with medium only, $^{***}P < 0.001$ compared with the cells treated with H_2O_2.

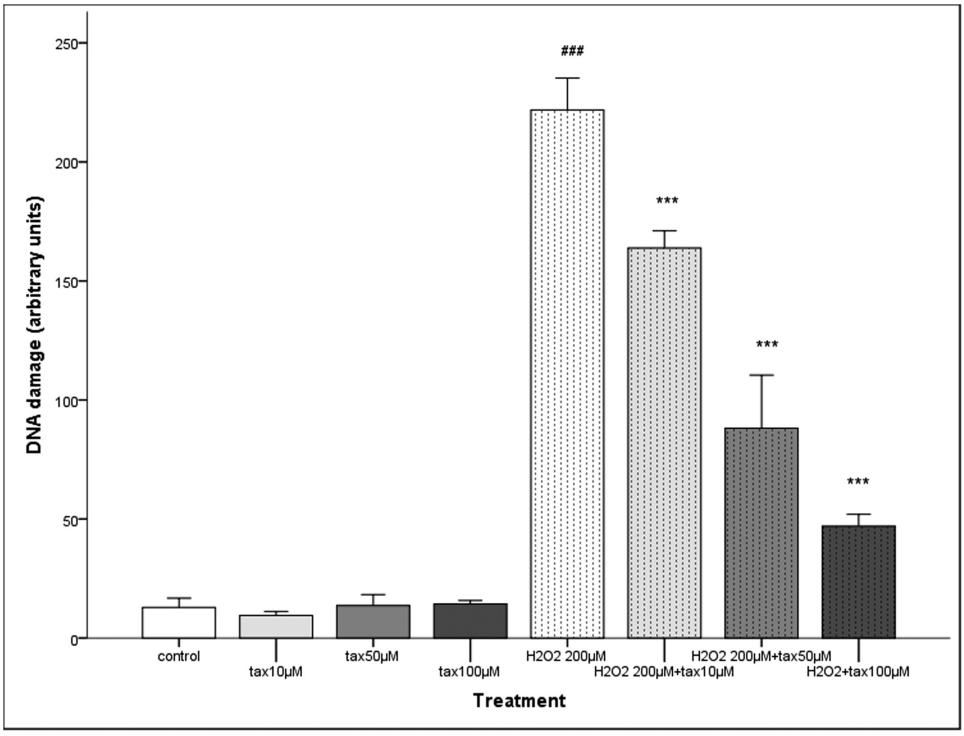

Figure 3. Antigenotoxic effect of taxifolin on H_2O_2-induced DNA damage. Values are shown as mean ± SEM. Tax, taxifolin, $^{###}P < 0.001$ compared with the cells treated with medium only, $^{***}P < 0.001$ compared with the cells treated with H_2O_2.

adherent cells. Taxifolin (10, 30, and 100 µM) was previously shown to reduce the viability of the breast cancer cells after 72h incubation using crystal violet assay, but also to decrease their migration and invasion by interfering with the β-catenin signaling pathway (33). The effects of taxifolin on the same pathways in trophoblast cells remain to be determined yet;

