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EXPERIMENTAL STUDY

Synergistic effects of quercetin and selenium on oxidative stress in endometrial adenocarcinoma cells

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ABSTRACT
OBJECTIVE: The effects of quercetin and selenium on oxidative stress in endometrial adenocarcinoma cells are unclear. In this study, the effects of quercetin and selenium on oxidative stress caused by both hydrogen peroxide and UV radiation in endometrial adenocarcinoma cells were examined.
METHODS: The viability of endometrial adenocarcinoma cells cultured *in vitro* and treated with different concentrations of quercetin and sodium selenite was measured using the MTT assay. Malondialdehyde (MDA) levels were investigated, and expression levels of BAD and p53 genes were analysed using real-time quantitative polymerase chain reaction. Acridine orange/ethidium bromide staining technique was applied to detect apoptosis. Mass attenuation coefficient of each quercetin and sodium selenite combinations was evaluated using Monte Carlo simulation.
RESULTS: The combination of quercetin and sodium selenite enhanced cell viability, and reduced MDA levels. The expression levels of BAD and p53 genes decreased by combined treatment with quercetin and selenium while showing synergistic effects in terms of gene expression. Fluorescent microscopic examination showed a decrease in apoptotic cells in endometrial adenocarcinoma cells treated with the combination of quercetin and selenium.
CONCLUSIONS: For the first time, selenium and quercetin have synergistic cytoprotective and radioprotective effects on oxidative stress caused by hydrogen peroxide in endometrial adenocarcinoma cells for the first time *(Tab. 1, Fig. 7, Ref. 39)*. Text in PDF *www.elis.sk.*
KEY WORDS: endometrial adenocarcinoma cells, malondialdehyde, Monte Carlo simulation, oxidative stress, quercetin, selenium.

Introduction

Quercetin is a flavonoid compound found in many plants and foods such as fruits, vegetables, red wine, propolis, rosehip and turmeric. Quercetin contributes significantly to brain development (1, 2). The most important feature of the quercetin compound is its high antioxidant activity in cells (3). Quercetin compound was reported as anti-inflammatory, antiviral, antioxidant, anti-carcinogenic and psychostimulant agent (4). In addition, quercetin plays an important role in the suppression of free radical formation, proliferation in tumour development, and metastasis-causing processes (5). Effects of several other antioxidant molecules on oxidative stress condition were investigated in several studies.

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Selenium (Se) is a trace element playing a significant role in living organisms (6). Selenium is found in organic and inorganic forms in the living systems (7). Selenium is available in the structure of glutathione peroxidase and iodothyronine deiodinase enzymes with high antioxidant activity. In this respect, selenium has a protective effect against harmful free radicals in cells (8). In humans, the characteristics of biological activity of selenium depend on its form and amount, while it protects the DNA structure against free radicals (9). Selenium-containing selenoproteins are involved in many important biological functions with different activities (10). These antioxidants play an important role in oxidative stress caused by various chemicals, but physical factors can also affect human health. For example, ionizing radiation from medical and industrial sources can damage the entire biological system. The exposure to ionizing radiation in living tissues may lead to different organ dysfunctions (11). Theoretically, all materials can be used as a radiation attenuator if they are designed with a particular slab thickness. Yet, the attenuation properties for ionizing radiation of employed material are dependent upon some material features such as density and chemical composition (12). Natural flavonoids are considered to be photo-protective compounds since they can absorb UV radiation. Flavonoids have a protective effect by eliminating free radicals and activating antioxidant enzymes in the biological systems (13). Quercetin was demonstrated to protect

from the damage caused by UV radiation in rats (5, 14). Moreover, Zhu et al (2017) demonstrated the protective effect of quercetin against UV radiation in keratinocyte cells (HaCaT) (15). However, the effects of quercetin and selenium on oxidative stress in endometrial adenocarcinoma cells are unclear. In this respect, Monte Carlo simulation was applied in order to examine the synergistic radiation reduction effect of the selenium and quercetin combinations, and both H_2O_2-induced oxidative stress and UV radiation were examined using endometrial carcinoma cells.

