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Comparison between daidzein and genistein antioxidant activity in primary and cancer lymphocytes

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Abstract

The main objective of this study was to compare the protective effect of daidzein and genistein against induced oxidative damage in Jurkat T-cell line and in peripheral blood lymphocytes of healthy subjects. After supplementation of cells with isoflavones (from 2.5 to 20 μmol/L in Jurkat T-cell and from 0.01 to 2.5 μmol/L in primary lymphocytes, 24 h), we determined DNA damage induced by hydrogen peroxide using the comet assay and lipid peroxidation evaluating malondialdehyde (MDA) production after ferrous ion treatment. Supplementation of Jurkat cells and primary lymphocytes with both isoflavones significantly increased DNA protection from oxidative damage at concentrations between 0.1 and 5 μmol/L ($P < 0.05$), and with just daidzein, at concentrations higher than 2.5 μmol/L, there was a decrease in the production of MDA ($P < 0.05$). Our results seem to support that daidzein is just as effective as genistein in protecting cells against oxidative damage especially with respect to DNA. Moreover, since the protective effect was found at concentrations reachable in plasma after soy consumption (less than 2 μmol/L), it can be assumed that the antioxidant activity of isoflavones could really contribute to the healthy properties of soy.

Keywords: Isoflavones; Lipid peroxidation; DNA damage; Jurkat T cells; Peripheral blood lymphocytes

Epidemiological studies have shown that a regular intake of soy foods is associated with a reduced risk of several chronic pathologies, such as coronary heart disease, cancer, and osteoporosis [1]. Different hypotheses have been suggested to explain these health benefits, such as the involvement of the two major isoflavones in soy foods, genistein and daidzein, whose different biological activities have been documented in vitro and in vivo studies. For example, it was demonstrated that genistein, and to a much lesser extent daidzein, inhibits tyrosine kinase [2–4], and malignant angiogenesis at physiological concentrations in vitro [5]. In addition, genistein was demonstrated to prevent activation of the redox-sensitive transcription factor, NF-κB in cancer cells in vitro [6], and in human blood lymphocytes [7]. This isoflavone also inhibits DNA topoisomerase I and II [8,9], and ribosomal S6 kinase [10], which may lead to protein-linked DNA strand breaks. Since it is believed that anticancer [11], anti-inflammatory [12], cardioprotective [13], and enzyme-inhibitory [9] effects of isoflavones might be related to their antioxidant activities, several studies have been undertaken to evaluate these properties. However, most of the research on isoflavones' antioxidant activity has been focused on genistein [14,15]. For example, it has been demonstrated that genistein inhibits hydrogen peroxide (H_2O_2) production due to tumour promoter in mouse skin cells [16] and the formation of 8-hydroxy-2′-deoxyguanosine following UV light irradiation [17,18]. Record et al. [19] in their in vitro study provided evidence that genistein is an effective scavenger of H_2O_2 but it is less effective against other peroxidative systems. Recent studies have considered the radical scavenging activity of isoflavones [19,20] demonstrating that genistein is more effective than daidzein in inhibiting oxidation of β-carotene linoleate in vitro [21], while

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significant inhibition of lipoprotein oxidation was found only for daidzein metabolites [15].

Daidzein was specifically studied in relation to the estrogenic property of its main mammalian metabolite, equol [22]. However, the high bioavailability of daidzein and the resulting reliable plasma concentration of this isoflavone [23–25] could suggest the occurrence of biological and antioxidant activities of daidzein independently from its conversion in equol.

The aim of this study was to compare the protective effects of daidzein with respect to those of genistein against oxidative damage to DNA and lipid membranes in Jurkat T cells and in primary human lymphocytes subjected ex vivo to oxidative stress.

Materials and methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) and Merck (Merck KgaA, Darmstadt, Germany). Standards of isoflavones (genistein and daidzein) were obtained from Extrasynthèse (Genay, France).

Cells

Immortalised and primary lymphocytes were used as cellular models. In fact lymphocytes, being involved in immune response, are normally subjected in vivo to oxidative stress [26–28]. Moreover, since lymphocytes are exposed immediately to variations of antioxidants in blood due to modification of dietary habits, they represent a reliable cellular line to study the effect of dietary antioxidants on cell protection [27,28].

Jurkat cell line

The human leukaemia T-cell line (Jurkat, clone E6-1), used as cellular model, was purchased from American tissue culture collection (ATCC) (Rockville, MD). Jurkat T cells were considered because they have membranes with markers similar to normal lymphocytes [29].

