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ORIGINAL ARTICLE

Study of the Potential Radiomitigator Effect of Quercetin on Human Lymphocytes

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Abstract— Several substances of synthetic and natural origin have been studied to determine their ability to protect the body from damage caused by ionizing radiation. Among these substances, quercetin has been shown to be a naturally occurring molecule with high radioprotective and radiomitigator potential due to its antioxidant properties. The objective of this work was to ascertain the potential radiomitigator effect of quercetin on chromosome aberration yield in lymphocytes of *in vitro*-irradiated human peripheral blood. At first, the DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical capture test was performed to determine the antioxidant activity of quercetin and to select the concentrations to be tested. The blood was irradiated at doses of 2.5, 3.5, and 4.5 Gy and lymphocytes were cultured with quercetin at preselected concentrations of 37.5 and 75 μM. Then, the slides were prepared for scoring

Highlights

- Quercetin demonstrated antioxidant action against the DPPH assay
- Irradiated lymphocytes exposed to quercetin (37.5 μM) showed a reduction in the number of dicentric chromosomes
- Quercetin at the concentration of 37.5 μM reduced the expression of INF-α
- Potential radiomitigator effect of the flavonoid quercetin on human lymphocytes

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unstable chromosome aberrations (dicentrics, rings, and fragments). The results showed that the lymphocytes irradiated and later exposed to quercetin presented a lower frequency of chromosomal alterations compared to the control sample which was irradiated and not exposed to quercetin. The results suggest a potential radiomitigator effect of the flavonoid quercetin on human lymphocytes exposed, *in vitro*, to ionizing radiation. This effect may be related to decrease in the release of cytokines (INF-γ, PGE2, IL-1β, IL6, IL-8) involved in the proinflammatory processes as well as downregulation of NF-kB and reduction of expression TGF-β.

KEY WORDS: lymphocyte; metaphase; quercetin; radiomitigator.

INTRODUCTION

Radiotherapy is the treatment employed in approximately 50% of patients diagnosed with some type of cancer [1], and together with surgery and chemotherapy, it represents an effective technique for achieving remission of cancer, accounting for about 5% of the total cost of treating the disease [2]. Data in the literature point to a cure rate of 40% in patients treated with radiotherapy [3], with better control of the symptoms and a reduction of the esthetic changes caused by surgery [4].

However, despite advances in the field of radiotherapy such as the development of imaging techniques, computerized planning systems, and a better knowledge of radiobiology [2], therapeutic strategies for the protection of non-target tissues are still necessary. Unwanted exposure of healthy tissues adjacent to the treated site still poses limitations for the radiotherapist in trying to deliver the optimum dose to the tumor tissue [5]. Thus, the continuing search for new radioprotective substances and radiomitigators, which are substances that may be administered before or after the irradiation process, respectively, remains necessary [6, 7].

Other reasons for the development of new radiomitigators is the need to protect those professionals occupationally exposed to ionizing radiation, such as aircraft pilots, workers at nuclear power plants, or even astronauts during long periods of exposure to cosmic rays. The search for new substances which may protect DNA is desirable for a better management of the risks of certain human activities involving occupational exposures to ionizing radiation.

Several products have already been tested to attenuate the adverse effects of ionizing radiation, such as WR-2721 and MPG (2-mercaptopropionyl glycine), developed especially during the Cold War as a precaution against the possibility of nuclear war. However, these compounds have a short shelf-life and are commonly associated with serious side effects such as vomiting, diarrhea, nausea, hypotension, hypocalcemia, nephrotoxicity, and neurotoxicity at clinically effective doses [8–10].

Antioxidant compounds of natural origin are considered a promising alternative to synthetic radioprotectors and radiomitigators because they promote the elimination of reactive oxygen species (ROS) produced by the interaction of radiation with the water present in tissues [9, 11], besides acting on other mechanisms that cause cell damage [6], such as cytokines tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-17 (IL-17), interferon-γ (INF-γ) among others that play crucial roles during the stages of the inflammatory cascade in cells [12]. Flavonoids are extremely effective antioxidant compounds of natural origin due to their rapid action on radicals produced by lipid peroxidation, a characteristic attributed to the presence of dihydroxyl groups (catechol group) in the B ring of the basic structure of flavonoids [13, 14].