In this study, the effects of quercetin and selenium on oxidative stress caused by H_2O_2 was examined using cell viability (MTT) assay, lipid peroxidation assay, gene expression analysis of BAD and p53 genes using real-time PCR, and fluorescence microscopy. Effects of UV radiation were tested and experimental results were confirmed by computational method of Monte Carlo N-Particle Transport Code System-extended (MCNPX). The synergistic effect of quercetin and selenium on Ishikawa cells was investigated experimentally, and the different selenium and quercetin combinations at different photon energies were analysed theoretically.

Materials and methods

Cell culture and selenium-quercetin treatments

Human endometrial adenocarcinoma cell line (Ishikawa) was used in the study. The cells were cultured in DMEM medium (Gibco, 11960044, UK) supplemented with 10 % foetal bovine serum (Gibco), 1 % penicillin/streptomycin and L-glutamat at 37 °C in a humidified atmosphere of 5 % CO_2. Ishikawa cells were grown in a 35-mm culture dishes for 24 h before the experiments. Various concentrations of quercetin and sodium selenite were applied to the cultured cells for 12 h, and 300 μM H_2O_2 was added into the cultures waiting for 3 h. Control group was treated neither with H_2O_2, nor with quercetin and selenium. A volume of 300 μL of H_2O_2 was used to induce oxidative stress in group 2. In groups 3 and 4, 100 μM of quercetin and 30 nM of selenium were supplemented into media before H_2O_2 treatment, respectively. The combination of 100 μM quercetin and 30 nM selenium before H_2O_2 treatment was used in group 5.

Cell viability assay

MTT assay was applied to investigate cell viability. A volume of 30 μL of 5 mg/mL of MTT reagent in phosphate buffered saline (PBS) was added to each well, and then replaced with 100 μL of DMSO. The optical density was measured using microplate reader (Thermo Scientific, USA) at 540 nm (16).

A separate experimental protocol was used to detect the UV-protective synergic effect of quercetin and sodium selenite. The effect of UV was investigated by exposing the cells under UV light for 30 minutes with the plate lid open. Then, MTT assay was performed again.

Evaluation of MDA levels by TBARS assay.

Thiobarbituric acid reactive substances (TBARS) assay was used to measure malondialdehyde (MDA) levels in the cells according to a previous procedure (17). Ishikawa cells were harvested with 0.25 % of trypsin and lysed with TRIzol reagent, and incubated for 30 min at 2–8 °C. All samples were added with 300 μL of TBARS acid reagent in 0.6 N of trichloroacetic acid solution, and incubated for 15 min at room temperature. Then, the samples were centrifuged at 12,000 g for 5 min. The standard was converted to MDA by adding 100 μL of TBARS standard (1 mL of 500 μM 1,1,3,3-tetramethoxypropane in deionized water) to 200 μL of TBARS acid reagent, and left for 30 min. A volume of 150 μL of standards and samples were added to a 96-well plate. Then, 75 μL of TBA reagent (thiobarbituric acid in deionized water) was added to each well, and incubated for 2 h at 65 °C. The absorbance of each well was measured using a microplate reader (ThermoScientific, USA) at 532 nm.

Real-time PCR

The total RNA was extracted using TRIzol reagent. The total RNA was used to synthesize cDNA using a thermal cycler (BIO-RAD, T100 Thermal Cycler). QRT-PCR (Roche Light Cycler, USA) was used to analyse gene expression levels using specific primers. The following primers were used in the study: for the BAD gene: R: 5´- GAGCAACATTCAGCAGG-3´and F: 5´- GTACGAACTGTGGCGACTCC-3´; p53 gene: F: 5´ AACTGCGGGACGAGACAGA 3´ and R: 5´ AGCTTCAAGAGCGACAAGTTTT3´; The relative quantification of gene expressions among the groups was analysed by the $2^{-\Delta\Delta CT}$ method (18).