Peripheral blood lymphocyte isolation

Human lymphocytes were isolated from the blood of four healthy subjects from the Blood Donors Center of the Istituto Nazionale Per lo Studio e la Cura dei Tumori. Blood was collected into tubes containing Histopaque-1077 and centrifuged at 400*g* at room temperature for 30 min. Cells, recovered from the gradient, were washed twice with phosphate-buffered saline (PBS) and suspended in culture medium.

Cell handling

Both Jurkat and primary lymphocytes were cultured in RPMI 1640 medium containing 100 ml/L heat inactivated newborn calf serum, 2 mmol/L L-glutamine, 1×10^5 IU/L penicillin, and 100 mg/L streptomycin. Moreover, 4 μg/ml phytohemoagglutinin (PHA) as mitogen was added to primary lymphocyte flasks.

Lymphocytes were grown in 275 ml flasks at 37 °C in a humidified atmosphere of 5% carbon dioxide/95% air and the medium was changed every 48 h, adjusting the cell number to 5×10^5 cells/ml after hemocytometer counts.

Experimental design

We first compared the effect of daidzein and genistein on Jurkat cells by using concentrations ranging from 2.5 to 20 μmol/L. Afterwards, trying to study a more physiological condition, we further confronted the protective effect of daidzein and genistein against oxidative damage in human primary lymphocytes supplemented with low concentrations of isoflavones (ranging between 0.01 and 2.5 μmol/L). In both trials, the DNA oxidative damage and membrane lipid peroxidation in control and supplemented cells were measured by the comet assay and the quantification of MDA[1] production, respectively.

Isoflavone supplementation

Genistein and daidzein were dissolved in tetrahydrofuran (THF) stabilised with 0.025% butylated hydroxytoluene (BHT) and added separately to the cell cultures to reach 2.5, 5, 10, and 20 μmol/L in Jurkat cells and 0.01, 0.05, 0.1, 0.25, 0.5, 1, and 2.5 μmol/L in primary lymphocytes. Control cells were similarly treated with the same amount of THF stabilised with 0.025% BHT (0-THF group). The supplementation was carried out at 37 °C in a humidified atmosphere of 5% carbon dioxide/95% air for 24 h in triplicate.

Oxidative treatments

After supplementation, the cells, washed with PBS, were embedded in agarose gel (see determination) and treated with 500 μmol/L H_2O_2 in PBS for 5 min to induce DNA oxidative damage [30]. To induce oxidative lipid damage, 100 μmol/L of ferrous ions (as $FeSO_4 \cdot 7H_2O$) in PBS for 15 min was used. Treated cells were then counted, centrifuged, resuspended in PBS, and stored at −80 °C for further analysis. We chose these concentrations of Fe^{2+} and H_2O_2 to produce detectable oxidative damage consistent with cell integrity as previously dem-

[1] *Abbreviation used:* D, daidzein; G, genistein; MDA, malondialdehyde.

onstrated [30,31]. Both H_2O_2 and Fe^{2+} are cause of oxidative stress to cells by means of hydroxyl radical production. In particular, H_2O_2 was chosen as oxidant specie to induce DNA oxidative damage because preliminary tests demonstrated that its oxidative efficiency on DNA is greater than Fe^{2+}, independently from the concentration, and the resulting damage is generally reproducible and quantifiable [30]. Since H_2O_2 is not a charged molecule and can pass through the phospholipids of cell membrane [32], it is believed that it generates, by Fenton reaction, OH^- radicals responsible for most of the injuries that take the form of strand breaks and oxidised bases. On the contrary, H_2O_2 even when used within a wide range of concentrations does not seem to induce MDA production [31], therefore it seems not to cause oxidative damage to lipids. It has been suggested that most of the lipid peroxidation observed in vivo is metal ion dependent, often involving iron and sometimes copper. The metal binding to the fatty acids of the cell membranes can promote lipid peroxide decomposition to chain-propagating alkoxyl radicals and the hydroxyl radical production by Fenton reaction. Thus, the evaluation of the ability to inhibit lipid peroxidation can be made by testing an antioxidant against metal ion stimulated lipid peroxidation in biological membranes [33].

Cytotoxicity test

The cytotoxicity of supplementation and oxidative treatments was assessed on cells by trypan blue exclusion assay [34]. The concentrations of isoflavones were selected to avoid any possible interference on cell viability.