Quercetin is a flavonoid widely found in vegetables, fruits, and cereals, possessing antioxidant and anti-inflammatory activity, being beneficial in the treatment of atherosclerosis, thrombosis, hypertension, and arrhythmias [15]. In addition, studies have confirmed the efficacy of quercetin in cancer prevention because of its influence on oxidative cellular balance and control of cell cycle phases [16, 17]. In view of this, the objective of the present work was to determine, *in vitro*, the potential radiomitigator effect of quercetin on human lymphocytes exposed to ionizing radiation.

MATERIALS AND METHODS

Determination of Antioxidant Activity

In order to establish the best concentration to be used in the *in vitro* assay with human lymphocytes, the antioxidant activity of quercetin (Sigma-Aldrich) was measured by the *in vitro* photochromic method of the stable free

radical DPPH (2,2-diphenyl-1-picryl-hydrazyl) (Sigma-Aldrich) as adapted by Miliauskas *et al*. [18]. The DPPH assay is a method used to quantify the antioxidant capacity of extracts and substances isolated through the 2,2-diphenyl-1-picryl-hydrazyl free radical scavengers [19]. In this method, quercetin solutions diluted in methanol were used at concentrations of 150, 75, 37.5, 18.6, and 9.3 μM (equivalent to 50, 25, 12.5, 6.2, and 3.1 μg/mL). Next, 40 μL aliquots of the samples were added to a number of wells in a 96-well microplate (Fig. 1). Subsequently, 250 μL of DPPH (stock solution of DPPH in 0.5 mM methanol) were added. The microplate remained protected from light for 25 min before reading the absorbance at 517 nm in a spectrophotometer (Perkin Elmer 552). DPPH and methanol were used for the negative control, whereas DPPH and gallic acid (Sigma-Aldrich) were used for the positive control, and the blank had no DPPH radical addition. Finally, the readings were performed in triplicate in the spectrophotometer.

Irradiation of Human Blood

The experiments, *in vitro*, with human blood were performed according to the procedures judged and approved by the Human Ethics Committee of the Centro de Ciências da Saúde of the Universidade Federal de Pernambuco (CEP/CCS/UFPE No. 475/2011). Peripheral blood samples were collected from healthy donors after the signing of an Informed Consent Document. Venipuncture was performed at the Instituto Materno-Infantil de Pernambuco (IMIP-Recife-PE, Brazil) using sterile vacuum tubes containing sodium heparin.

Immediately after blood collection, the samples were irradiated in a linear accelerator (Siemens, Primus—energy of 6 MV and dose rate of 200 cGy/min). The blood samples were placed into a phantom hydrogel with density similar to human tissue of approximately 1 g/cm^3 and positioned in the center of the irradiation field at a distance of 100 cm between the origin of the X-ray beam and the surface of the block. The doses used were 2.5, 3.5, and 4.5 Gy, chosen according to the criteria established by the International Atomic Energy Agency [20], close to human LD_{50} (2.5–3 Gy).

After irradiation, the blood samples were kept at 37 °C for 2 h, in order to simulate an *in vivo* condition in a real scenario where there is enough time for DNA repair before blood sampling [20]. Then, the irradiated material was exposed to the flavonoid quercetin at the pre-established concentrations and blood cultures set up.

Culture of Lymphocytes

For the lymphocyte culture, flasks (25 cm^2, TPP, Switzerland) were used containing 3.96 mL of RPMI 1640 culture medium (Sigma-Aldrich) with 40 μL of the flavonoid quercetin (concentrations of 37.5 and 75 μM)

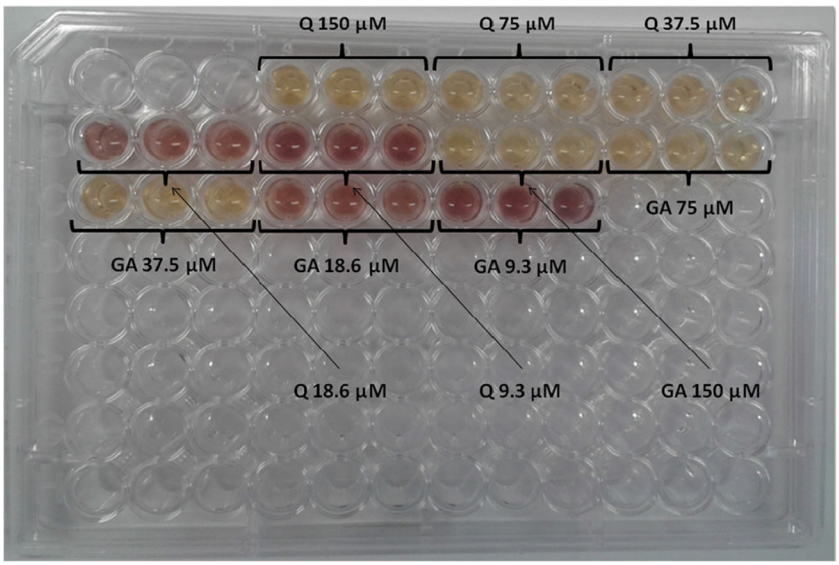

Fig. 1. Antioxidant activity of quercetin against DPPH molecules. Q indicates the samples that present quercetin at different concentrations and GA indicates the samples with gallic acid plus their respective concentrations.