Fluorescence microscopy

Morphological assessment of apoptotic cells was performed using the AO/EB double-staining method (19). Ishikawa cells were collected using trypsin (Gibco, Lot no. 1951049, Canada), and diluted with DMEM (Gibco, Lot no. 1979018, UK) medium reaching 10^5 cells per mL. The cells were transferred to each well and left in CO_2 incubator for 24 h at 37 °C. After 24 hours, 5 M of H_2O_2 was added to all wells with the exception of the control group. All the medium in the wells was removed after 5 h, and 2 mL of the compounds (sodium selenite and/or quercetin) were added into the wells. The wells were transferred to CO_2 incubator again and left there for 12 h. A volume of 100 μL of acridine orange was added into 900 μL of sterile PBS (VWR, Lot No. 1206C155). A volume of 10 μL of ethidium bromide (EtBr) solution was added into 900 μL of sterile PBS, and mixed with acridine orange solution in a 1: 1 ratio to prepare the fluorescent staining dye. The whole medium was removed from the wells, and a volume of 40 μL of staining dye was added into the each well. A fluorescence microscope (SOIF Optical/MF52) was used to visualize Ishikawa cells using 20X objective and 10X ocular.

Monte Carlo simulation for radiation attenuation properties

A Monte Carlo simulation approach can offer an alternative problem solving for extremely complex physical problems pertinent to the transport of different types of radiation passing through matter, such as gamma and neutron rays. Among some reputable and well-known Monte Carlo codes that are generally utilized in nuclear physics and medical physics problems, MCNPX has attracted major attention of researchers (20–24).

In this study, to calculate the linear attenuation coefficient (μ) of investigated selenium and quercetin combinations, the simulation set-up and basic scheme are designed by considering the Beer-Lambert law ($I = I_0 e^{-\mu x}$), where I is the intensity of photons transmitted across some distance x, I_0 is the initial intensity of photons, μ is the linear attenuation coefficient and finally x is the distance travelled. Certain simulation equipment such as isotropic point source of radiation, selenium and quercetin combinations as attenuator material for primary radiation beam, lead collimator, detection field to record the attenuated radiation beam from selenium and quercetin combinations and lead block to avoid detection field from scattered radiation were designed in input file defined in the MCNPX input file in order to investigate the radiation attenuation properties of generated combinations (22–23). The primary radiation beam originated from the isotropic point source was directed onto the selenium and quercetin combination. The detection field (F4 Tally Mesh) was put on the same line at a distance 70 cm from isotropic point source of radiation. The investigated selenium and quercetin combination was located between the source and the detector at a distance of 50 cm from the source. In this study, F4 tally mesh was used to count the number of photons entering the detector per $MeVcm^2s^{-1}$.

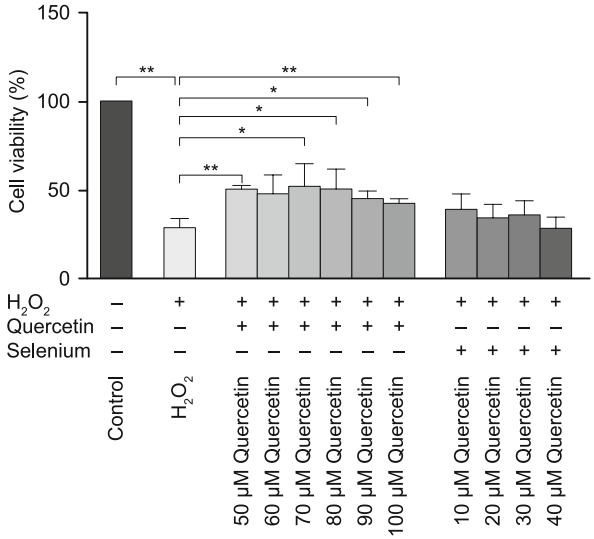

Fig. 1. Cell viability results using MTT assay.