Determination of DNA damage by comet assay

We quantified DNA damage with the single cell gel electrophoresis or comet assay that evaluates specifically single strand breaks and alkaline-labile sites in any eucaryotic cells. The assay was applied as previously described [35]. Typically, 50 cells for each slide were electronically captured at random and analysed for fluorescence intensity with a comet analysis program supported by the image processing environment Visilog 4 (Noesis, Orsay, Cedex, France). DNA damage was expressed as % DNA in tail.

Determination of MDA

MDA was determined as previously reported by Erba et al. [31] on 10 ml of cell suspensions (5×10^5 cells/ml). After supplementation and Fe^{2+} treatments, cells were centrifuged, resuspended in 1 ml PBS, and sonicated at 40% power for 20 s 5 cycle (Sonopuls, Bandelin, Berlin, Germany). Afterwards, 400 μl of the sample was added with 400 μl of 2% H_3PO_4 and 200 μl thiobarbituric acid (TBA 41.6 mmol/L). This solution was boiled for 1 h and cooled for 2–3 min, then 500 μl of a 1:12 mixture of 2 N NaOH/MeOH was added; the resulting adduct ($MDA\text{-}TBA_2$) was separated in HPLC. The HPLC analysis was performed using a model 501 pump (waters) equipped with a Rheodyne injector (loop 20 μl) connected with a UV/Vis detector model 486 (waters) and chromatograms were analysed by a software Azur 3.0 (Datalis, Saint Martin d'Heres, France). Separation of MDA was obtained on YMC-pack ODS-AQ, 5 μm (250 × 4.6 mm) column (YMC, Kyoto, Japan) eluted isocratically at 0.5 ml/min with a 60:40 mixture of 10 mmol/L phosphate buffer, pH 6.8/methanol.

The standards of MDA were prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane as reported by Esterbauer et al. [36]. A calibration curve was used to calculate MDA concentration, and results were expressed as nanogram MDA/10^6 cells.

Statistical analysis

Statistical analyses were performed on a personal computer using the Statistical Software (Stat Soft, Tulsa, OK). A repeated measures ANOVA design with treatment as independent factor was used to investigate the effect of the different experimental conditions. For each analysis, the treatments were as follows: control (0-THF), control + oxidation (0-THFox), supplementation with isoflavone (suppl), supplementation + oxidation (suppl + ox). Differences between means were further evaluated by the least significant differences test and P values <0.05 were considered as significant. Data are expressed as means ± SD.

Results

Cytotoxicity test

The supplementation with different concentrations of isoflavones and the oxidative treatments were not cytotoxic and did not affect significantly cell morphology. The treatment of cells with THF (0-THF group) in the same amount used to solubilize the isoflavones did not produce any effect compared with control cells without THF (0 group).

DNA damage

Table 1 shows the effect of isoflavone supplementation on DNA damage in Jurkat T cells. Percentage DNA in tail (means ± SD) was not significantly affected by the type of treatment (from 2.5 to 20 μmol/L). H_2O_2-induced oxidative stress significantly increased the level of DNA strand breaks in control cells ($P < 0.05$). After oxidation, the cells supplemented with daidzein and genistein

Table 1
Values of DNA damage and MDA production in control and supplemented Jurkat T cells (24 h incubation) with and without oxidative treatment (see Materials and methods)

	DNA (% DNA in tail)		MDA (ng/10e6 cells)	
	C	C + ox	C	C + ox
Control (μmol/L)				
0	5.0 ± 0.3	61.9 ± 4.3	26.6 ± 6.2	93.2 ± 0.3
0-THF	6.0 ± 0.9	64.0 ± 3.2	28.8 ± 2.0	89.7 ± 2.4
	Suppl.	Suppl. + ox	Suppl.	Suppl. + ox
Daidzein (μmol/L)				
2.5	4.0 ± 0.5	42.0 ± 7.2[a]	23.4 ± 1.9	62.3 ± 11.6[a]
5	3.6 ± 0.3	41.0 ± 2.5[a]	21.5 ± 2.0	54.4 ± 11.1[a]
10	5.0 ± 0.8	57.0 ± 9.8	22.7 ± 2.1	51.4 ± 10.4[a]
20	6.0 ± 0.3	55.0 ± 3.9	28.1 ± 2.3	56.8 ± 11.3[a]
Genistein (μmol/L)				
2.5	5.0 ± 0.4	39.0 ± 0.1[a]	25.2 ± 2.3	80.2 ± 8.5
5	7.0 ± 0.6	47.0 ± 2.3[a]	24.8 ± 2.7	87.7 ± 1.2
10	7.0 ± 0.5	59.0 ± 1.4	24.9 ± 1.9	73.1 ± 14.5
20	3.0 ± 0.3	56.0 ± 2.8	25.0 ± 2.9	90.3 ± 9.4