(Sigma-Aldrich), 0.5 mL of fetal bovine serum (Cultilab/Campinas-SP), 0.4 mL of the whole blood sample, and 0.1 mL of phytohemagglutinin (Gibco/BRL). The cells were cultured for 48 h, with 100 μL of 2.5% colchicine (Sigma-Aldrich) added at 45 h according to the protocol of the International Atomic Energy Agency [20].

After cell culture, the samples were removed from the incubator and transferred to conical tubes and centrifuged for 6 min at 300×*g*. After centrifugation, the supernatant was removed. Subsequently, approximately 6 mL of potassium chloride (KCl) at a concentration of 0.56% *w*/*v* was added and the samples were placed in a water bath at 37 °C for 10 min, then the tubes were centrifuged for 6 min at 300×*g*. Again, the supernatant was removed and 6 mL of fixative solution consisting of methanol and acetic acid (3:1, respectively) were added. The sample was homogenized and centrifuged for 6 min at 300×*g*. This process was repeated until the bottom became clear (approximately twice), and 6 mL of fixative were added in the last process.

Preparation of the Slides

The cells were resuspended in 1 mL of fixative, and 50 μL were dropped onto the slide and dried at room temperature.

The dried slides were immersed in 5% Giemsa solution for 2 min, and dried at room temperature.

Analysis of Chromosomes

Chromosomes were analyzed using an optical microscope, with the × 10 objective to locate cells in metaphase and the × 100 objective to analyze the chromosomes. Unstable chromosomal aberrations (dicentric chromosomes and rings) were scored in 500 metaphases following the well-established criteria described in IAEA [20].

Peripheral Blood Mononuclear Cells Cultures

Peripheral blood mononuclear cells (PBMCs) (1×10^6 cell/mL) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, HEPES 10 mM (Gibco/BRL), and penicillin (10.000 U/mL)/(streptomycin 10.000 μg/mL) (Gibco/BRL). Cells were stimulated with PMA (Sigma-Aldrich) + ionomycin (Sigma-Aldrich) and treated with quercetin at concentrations of 37.5 and 75 μM. The cells were incubated for 48 h at 37 °C in humidified 5% CO_2 incubator.

Cytokine Titration

Cytokines of cultures supernatants were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. In culture supernatants from PBMCs, IFN- (BD Bioscience), IL-17A (eBiosciences), and TNF-α (BD Bioscience) were determined. Groups were identified as control (C), containing only cells, and those submitted to pro-inflammatory stimulus (PMA + iono), as well as those exposed to quercetin in two different concentrations, that is 37.5 and 75 μM (named $Q_{37.5}$ and Q_{75}, respectively). The lower limits of detection for the ELISA analyses were as follows: 4.6875 pg/mL for human IFN- , 3.90625 pg/mL for human IL-17A, and 7.8125 pg/mL for human TNF-α.

Statistical Analysis

For statistical analysis, the Student's *t* test was performed using GraphPad Prism 5.01 software. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Antioxidant Activity of Quercetin by DPPH Assay

In Fig. 1, the results are shown of the antioxidant capacity test of quercetin (Q) after interaction with DPPH radicals, where gallic acid (GA) was used as a positive control substance.

At the end of the 25-min interval, the microplates were kept in a darkroom, and the variation in the coloration of the wells containing gallic acid as well as those with quercetin may be observed. Macroscopically, the result of antioxidant activity was positive in the test against DPPH, confirmed by spectrophotometry at a wavelength of 517 nm.

The DPPH test has been widely used to investigate substances that may have an antioxidant action since this molecule has an unpaired electron in its structure, which increases its reactivity [21]. The DPPH solution demonstrates a visible dark purple color, so the antioxidant activity can be characterized by the progressive discoloration of the solution which may present a yellow tint [22].

