Protective effects of hesperidin against genotoxicity induced by ^{99m}Tc-MIBI in human cultured lymphocyte cells ☆

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Abstract

Introduction: Radiopharmaceuticals have been widely used as nuclear tracers for myocardial perfusion imaging. The purpose of this study was to investigate the radioprotective effects of hesperidin as a flavonoid which protects against the genotoxic effects of ^{99m}Tc-MIBI in human cultured lymphocytes.
Methods: Whole blood samples from human volunteers were incubated with hesperidin at doses of 10, 50 and 100 µmol. After 1 h of incubation, the lymphocytes were incubated with ^{99m}Tc-MIBI (200 µCi/2 ml) for 3 h. The lymphocyte cultures were then mitogenically stimulated to allow for evaluation of the number of micronuclei in cytokinesis-blocked binucleated cells.
Results: Incubation of lymphocytes with ^{99m}Tc-MIBI at this high dose induces additional genotoxicity and shown by increases in micronuclei frequency in human lymphocytes. Hesperidin at these doses significantly reduced the micronuclei frequency in cultured lymphocytes. The maximum protective effect and greatest decrease in micronuclei frequency occurred when cultures were incubated with a 100-µmol dose of 65% hesperidin.
Conclusion: This study has important implications for patients undergoing nuclear medicine procedures. The results indicate a protective role for hesperidin against the genetic damage and side effects induced by radiopharmaceutical administration.
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Keywords: Radiopharmaceutical; Hesperidin; Genotoxicity; ^{99m}Tc-MIBI; Micronucleus

1. Introduction

Humans are probably exposed to ionizing irradiation through both external and internal contamination. In internal contamination, radioactive substances accumulate and cause irradiation of critical organs. Ionizing radiation passing through living tissues generates free radicals. Interactions of free radicals with DNA can induce DNA damage leading to mutagenesis and carcinogenesis [1]. One significant source of internal irradiation stems from the use of radiopharmaceuticals in nuclear medicine procedures. Radiopharmaceuticals, which are often used for diagnostic and therapeutic purposes, contain at least one radionuclide and a nonradioactive ligand. Due to its ideal imaging energy, short physical half-life and ability to readily bind to a variety of compounds, technetium-99m (^{99m}Tc) is widely used for diagnostic imaging in nuclear medicine. Technetium forms chelates with different ligands, and the resulting complex is transferred to the target organ for image capture. ^{99m}Tc emits gamma rays (140.5 keV) as well as low-energy Auger and conversion electrons. Tc-99m methoxy-isobutyl-isonitrile (^{99m}Tc-MIBI) is taken up by human peripheral blood lymphocytes, which are highly radiosensitive [2–4]. Taibi et al. [5] showed that ^{99m}Tc-MIBI induces genotoxic effects and apoptosis at an absorbed dose of 0.1 Gy (250 µCi) in human peripheral blood lymphocytes. This absorbed dose

from ^{99m}Tc-MIBI is more than that usually caused by nuclear medical diagnostic procedures [6].

Recently, we demonstrated that the flavonoid hesperidin has powerful protective effects against DNA damage induced by gamma irradiation in mice. It reduced the micronuclei frequency caused by external gamma irradiation in the mice [7]. Hesperidin has been reported to have many biologically protective properties, including anti-inflammatory, antimicrobial, anticarcinogenic, antioxidant and capillary strengthening effects [8]. Daflon, which contains hesperidin and a flavone called diosmin, is used to treat chronic venous insufficiency in Europe [9] and has been helpful in other chronic diseases. Although many studies have evaluated natural products as radioprotective agents in animals and humans, little is known about the effects of radioprotective agents against damage from radiopharmaceuticals.

The aim of this study was to determine the radioprotective effects of hesperidin against the genotoxicity induced by ^{99m}Tc-MIBI in human peripheral blood lymphocyte cells in vitro.

2. Material and methods

2.1. Chemicals and preparation of ^{99m}Tc-MIBI

All chemicals were obtained from Merck and Sigma, and were used without further purification. Hesperidin was prepared in sterile water and DMSO (0.25%). ^{99m}Tc, in the form of sodium pertechnetate (Na^{+99m}TcO$_4^-$), was eluted from an in-house ^{99}Mo/^{99m}Tc generator system with normal saline solution. A commercial sestamibi kit (AEOI, Tehran, Iran) was used, and the labeling and quality control procedures were performed according to the manufacturer's instructions.