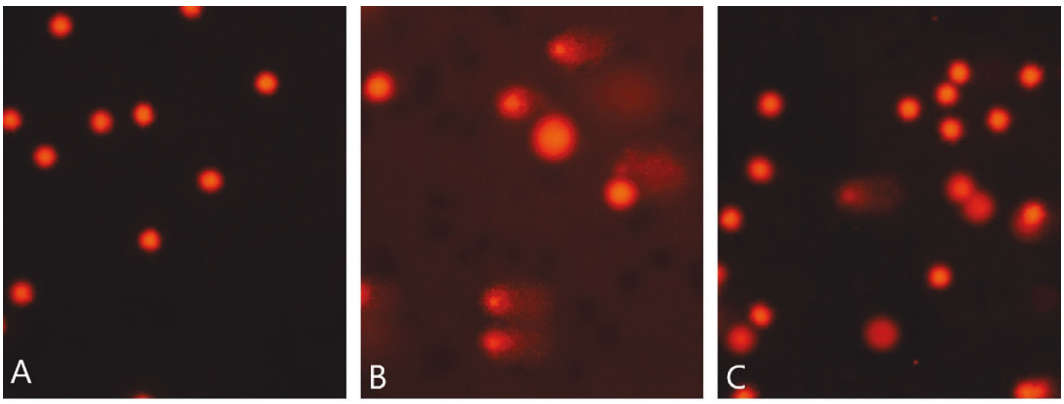

Figure 4. Comets in (a) control HTR-8/SVneo cells, (b) cells exposed to H_2O_2, and (c) cells pretreated with 100 μM taxifolin prior to H_2O_2 exposure.

however, from our results, it can be concluded that taxifolin at a concentration of 150 μM could affect trophoblast cell viability and reduce the number of adherent cells.

A model of oxidative stress was established by exposing the HTR-8/SVneo cells to H_2O_2, in order to evaluate cytoprotective and antigenotoxic effects of taxifolin in concentrations of 10, 50, and 100 μM, which showed no cytotoxic effects in the previous experiment. Pretreatment of HTR-8/SVneo cells with all three studied concentrations showed a significant increase in cell viability, indicating a cytoprotective effect of taxifolin on H_2O_2-induced oxidative damage in HTR-8/SVneo cells.

While antioxidant, anti-inflammatory, hepatoprotective, and cardiovascular effects of taxifolin have been widely studied (21–23), data on its genotoxic and antigenotoxic effects are scarce. Zhanatev *et al.* (26) have evaluated genotoxicity of taxifolin in mice, while Zivkovic *et al.* (34) examined the effects of dihydroquercetin (taxifolin) on human blood cells, but, to the authors' best knowledge, no study evaluated its effects on human trophoblast cells. Therefore, genotoxic and antigenotoxic potential of taxifolin was evaluated. The obtained results show a lack of genotoxicity on HTR-8/SVneo cells, adding further evidence of its safety. Pretreatment with taxifolin attenuated the DNA damage induced by H_2O_2, demonstrating marked genoprotective effects. The highest studied concentration (100 μM) showed the most prominent effect on H_2O_2-induced DNA damage, indicating that free radical scavenging could be one of the underlying mechanisms. The results of chemical studies of taxifolin showed that it possesses marked reducing ability, radical scavenging, and metal-chelating activities (35). Indeed, other authors showed that the mechanism of taxifolin's protective antioxidant effect in bone marrow-derived mesenchymal stem cells *in vitro* is the result of OH-scavenging activity (36). Additionally, the study by Zeng *et al.* (37) showed that the same antioxidant mechanisms of taxifolin are effective both *in vitro* and *in vivo*.

The obtained results showed that studied concentrations (1–150 μM) of taxifolin did not demonstrate genotoxic effects on HTR-8/SVneo cells, nor cytotoxicity up to the concentrations of 100 μM. Furthermore, cytoprotective and genoprotective effects of taxifolin against H_2O_2-induced oxidative stress in human trophoblast cells were demonstrated, where pretreatment with taxifolin (10, 50, and 100 μM) significantly attenuated oxidative DNA damage and reduced H_2O_2-induced cytotoxicity. These findings suggest that taxifolin could exert a protective role in preventing pregnancy complications related to oxidative stress. However, the current study has some limitations. One of them is the use of cell lines instead of primary cells, which would provide more relevancy to the research, as well as enable the obtained data to be utilized as a preventive measure of pregnancy disorders. Therefore, this preliminary study provides a framework for further mechanistic studies in primary trophoblast cells, which would help understand the exact molecular mechanism behind taxifolin's protective effects.

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Conflict of interest statement: The authors declared no conflict of interest.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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