Statistical analysis

All experimental data were expressed as mean +/– S.D. of replicated values. Data were analysed by one-way analysis of variance (ANOVA), followed by Student t-test.

Results

Cytotoxicity results

Figure 1A shows the effects of quercetin at various concentrations and sodium selenite when they are used individually. Figure 1B shows cell viability results affected by the combination of quercetin and selenium using Ishikawa cells. Hydrogen peroxide treatment led to a significant decrease in cell viability corresponding to around 30 % of cell viability when compared to the control group ($p < 0.05$). A gradual increase in concentrations of quercetin from

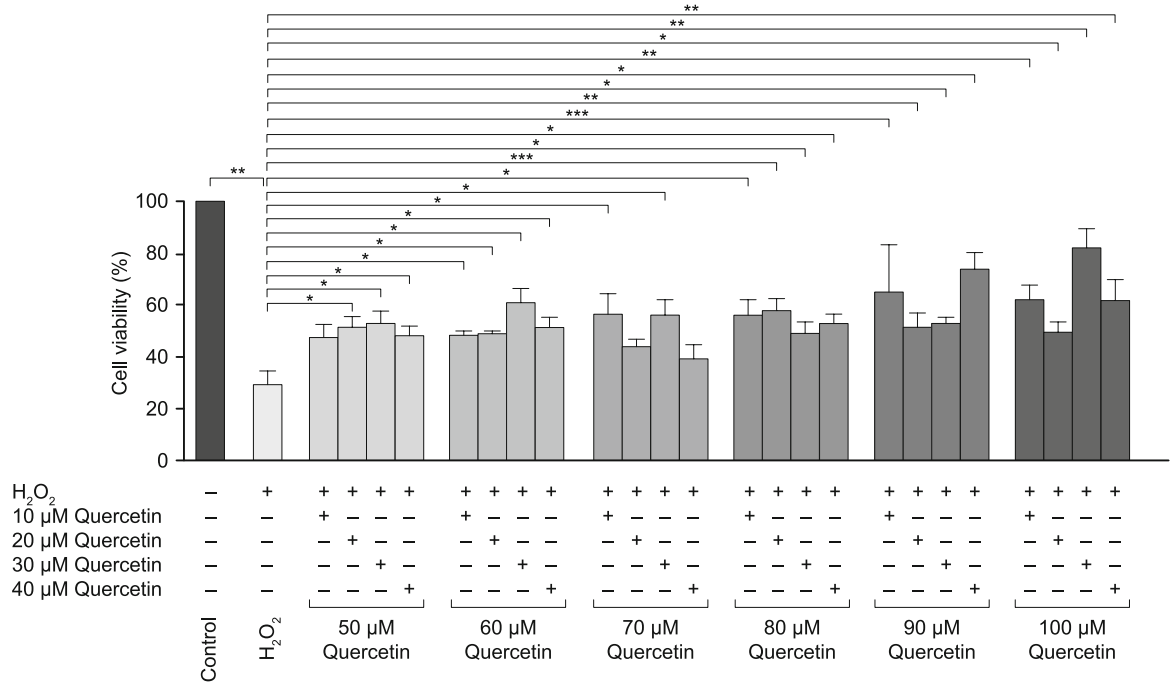

Fig. 2. Cytotoxicity results affected by UV treatment on Ishikawa cells, and synergistic effect of quercetin and selenium.

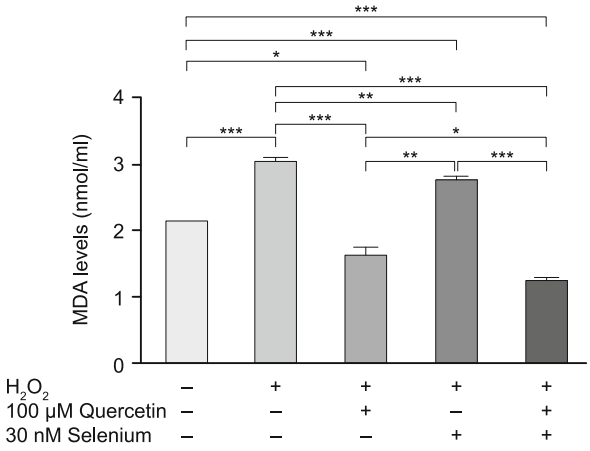

Fig. 3. MDA levels affected by quercetin and selenium in Ishikawa cell culture.