The best antioxidant action of quercetin is shown as histograms in Fig. 2. Besides this, other assay, such as ABTS and ORAC, can be used to determine the antioxidant activity of quercetin [23–25].

The analysis of Fig. 2 showed that quercetin and gallic acid, when used at a concentration of 9.3 μM,

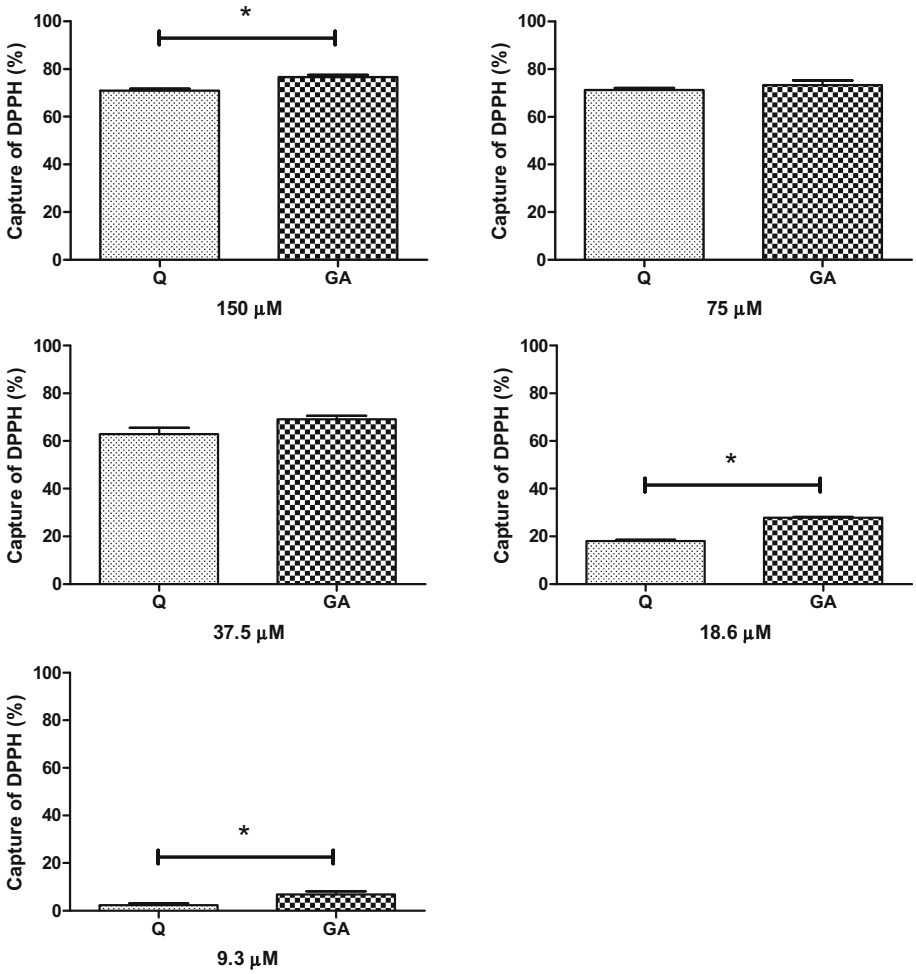

Fig. 2. A test performed with quercetin and gallic acid against the oxidative action of DPPH. The data are expressed as mean ± standard error, $p < 0.05$. Student's *t* test was used.

showed reactivity with DPPH lower than 20% and the antioxidant activity of quercetin was lower than that of gallic acid. In the samples where a concentration of 18.6 μM was used, the results showed that the percentage of reactivity to oxidative species, gallic acid, and quercetin were 27.8 and 18%, respectively. The statistical analysis showed that quercetin showed a significantly lower reactivity than that observed with gallic acid. In the samples where a concentration of 37.5 μM was tested, it was found that both gallic acid and quercetin showed a percentage of reactivity over DPPH above 60%, and no significant difference between the two was found. With regard to the samples where a concentration of 75 μM was used, quercetin and gallic acid did not show significant differences in the percentage of reactivity. For quercetin, 71.3% free radical reactivity was observed, very close to that of gallic acid, which showed reactivity of 73.3%. In the samples where the concentration of the substances tested was 150 μM, the statistical analysis showed that the samples containing gallic acid had an antioxidant action against DPPH significantly higher than that demonstrated by quercetin; however, in both cases, the percentage of reactivity was higher than 70% on oxidative species.