2.2. Irradiation protocol

The study protocol was approved by the ethics committee of the university. After obtaining written informed consent, 12-ml blood samples were collected in heparinized tubes from three healthy, nonsmoking male volunteers, aged 25–35 years. The blood was divided into six 2-ml tubes, one for each of the six study groups: control; ^{99m}Tc-MIBI only; hesperidin at doses of 10, 50 and 100 µmol (final concentrations) with ^{99m}Tc-MIBI; and hesperidin only. First, blood samples were incubated with hesperidin for

1 h. Then, 200 µCi of ^{99m}Tc-MIBI was added to the blood samples and incubated for 3 h. After incubation, RPMI 1640 medium was added to each tube and the cultures were centrifuged at 1200 rpm for 8 min. To separate ^{99m}Tc-MIBI from the whole blood, the upper (less dense) solution was removed and blood was transferred for micronucleus assay.

2.3. Micronuclei assay

One-half milliliter of each sample (control and irradiated groups) was added to 4.5 ml of RPMI 1640 culture medium (Gibco, USA), which contained 20% fetal calf serum, 0.1 ml/5 ml phytohemagglutinin (Gibco), antibiotics (penicillin 100 IU/ml , streptomycin 100 µg/ml) and 2 mM glutamine (Sigma, USA) at final concentration. All cultures were set up in duplicate and incubated at 37±1°C in a humidified atmosphere of 5% CO$_2$/95% air. Cytochalasin B (Fluka, final concentration: 30 µg/5 ml) was added after 44 h of culture incubation. At the end of 72 h of incubation, the cells were collected by centrifugation and resuspended in 0.075 M cold potassium chloride for 8 min at 1000 rpm. They were then immediately treated with a fixative solution three times (methanol/acetic acid, 6:1). Fixed cells were dropped onto clean microscopic slides, air-dried and stained with Giemsa solution. All slides were coded by an individual other than the scorer and evaluated at 40× magnification for the micronuclei frequency in cytokinesis-blocked binucleated cells with well-preserved cytoplasm. To be scored as micronuclei, candidates had to have a diameter of between 1/16 and 1/3 of the main nuclei, be nonrefractile and not be linked to or overlap with the main nuclei [10]. At each blood collection time, 1000 binucleated cells from duplicate irradiated and control cultures from each volunteer were examined to record the frequency of micronuclei.

2.4. Statistical analysis

At each blood collection, the prevalence of micronuclei was recorded for each volunteer. The data were analyzed using ANOVA with Tukey's HSD post hoc test.

3. Results

The percentage of micronuclei in the lymphocytes of volunteers treated with 200 µCi of ^{99m}Tc-MIBI was

Table 1
The percentages of micronuclei induced in vitro by ^{99m}Tc-MIBI (TC) on cultured blood lymphocytes from human volunteers and protective effects of hesperidin (HES)

Volunteer group	Binucleate cells with micronuclei (%)*					
	Control	TC	TC+HES10	TC+HES50	TC+HES100	HES100
Volunteer 1	0.7	3.8	3.2	1.3	1.3	0.5
Volunteer 2	0.7	3.3	3.8	1.5	1.1	1.0
Volunteer 3	1	3.9	4.0	2.0	1.5	1.4
Mean±S.D.	0.8±0.17	3.67±0.32	3.67±0.42	1.6±0.36	1.3±0.2	0.97±0.42

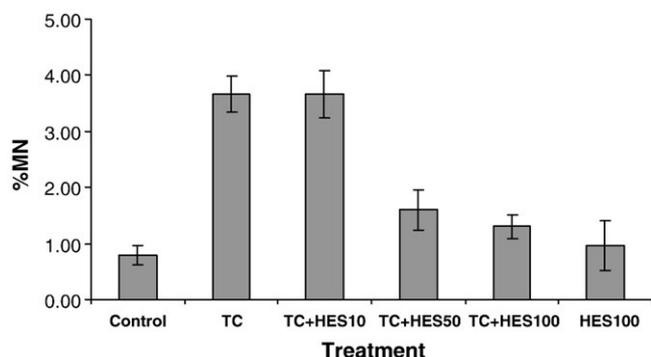* One thousand BN cells were examined in each culture.

Fig. 1. In vitro protection by hesperidin (HES) at different concentrations (10, 50 and 100 µmol) against radiation-induced genetic damage induced by ^{99m}Tc-MIBI (TC) in cultured whole blood lymphocyte. The data represent the average±S.D. of three human volunteers. *P*<0001: Control sample compared with similarly irradiated lymphocytes from the blood sample treated with TC. *P*<001: TC sample compared to TC-HES50 and TC-HES100 samples. *P*<001: TC-HES10 sample compared to TC-HES50 and TC-HES100 samples. *P*>.05: TC-HES50 sample compared to TC-HES100 sample.