50 to 100 µM increased the cell viability percentage of Ishikawa cells after treatment with hydrogen peroxide when compared to experimental groups (H_2O_2 treatment solely). On the other hand, addition of sodium selenite in the range of 10 – 40 nM into 50 µM (~15 µg/mL) of quercetin increased cell viability up to 60 %. The following increase in the concentration of quercetin up to 100 µM in combination with sodium selenite especially at 30 nM concentration significantly protected Ishikawa cells leading to a survival of almost 90 %.

Figure 2 shows effects of UV on Ishikawa cells using MTT assay. Treatment with H_2O_2 significantly reduced cell viability of Ishikawa cells exposed to UV light. Quercetin led to an increase in cell viability when compared to H_2O_2-treated cells, and its combination with sodium selenite moderately affected it.

Lipid peroxidation levels

Figure 3 shows malondialdehyde (MDA) levels and corresponding lipid peroxidation levels affected by quercetin and selenium treatment. Hydrogen peroxide caused a significant increase in MDA levels from 2 nmol/mL to 3 nmol/mL ($p < 0.0001$), while quercetin treatment at the concentration of 100 µM significantly decreased MDA levels. The amount of 100 µM quercetin and 30 nM sodium selenite treatments reduced the MDA levels when compared to H_2O_2 group. The sodium selenite treatment, when applied as a sole agent, did not show a significant effect as shown with quercetin. However, the combination of 100 µM quercetin and 30 nM sodium selenite decreased MDA levels almost down to 1 nmol/mL, thus improving the antioxidant feature of quercetin as a sole compound.

Real-time PCR results

Quantitative PCR analysis was performed to examine the gene expression levels of BAD and p53 in response to quercetin and selenium treatments against oxidative stress caused by H_2O_2. While 300 µM H_2O_2 administration increased BAD and p53 gene expression, 100 µM quercetin and 30 nM sodium selenite co-administration decreased their expression. The expression level of Bad gene increased the chance up to 3-fold when compared to control group (1-fold). The 100 µM-quercetin treatment decreased BAD gene's expression while a more significant decrease was shown when quercetin was combined with selenium (Fig. 4A). The p53 gene expression levels were not significantly affected by quercetin or sodium selenite when applied as sole agents while the combination of quercetin and selenium significantly lowered p53 gene expression results showing a quite similar change as shown in control group (Fig. 4). BAD gene's expression levels were decreased by the quercetin+sodium selenite treatment which was increased by hydrogen peroxide treatment (Fig. 4).