After analyzing the tests performed at the five quercetin concentrations, 37.5 and 75 μM were chosen to be used during the cell culture assays of lymphocytes exposed to ionizing radiation. These concentrations showed a high reactivity (both higher than 60%) against the radical DPPH, showing no significant difference in relation to the gallic acid activity. Patil *et al*. [26] observed after the DPPH assay that the required concentration of quercetin to inhibit the action of 50% of the free radical molecules was 8.09 μg/mL

(24.3 μM). This result was proportionally similar to that observed in the experiments of the present study, where a percentage higher than 60% of quercetin interaction with DPPH was obtained at 37.5 μM (12.5 μg/mL).

Devipriya *et al*. [27], in an experiment conducted *in vitro* with leukocyte cells and using quercetin at concentrations ranging from 3 to 48 μM, observed that the quercetin concentration of 24 μM produced a free radical interaction of more than 50%. Similar results were obtained in experiments at the 37.5 μM concentration in the present study.

In addition, studies conducted by Dias *et al*. [28] evaluated the effect of quercetin on oxidative stress, *in vivo*, observing that diabetic rats receiving quercetin presented a significant decrease in the production of reactive oxygen species.

Radiomitigator Activity of Quercetin

To evaluate the radiomitigator action of quercetin, blood samples were obtained by venous puncture, where one sample was not irradiated (control) and the other was divided into aliquots and *in vitro* irradiated with the absorbed doses of 2.5, 3.5, and 4.5 Gy of high-energy X-rays (LINAC 6MV) (Fig. 3). No noticeable changes in lymphocyte morphology were seen under the light microscope despite the doses of radiation used (Fig. 3).

After cell culture to obtain metaphases, all the samples that had been treated with quercetin at the 75 μM concentration, irrespective of the dose of ionizing radiation received, did not contain metaphases, showing that this concentration is toxic. In the non-irradiated control sample (C), no chromosomal aberrations were observed, such as dicentric and ring-shaped chromosomes. In relation to the control samples irradiated at doses of 2.5 Gy (C_I) and 3.5 Gy (C_{II}) (Table 1), an unexpected decrease of dicentrics was observed from C_I to C_{II}, possibly due to the Poisson distribution of damage among cells, what may be the cause of this statistical fluctuation. However, in the samples irradiated (Table 1) at the highest dose (4.5 Gy–C_{III}), a higher yield of chromosomal aberrations was observed compared to the other samples.

During scoring, it was possible to observe a greater number of dicentric chromosomes (Fig. 4c, d) in relation to ring-shaped chromosomes, regardless of the use of quercetin, between the control samples submitted to radiation and the irradiated samples exposed to quercetin. Moreover, the possibility of two breaks occurring on a chromosome giving a ring-shaped is less than they occur on distinct chromosomes forming a dicentric chromosome. These chromosomal changes are triggered by the interaction of ionizing radiation with the DNA molecule and are characterized by the breakage of the double helix or even inadequate repair of the molecule, where breaks in two different chromosomes can produce a dicentric chromosome, characterized by the presence of two centromeres, accompanied by an acentric fragment. When the same chromosome suffers two breaks, the presence of ring-shaped chromosomes can be observed, also accompanied by an acentric fragment (Fig. 4e, f) [29–31].

Table 1 shows the aberration yields from the samples irradiated at the doses of 2.5, 3.5, and 4.5 Gy in the absence