Values are reported as means ± SD ($n = 3$ independent experiments performed).
[a] Different from 0-THF control cells ($P < 0.05$).

showed a lower level of DNA damage compared to 0-THFox cells at concentrations of 2.5 and 5 μmol/L ($P < 0.05$). The reduction of DNA strand breaks with respect to 0-THFox was about 39 and 27% after 2.5 and 5 μmol/L genistein and about 34 and 36% after 2.5 and 5 μmol/L daidzein supplementation, respectively. With regard to the effect of isoflavone supplementation on oxidatively induced DNA damage in human primary lymphocytes, DNA strand breakage in control and supplemented cells was not significantly different (Fig. 1), while hydrogen peroxide-mediated oxidative stress significantly increased DNA damage in control cells

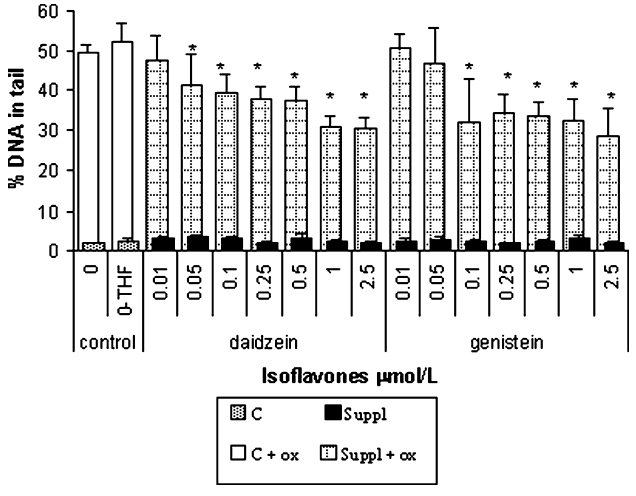

Fig. 1. DNA damage evaluated by the comet assay and expressed as % DNA in tail in supplemented (suppl) and oxidised (ox) primary lymphocytes (H_2O_2, 500 μmol/L for 5 min) after supplementation with different concentrations of isoflavones. Values are means ± SD, $n = 3$ independent experiments performed. Different from 0-THF control cells ($^*P < 0.05$).

($P < 0.05$). After oxidation, the cells supplemented with genistein at concentrations between 0.1 and 2.5 μmol/L showed a lower level of DNA damage compared to control cells ($P < 0.05$). DNA damage reduction with respect to 0-THFox was about 45% after 2.5 μmol/L, 33 and 38% after 0.1 and 0.25 μmol/L, and 35 and 37% after 0.5 and 1 μmol/L, respectively. The doses 0.01 and 0.05 μmol/L did not show any protective effect even if a reduction was noted.

Concerning the supplementation with daidzein to primary lymphocytes, we observed that this isoflavone exerted a significant protective effect against DNA oxidative damage at concentrations included between 0.05 and 2.5 μmol/L ($P < 0.05$). The decrease of oxidative injury was about 20% for 0.05 μmol/L, 24% for 0.1 μmol/L, 27% for 0.25 μmol/L, 28% for 0.5 μmol/L, 40% for 1 μmol/L, and finally 41% for 2.5 μmol/L with respect to 0-THFox. At 0.01 μmol/L, the protection against DNA damage was not significant.

MDA production

In Table 1 are presented the amounts of MDA produced after oxidative treatment in supplemented and control Jurkat T cells. The use of THF did not affect MDA production with respect to control cells (0-THF vs 0 group). Genistein and daidzein supplementation did not produce any significant increase of MDA concentration compared to 0-THF control cells. After oxidative treatment, MDA production was not significantly different in cells supplemented with genistein with respect to control cells (0-THFox). On the contrary, lower levels of MDA were found in cells supplemented with daidzein ($P < 0.05$). The reduction of MDA production with