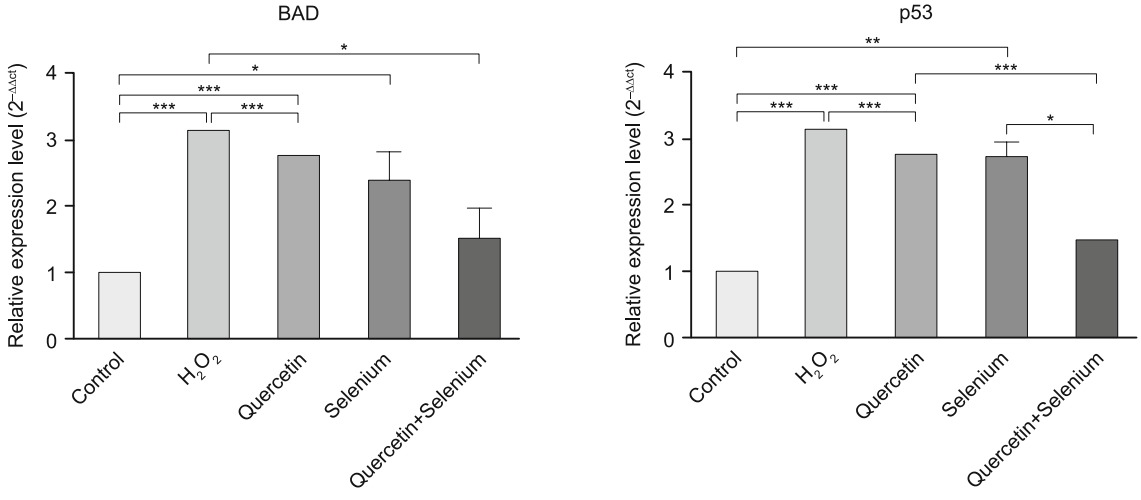

Fig. 4. Relative gene expression levels of BAD and p53 c affected by quercetin and selenium.

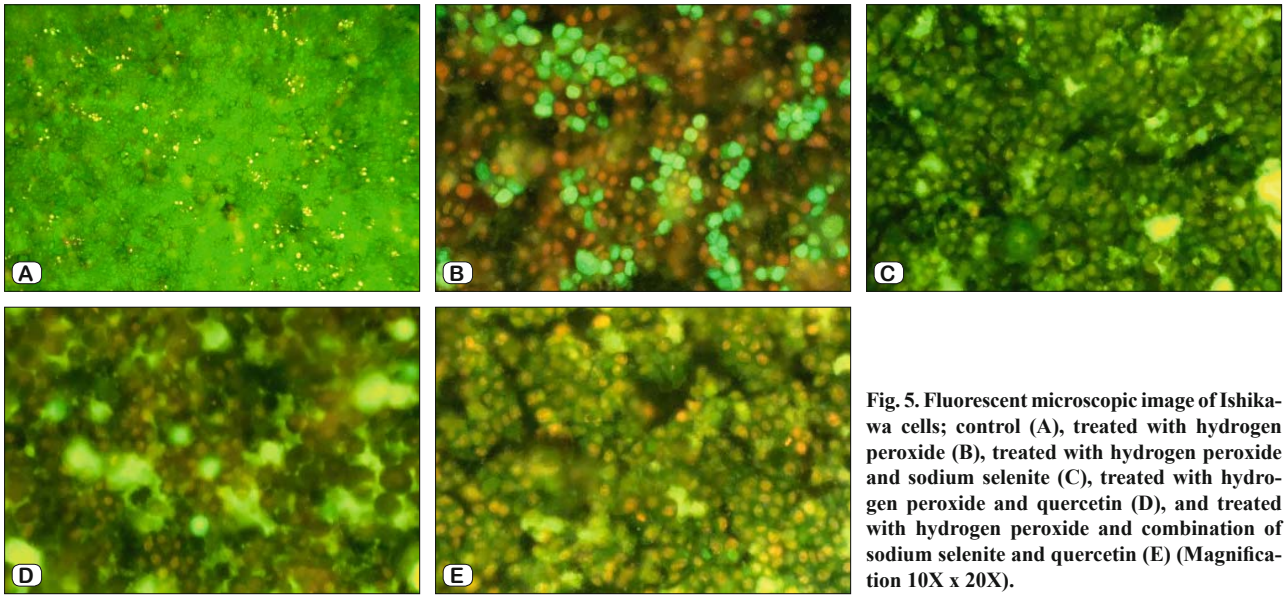

Fig. 5. Fluorescent microscopic image of Ishikawa cells; control (A), treated with hydrogen peroxide (B), treated with hydrogen peroxide and sodium selenite (C), treated with hydrogen peroxide and quercetin (D), and treated with hydrogen peroxide and combination of sodium selenite and quercetin (E) (Magnification 10X x 20X).