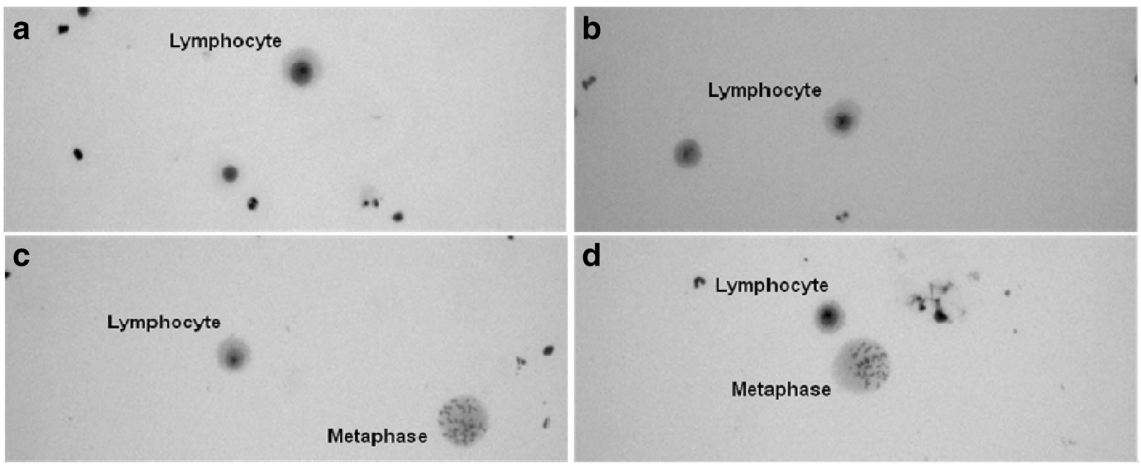

Fig. 3. Photographic images of lymphocyte morphology (× 400). **a** Non-irradiated. **b**, **c** Subjected to radiation in the absence of quercetin. **d** Irradiated and exposed to quercetin.

Table 1. A number of Chromosomal Alterations

Groups	Metaphases	Dicentrics	Rings
C	500	0	0
C_I	500	46	1
C_{II}	500	39	9
C_{III}	500	102	8
Q_I	500	14	0
Q_{II}	500	26	1
Q_{III}	500	109	8

C control, *Q* quercetin

(C_I, C_{II}, and C_{III}) and presence (Q_I, Q_{II}, and Q_{III}) of quercetin at a concentration of 37.5 μM and the control sample irradiated alone (C). Sample Q_I presented a decreased of 69.6% in the frequency of dicentric chromosomes compared to the irradiated sample without quercetin (C_I). It can also be observed that, in the C_I sample, a ring-shaped chromosome was found. The data from the comparison pairs Q_{II} and C_{II} show a similar trend in reduction (33%) in aberration frequencies in the sample treated with quercetin. However, no reduction was noted at the highest radiation dose, Q_{III} and C_{III}, where the aberration frequency was slightly, but statistically insignificantly, higher in the sample with quercetin.

In order to confirm the radiomitigator effect of quercetin, in reducing the inflammatory process, pro-inflammatory cytokines were measured after *in vitro*