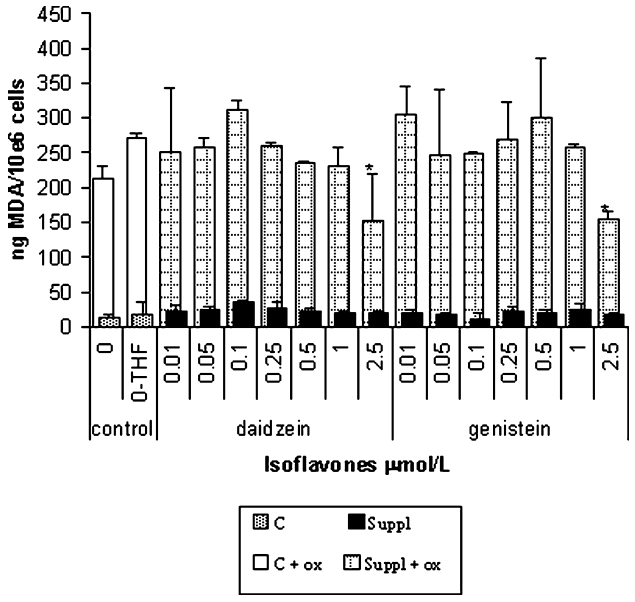

Fig. 2. Malondialdehyde (MDA) production, expressed as ngMDA/10^6 cells, in supplemented (suppl) and oxidised (ox) primary lymphocytes (Fe^{2+}, 100 µmol/L for 15 min) after supplementation with different concentrations of isoflavones. Values are means ± SD, $n = 3$ independent experiments performed. Different from 0-THF control cells ($^*P < 0.05$).

respect to 0-THFox was about 30 and 39% after the concentration of 2.5 and 5 µmol/L, and about 43 and 37% after the concentration of 10 and 20 µmol/L, respectively.

Concerning the effect of isoflavone supplementation on lipid peroxidation in human primary lymphocytes, MDA analysis showed that genistein and daidzein supplementation did not produce any significant increase of MDA concentration compared to control cells as shown in Fig. 2. On the contrary, after oxidative treatment MDA concentration was significantly lower ($P < 0.05$) only in cells supplemented with daidzein or genistein 2.5 µmol/L with respect to oxidized control cells (0-THFox). The reduction of MDA production with respect to 0-THFox was about 43% for genistein and 44% for daidzein. The other concentrations of daidzein and genistein did not decrease significantly MDA production although slight reductions were found.

Discussion

In this study, we investigated and compared the protective effects of daidzein and genistein supplementation against oxidative damage in Jurkat T cells and primary lymphocytes. We first supplemented Jurkat cells with isoflavones using a relatively wide range of concentrations (from 2.5 to 20 µmol/L) to test the effect of supplementation both on cell viability and the antioxidant activity of daidzein and genistein. Afterwards, since our results on Jurkat cells showed that the concentrations able to protect the cells from oxidative damage were the lower doses and trying to study a more physiological condition, we further used a cellular model consisting of human primary lymphocytes, supplemented with low and physiological concentrations (ranging between 0.01 and 2.5 µmol/L). Isoflavones are present in plasma especially as glucuronides and sulphates. However, about 20–30% of circulating isoflavones are in their unconjugated forms [37]. In our study, we used the aglycones to supplement primary and cancer cells. On the whole, our study supports the hypothesis that, not only genistein but also daidzein at physiological concentrations, is able to increase cell protection against oxidative stress, as demonstrated by the decrease of oxidative damage to DNA and lipid membranes.

About these biomarkers we evaluated the protective effects of isoflavones from damage induced by H_2O_2 and iron ions, two of the main oxidant species responsible for in vivo oxidative stress. In the Fenton reaction, reducing metal ions interact with H_2O_2 to produce hydroxyl radicals, which are the most reactive oxidant in the chain of free radical reactions that leads to tissue injury [38]. Challenge with iron ions showed different damaging effect on lipids and DNA. In particular, the degree of DNA damage due to iron ions was lower than that generally produced by H_2O_2 treatment [30]. This observation may be explained by considering that H_2O_2 can easily diffuse across cell membranes while iron ions cannot. Further, hydroxyl radicals react so rapidly that they do not diffuse more than a few angstroms from where they are generated [38]. However, many different oxidant species produced by lipid peroxidation are able to reach the nucleus. For example, it has been reported that lipid peroxidation products, particularly MDA, can induce DNA damage [39].