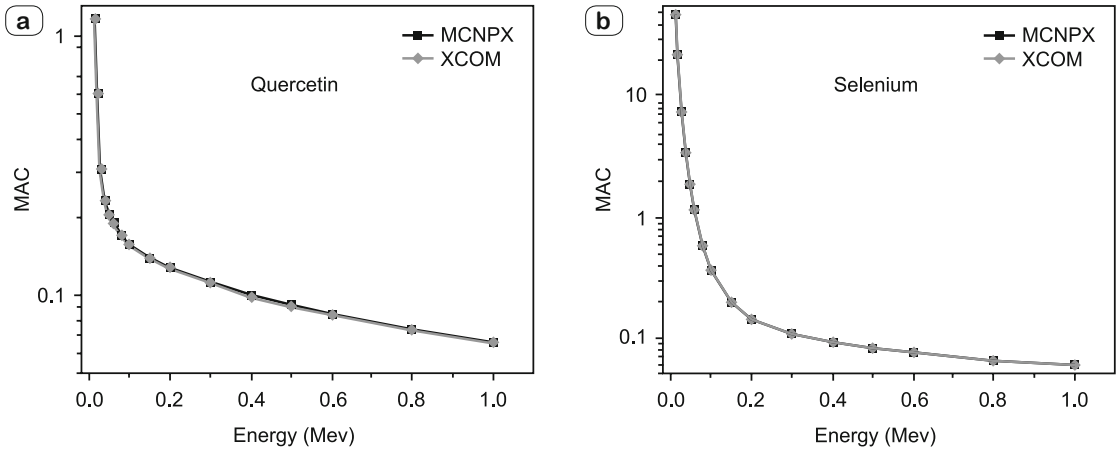

Fig. 6. Comparison of MCNPX and XCOM results for quercetin (a) and sodium selenite (b).

Fluorescent microscopic examination showed an increase in necrotic cells in Ishikawa culture treated with H_2O_2 as highlighted in red in Figure 5 while both sodium selenite and/or quercetin ameliorated the deleterious effects of H_2O_2. These results indicate that sodium selenite and/or quercetin can protect Ishikawa cells against oxidative stress caused by H_2O_2.

Radiation attenuation results

The radiation attenuation coefficients include highly significant data for the attribute of radiation attenuation. In this study, mass attenuation coefficients (μ/ρ) in values of selenium, quercetin and selenium-quercetin combinations were calculated utilizing MCNPX simulation code to validate the results with standard WinXcom data (31). The variations in mass attenuation coefficient (μ/ρ) values as a function of the photon energy for sodium selenite, quercetin, and quercetin-sodium selenite combinations are presented graphically in Figures 6 and 7. It is obvious from Figure 6 that WinXCom data are in good agreement with those of MCNPX code. Therefore, it was considered as a validated input for next calculations. Further, we have modelled 4 different quercetin+sodium selenite combinations namely QS1, QS2, QS3 and QS4 while considering their chemical compositions as well as their densities in different molar fractions (Tab. 1). The calculated

Tab. 1. 4 different quercetin+sodium selenite combinations namely QS1, QS2, QS3 and QS4.

Composition	Sample ID
99.9943 % quercetin + 0.005690 % sodium selenite	QS1
99.98858 % quercetin + 0.0114 % sodium selenite	QS2
99.98286 % quercetin + 0.0171 % sodium selenite	QS3
99.97724 % quercetin + 0.022758 % sodium selenite	QS4