3.67±0.32%, while the percentage in nontreated control lymphocytes was 0.80±0.17% (Table 1). The frequency of micronuclei (an indication of the genotoxic effects of internal irradiation) after preincubation with hesperidin at doses of 10, 50 and 100 µmol was 3.67±0.42, 1.6±0.36 and 1.3±0.2, respectively (Fig. 1). The data demonstrate that the frequencies of micronuclei found in the hesperidin-treated samples were significantly lower than in the samples cultured with ^{99m}Tc-MIBI only. Whole blood samples incubated with 50 and 100 µmol of hesperidin, and then exposed in vitro to ^{99m}Tc-MIBI radiation, exhibited a significant decrease in micronuclei frequency compared to those samples incubated with 10 µmol of hesperidin only (*P*<0001). Total micronuclei values were 56% and 65% less in the samples treated with hesperidin at concentrations of 50 and 100 µmol, respectively, than in controls (Table 1). Hesperidin did not show any protective effect at a concentration of 10 µmol. Hesperidin alone did not cause genotoxicity in cultured lymphocytes at concentrations of 100 µmol.

A typical depiction of binucleated cells with micronuclei is shown in Fig. 2.

4. Discussion

In this study, we show that hesperidin significantly protects against genotoxicity induced by the radiotracer ^{99m}Tc-MIBI in lymphocytes. In vitro incubation of human blood with the natural compound hesperidin reduced the frequency of micronuclei induced by internal irradiation by the radiopharmaceutical ^{99m}Tc-MIBI. Measures of genotoxicity have been used to estimate the risk of damage induced by internal irradiation from the radiopharmaceutical [5].

Genotoxic agents can cause cancer, hereditary disorders and abnormalities in developing embryos [11].

In this study, we have observed genotoxic effects induced by ^{99m}Tc-MIBI as shown by increases in lymphocytes at a dose of 200 µCi. Taibi et al. [5] studied the effect of increasing ^{99m}Tc-MIBI activity in vitro (corresponding to absorbed doses ranging from 1 µGy to 1 Gy) on healthy human lymphocytes. The micronuclei frequency was similar in control cultures and lymphocyte cultures exposed to doses of 10 µGy, 100 µGy and 1 cGy. A significantly higher frequency of micronuclei was observed after exposure to a dose of 10 cGy (corresponding to 250 µCi of ^{99m}Tc-MIBI) [5]. Although we observed an increase in the micronuclei frequency in lymphocytes treated with ^{99m}Tc-MIBI, this dose of radiotracer was much higher than the usual dose used for diagnosis of patients in the clinical setting. Bolus injections of 700 MBq (19 mCi) of ^{99m}Tc-MIBI exhibited peak activity in the blood after 30 s with about 0.00040 fraction of injected dose absorbed per milliliter [6]. Whole blood activity was about 7.6 µCi/ml. ^{99m}Tc-MIBI was rapidly cleared from the blood with a total clearance rate of more than 4 L/min. ^{99m}Tc-MIBI reached its minimum blood concentration of about 0.00001 fraction of radioactive dose 5 min after injection [6].

A recent study reported that the micronuclei frequency does not significantly increase in patients undergoing perfusion imaging using ^{99m}Tc-MIBI injection as compared to controls [12]. In that study, patients received a high dose of 40 mCi ^{99m}Tc-MIBI for myocardial diagnosis and there was no genotoxicity observed in the cultured lymphocytes 48 h after [12]. In the current study, we used a high blood concentration of ^{99m}Tc-MIBI (200 µCi/ml of whole blood) for a model of internal irradiation similar to that in the Taibi et al. [5] study. ^{99m}Tc-MIBI was chosen for several reasons. First, over 80% of radiopharmaceuticals currently used in diagnostic nuclear medicine are labeled with ^{99m}Tc, which has optimal nuclear properties for nuclear medicine imaging [3]. Decaying technetium-99m not only emits gamma rays, but also emits Auger electrons that cause
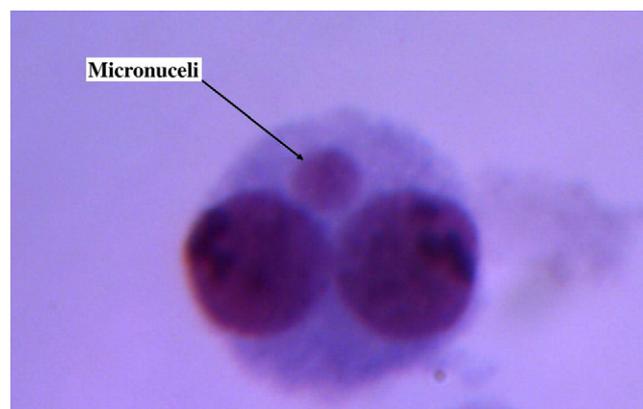