In the present study, treatment with H_2O_2 caused significant DNA damage in control cells. On the contrary, lymphocytes previously supplemented with daidzein or genistein (0.1–5 µmol/L) were protected from oxidative injury. The antioxidant activity of genistein with respect to DNA damage has been previously investigated: at doses lower than 50 µmol/L, it has been reported that genistein inhibits carcinogenesis induced by H_2O_2 and protects DNA against UV light and Fenton reaction induced oxidative damage [17]. On the contrary, at concentrations higher than the physiological range(that is less than 2 µmol/L), the protective activity is lost or the H_2O_2-induced DNA strand breaks is increased [40,41]. Pro-oxidant effects have been reported for very high concentration, also genotoxic effects of isoflavones have been reported for concentrations higher than those reachable in plasma following soy intake [40]. In accordance with studies present in the literature, data obtained with Jurkat cells showed that the DNA protective effect of genistein is lost at concentrations higher than 10 µmol/L. The effect of daidzein does not seem to

be different from that of genistein since the DNA oxidative damage was not different from control cells after 10 and 20 μmol/L of daidzein. On the other hand, the protective effect of daidzein against DNA oxidative damage in primary lymphocytes seems even more effective with respect to genistein taking into account that daidzein decreased DNA damage even at the lowest concentration used in this study (0.05 μmol/L) while genistein did not show any effect. This could be ascribed to the molecular structure of daidzein that may have affected bioavailability of this compound to cells or alternatively there might be a higher biological activity of daidzein with respect to genistein.

There are few studies on the protective effect of isoflavones on lipid peroxidation at physiological concentrations, whereas the effects of high doses have been reported [42]. The protective mechanism of isoflavones on lipid peroxidation was studied by Tikannen and Adlercreutz [43] on LDL artificially enriched with genistein and daidzein: LDL modified in this way became less sensitive to oxidative damage than native LDL. However, Kerry and Abbey [44] did not demonstrate this incorporation in vivo. Our results showed that the supplementation with genistein did not improve protection of Jurkat T cells from Fe^{2+} injury. Also, in primary lymphocytes the supplementation with this isoflavone did not modify MDA production except for the highest dose used (2.5 μmol/L). This different antioxidant activity of genistein at 2.5 μmol/L could be related to the cellular model, or dependent on a different expression of membrane receptors [45], cellular metabolism or different sensitivity of primary lymphocytes with respect to leukaemia cells as supposed by Chen et al. [46]. Differently from genistein, daidzein demonstrated a protective effect against lipid peroxidation in Jurkat cells, decreasing MDA production at concentrations ranging between 2.5 and 20 μmol/L. The protection was not dose-dependent and this could be due to problems related to the mechanism of uptake. Daidzein, different from genistein, lacks the hydroxyl group in position 5 so giving a lower steric hindrance. This could be the cause of a higher concentration of daidzein next to the membrane surface. In effect, due to the lipophilicity of daidzein, an interaction with membrane phospholipids was demonstrated in vitro, while this was not observed for the structurally closely related isoflavone, genistein [47]. On the contrary, a better metal chelating activity of daidzein than genistein does not seem sustainable considering that it is generally recognised that there is a direct correlation between the number of hydroxyl groups per molecule and the ability to chelate metal ions [48]. Data in the literature support the weakly chelating activity of isoflavones with respect to iron and copper [49,19] while the lipid radical scavenging due to the hydrogen atom donation from isoflavone to peroxy radical, is sustained by Patel et al. [49]. Possibly, different mechanisms of action could occur: for instance, deactivation of the reaction cascade resulting in production of MDA or other products of lipid peroxidation. Therefore, we cannot exclude that other compounds occurring as a consequence of membrane lipid peroxidation may be modulated by isoflavones.

In conclusion, our study sustains the fact that there is a protective effect of daidzein and genistein against cellular oxidative damage. In particular, our results emphasise that not only genistein but even daidzein exerts antioxidant activity especially with respect to DNA oxidative injury. The protection against lipid peroxidation was demonstrated at concentrations of isoflavones reachable in plasma after habitual consumption of soy products, while DNA protection was shown at levels comparable to those present in plasma of not regular soybean consumers. [50,51]. Consequently, we could suppose that the positive health effect of soy consumption shown by epidemiological studies on an eastern population could at least be partially related to the antioxidant activity of isoflavones. It would be interesting to investigate whether the protective doses of isoflavones shown in this study could be reached in plasma after intake of other legumes, which contain isoflavones, normally consumed in the Mediterranean area.

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