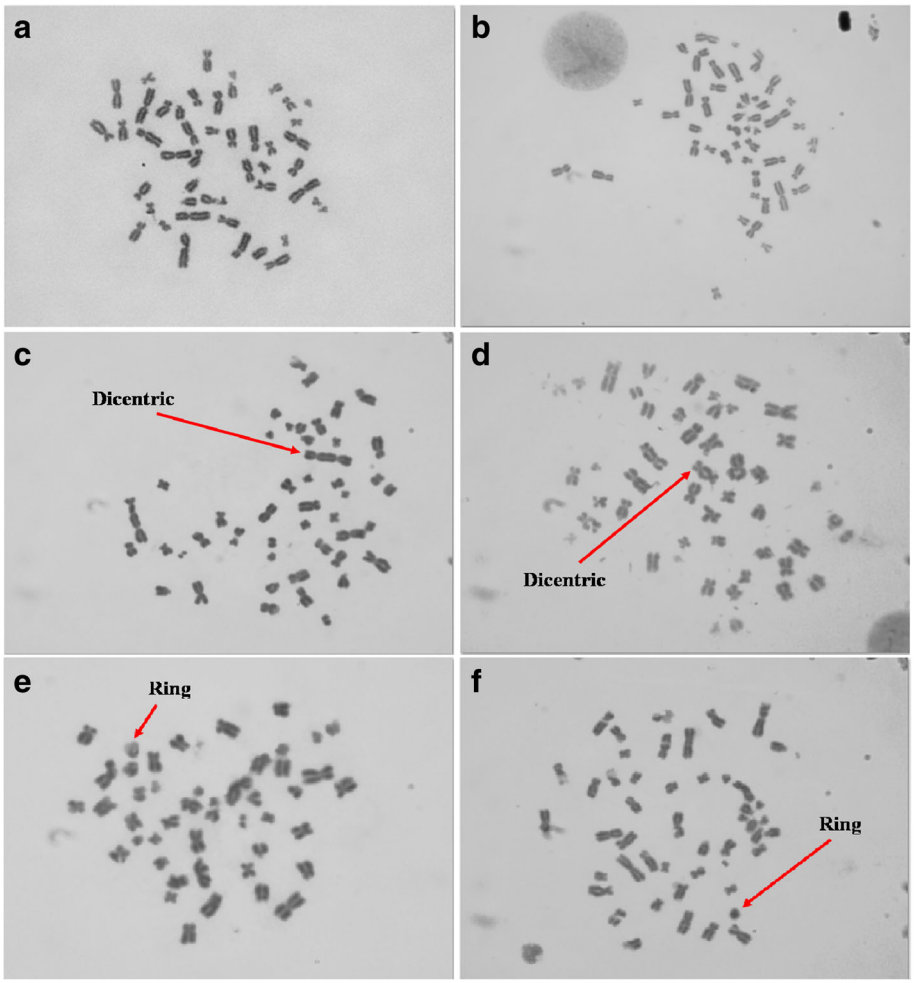

Fig. 4. Metaphases observed under the optical microscope (× 1000). In images **a**, **b** (both non-irradiated control), two normal metaphases are observed; in **c**, **d** (respectively, C_{III} and Q_{III}), two dicentric chromosomes can be seen; images **e**, **f** (respectively, C_{III} and Q_{III}) present two chromosomal aberrations in ring form.

irradiation (dose of 3.5 Gy) of peripheral lymphocytes (Fig. 5). The quantification of cytokines showed that for interleukin-17 (IL-17), there was no significant reduction ($Q_{37.5}$) in its expression when compared to the group of cells that received the pro-inflammatory stimulus (PMA + iono). Also, the dosage of tumor necrosis factor alpha (TNF-α) showed no reduction with the addition of quercetin when compared to cells which were exposed to PMA + iono. However, the expression of interferon-γ (IFN-γ) showed a significant decrease ($p < 0.001$) in the cells submitted to $Q_{37.5}$ and PMA + iono when compared to the group exposed only to PMA + iono.

The results showed that quercetin at the 37.5 μM concentration showed a radiomitigator effect for reducing the yield of dicentric chromosomes after irradiation. This radiomitigator effect may be related to the decrease in the release of INF-γ as demonstrated in Fig. 5; furthermore, other cytokines may have decreased expression, such as prostaglandin E2 (PGE2), interleukin-1 beta (IL-1β), and interleukin-8 (IL-8), all cytokines involved in the proinflammatory processes as well as downregulation of nuclear factor kappa B (NF-kB) and reduction of expression transforming growth factor-beta (TGF-β) [32]. In addition, studies on chrysin, a type of flavonoid that has a quercetin-like molecular structure, have been shown to evaluate immunoregulatory and anti-inflammatory properties that have been shown to reduce serum levels of TNF-α, IL-1β interleukin-17 (IL-17), and INF-γ [33]. However, Silva *et al.* [34] demonstrated that the extract of *Macrosiphonia longiflora* (rich in flavonoids such as quercetin) has a potent anti-inflammatory activity in both the *in vivo* and *in vitro* models of acute inflammation. The anti-inflammatory effect is partly related to the inhibition of IL-1β, IL-10, and nitric oxide releases, but independent of TNF-α and IL-17 modulation [34].

A possible explanation for the lack of the mitigation effect from quercetin seen at 4.5 Gy may be found in the work of Alghamian *et al.* [35] who used an irradiated human astrocytoma cell line. We suggest that the effect of ionizing radiation at the highest dose used in the present work may be related to cell cycle arrest at G2/M, consequently inhibiting cell proliferation as well as the formation of metaphases. This would agree with the idea that quercetin influences the apoptotic processes that select out damaged cells at cell cycle checkpoints. Peng *et al.* [36], after exposure of mice to quercetin, observed a significantly