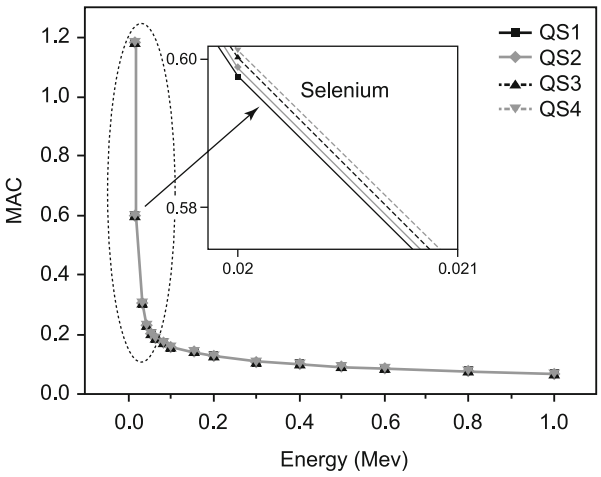

Fig. 7. Comparison of mass attenuation coefficients of quercetin-sodium selenite combinations. QS1: 100 µM+10 nM Se; QS2: 100 µM+20 nM Se; QS3: 100 µM+30 nM Se; QS4: 100 µM+40 nM Se.

(µ/ρ) values of the quercetin+sodium selenite combinations versus the photon energy using MCNPX code are presented in Figure 7.

For all combinations, the (µ/ρ) values decrease with the increasing incidents of photon energy. However, the maximum (µ/ρ) values were observed for QS4 sample. Furthermore, it is to be noted that the attenuation attitudes of each quercetin+sodium selenite combinations were observed to be similar, which is certainly due to similar elemental combinations in the sample parts. However, QS4 has the highest mass attenuation coefficients in the used photon energies. This can be explained by the higher elemental mass fraction value of selenium (Se) in QS4 sample.

Discussion

In this study, the effects of quercetin and selenium on oxidative stress caused by hydrogen peroxide in Ishikawa cell line were examined. Both antiproliferative and anti-radioactive effects of quercetin in oxidative stress-induced Ishikawa cells increase with selenium. Increasing concentration of quercetin protects Ishikawa cells after H_2O_2 treatment, and based on MTT assay results, it could be suggested that quercetin and sodium selenite may have a synergistic effect in protecting the cells against oxidative stress. Similarly, Chen et al (2018) reported that 5 µg/mL quercetin increased cell viability from 20 % up to 80 % in a model of H_2O_2-induced oxidative stress at concentration level of 1000 µM in intestinal porcine enterocyte cells (25). Quercetin and sodium selenite combination has a moderate effect on Ishikawa cells' viability exposed to UV radiation and H_2O_2 treatment. Oliviera et al (2018) reported that antioxidant compounds including quercitrin in *Nectandra hihua* ethanolic extract protects against UVB-induced oxidative stress in L929 fibroblasts (26). Stellavato et al (2018) reported that the treatment with a mixture of hyaluronic acid, minerals, amino acids, and vitamins protected the immortalized human keratinocyte cells exposed to UV-A and H_2O_2 (27). In this study, lipid peroxidation levels decreased significantly by the treatment of both quercetin and sodium selenite, while the combination of both compounds showed a synergistic affect for the protection of Ishikawa cells. Previously, decreased MDA and lipid peroxidation levels caused by a selenium polysaccharide were reported in cultured rat pheochromocytoma (PC12) cells after H_2O_2 exposure through inhibiting the oxidative stress (28). The expression levels of BAD and p53 genes were lowered by the combined treatment of quercetin and selenium showing synergistic effects in terms of gene expression. Shang et al (2018) reported that quercetin increased pro-apoptotic protein of BAD gene in human gastric cancer AGS cells (29). Synergistic protective effects of selenium in combination with other compounds such as taurine were also reported showing downregulation of p53 gene in myocardial infarction in rats (30). Muecke et al (2018) reported that selenium supplementation showed promising results concerning radioprotection in tumour patients (32). It was previously suggested that p53 stimulate BAD and promote apoptosis through cytochrome c release from mitochondria in cancer cells (33). Protective effects of selenium and quercetin with other antioxidants were previously reported in biological systems (34-39). The combination of both molecules in chemotherapy treatment may reduce the damage and accelerate the repairing process caused by chemotherapy. In conclusion, for the first time, the combination of selenium and quercetin has synergistic effects on oxidative stress in Ishikawa cells.

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