Fig. 2. A typical binucleated lymphocyte with micronuclei in our study.

subcellular side effects due to the extremely short range of action in the cell [2]. Second, myocardial perfusion scintigraphy is a routine nuclear diagnostic test for cardiac disease. In patients undergoing bone scintigraphy, additional induction of chromosomal aberrations by ^{99m}Tc-hexamethylene diphosphonate (^{99m}Tc-HDP) was not observed. ^{99m}Tc-HDP has also been reported to not cause any genotoxic effects after intravenous administration at doses of 20–25 mCi [13].

The genotoxic effects induced by radiopharmaceuticals were evaluated by incubating human lymphocytes with ^{99m}Tc during ^{99m}Tc-labeling of lymphocytes. In a study aimed at determining whether ^{99m}Tc induced genotoxicity after labeling of lymphocytes, researchers observed that increasing dose of ^{99m}Tc resulted in proportional increases in micronuclei in lymphocytes at doses of 0.236, 0.435 and 0.580 mCi per 10^7 of isolated lymphocyte cells [4]. The 0.580-mCi dose of ^{99m}Tc corresponded to 1 Gy of 250-keV X-ray emission; the frequency of micronuclei was 5.5% and 0.68% after 72 h of incubation of lymphocytes with ^{99m}Tc at a dose of 0.580 mCi per 10^7 for the samples and controls, respectively [4]. Patients exposed to clinically used doses of technetium-99m did not show any chromosomal aberrations or mutations in peripheral blood lymphocytes [14]. Internal radiation from different radionuclides, as well as external radiation of various types, can induce genotoxicity, which increases the risk of cancer. Dose–response models in animals looking at the effects of high doses may be used to assess the risks associated with ionizing radiation emitted by radiolabeled compounds in humans [11].

Hesperidin has a molecular weight of 610.5 and is found mainly in the citrus family. It has been reported to have several beneficial health effects, including chemoprotection against carcinogenesis in the colon [15,16] and reduction of oxidative stress in the rat liver and kidney [17]. The vasoprotective and venotonic effects of hesperidin make it a potential therapeutic agent for patients. Daflon (composed of hesperidin plus diosmin) is used to treat chronic venous insufficiency in Europe [9]. Ionizing radiation produces free radical damage in DNA and induces genotoxic effects and apoptosis. Free radical scavenging is apparently responsible for the inhibitory effects of flavonoids, such as rutin, morin, quercetin and genestin, on the clastogenic activity induced by gamma radiation in mice [18].

The molecular mechanisms underlying the radioprotective effects of hesperidin are not clear. Hesperidin has been shown to have antioxidant activity against the cellular oxidative stress associated with neurodegenerative diseases. This flavonoid also attenuated decreases of glutathione peroxidase and glutathione reductase activity and decreased DNA damage in H_2O_2-induced PC12 cells [19], and also inhibited low-density lipoprotein oxidation [20]. Oral administration of hesperidin has protective effects against gamma radiation-induced hepatocellular damage and oxidative stress in rats [21]. The pharmacokinetic parameters of hesperidin were measured for total hesperidin in healthy humans after the consumption of orange juice [22]. In that protocol, administration of orange juice corresponded to 192 mg hesperidin, with a peek plasma concentration (C_{max}) of 1.05 mmol/L of hesperitin (aglycone of hesperidin). In our study, the concentration of hesperidin (molecular weight, 610.5) was 6.1, 30.5 and 61.05 µg/ml of incubated whole blood for 10, 50 and 100 µmol, respectively. In this study, we showed that hesperidin protects human lymphocytes against genotoxicity induced by internal irradiation. For all three volunteers, the ^{99m}Tc-MIBI-treated lymphocytes incubated with hesperidin showed a reduction in micronuclei frequency as compared to ^{99m}Tc-MIBI-treated samples without hesperidin. This finding suggests that hesperidin acts effectively as a free radical scavenger as such radioprotection is concentration dependent. Hesperidin treatment at 100 µmol provides maximum leukocyte protection.

Since hesperidin has been used extensively as a phlebotropic drug, and with regards to potential radioprotective effect, it may be a useful therapeutic candidate for patients undergoing nuclear medicine procedures.

Acknowledgment

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