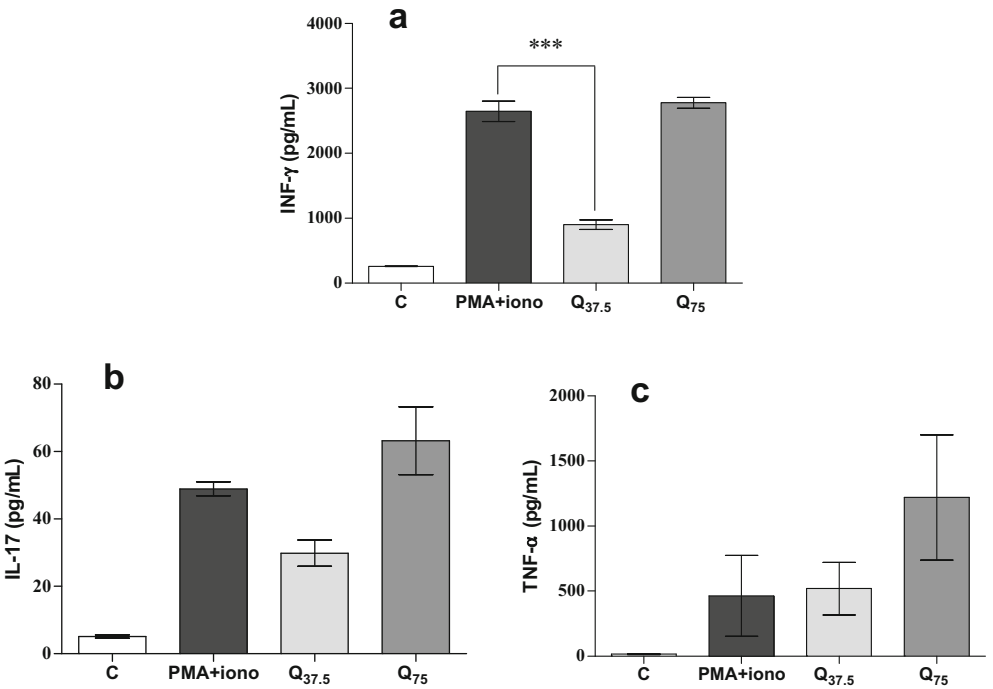

Fig. 5. Expression of INF-γ (**a**), IL-17 (**b**), and TNF-α (**c**), in irradiated cells also exposed to PMA + iono, and to PMA + iono together with quercetin in two different concentrations ($Q_{37.5}$ and Q_{75}). Results were expressed as mean ± standard error of the mean. Differences were considered significant when $p < 0.05$ (***$p < 0.001$).

diminished production of proinflammatory cytokines, including TNF-α, interleukin-6 (IL-6), and IL-1β, and inhibition of the activation of the NF-κB and mitogen-activated protein kinases (MAPK) signaling pathways as well as the expression of apoptotic proteins. Furthermore, in a study conducted to evaluate the potential anti-inflammatory action of the *Scutia buxifolia Reissek* stem barks extract, it was observed that the compound acted reducing the levels of IL-1, IL-6, TNF-α, and INF-γ in mice and that this effect is possibly associated with the presence of some components, such as quercetin, gallic acid, and rutin [37].

Additional evidence comes from work with macrophages exposed to quercetin-loaded silica nanoparticles that demonstrated a significant reduction in the number of proinflammatory cytokines, such as IL-1 β, IL-6, and TNF-α [38].

Another study by Yu *et al*. [39] analyzed the suppressive effect of quercetin on interleukin-2 (IL-2) and IFN-γ, noting that when it is used at a concentration of 20 and 40 μM, a significant inhibition of cytokines was observed compared to the control sample.

Studies in neural cells showed that quercetin significantly induced the activation of cell survival kinases, such as Akt (protein kinase) and protein kinase D1 (PKD1). In addition, it has been found that PKD1 acts as an upstream regulator of Akt in quercetin-mediated neuroprotective signaling [40]. Moreover, studies conducted by Tangsaengvit *et al*. [41], with neural cells submitted to quercetin, showed a neuroprotective effect through inhibition of cholinesterase and clearance of free radicals.

In tests done with rats exposed to ionizing radiation for induction of cutaneous lesions and fed with quercetin supplementation, it was demonstrated that the flavonoid reduced the expression of TGF-β in fibroblasts. Thus, quercetin was capable of mitigating radiation-induced skin fibrosis [42].

In experiments performed with macrophages in culture submitted to a lipopolysaccharide of *Escherichia coli* O111:B4 and exposed to quercetin, inhibition was found in the production of proinflammatory mediators, such as inducible nitric oxide synthase (iNOS)-mediated NO, cyclooxygenase-2 (COX-2), and cytokines (IL-1β, IL-6, and TNF-α) as well as the expression of cell surface molecules including CD80, CD86, and major histocompatibility complex (MHC) classes I/II protein [43]. In addition, the authors suggested that quercetin may also influence the mechanisms involved in the inhibited MAPK and NF-κB signaling activation [37, 43].

Therefore, the present research has shown evidence that quercetin is a potent radiomitigator of the effects of ionizing radiation, in terms of reducing the yield of dicentrics, but this was only observed in the cases of doses lower than 4.5 Gy.

CONCLUSION

In the present study, quercetin presented, *in vitro*, a radiomitigator effect against ionizing radiation chromosomal damage in human peripheral lymphocytes. This effect is possibly related to a decrease in the production and release of proinflammatory cytokines as well as inhibition of activation of the inflammatory signaling pathways.

COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest. The authors declare that they have no conflict of interest.

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