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Cytogenetic and immunological efficacy of nicotiflorin and rutin combination on gamma irradiated rats

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► ***Original article***

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ABSTRACT

***Background*:** Cytogenetic and immunological damages after ionizing radiation exposure are critical factors that lead to many different events and consequences cascade reactions starting from inflammation, ending with cell damage. These biological events if not well controlled will lead to deleterious effects and cancer. The present study aimed to evaluate the efficacy of the Nicotiflorin and Rutin (NR) combination against drastic effects resulted from γ-irradiation. ***Materials and Methods*:** Rats were divided into four groups. (Control) group, (NR) group: treated with 20mg/kg body weight orally daily one dose for two weeks, (Irrad) group: rats exposed to 6Gy as a single dose, and (NR+ Irrad) group which treated with NR combination before irradiation. Detection of DNA damage was done with the Micronucleus test (MN) and Comet assay. Immunological responses were detected by assessing inflammatory cytokines Interluekines-1β (IL-1β), tumor necrosis factor (TNF-α), and homeostasis maintenance cytokines (IL-6 & IL-10). ***Results*:** Irradiated group recorded a significant increase in micronuclei (MNi) incidences and significant DNA fragmentation. As well, immunological parameters displayed a significant increase in all measured interleukins except IL-10 which recorded a significant decline. The group injected with NR before irradiation showed significant improvement in all measured parameters. ***Conclusion*:** The efficacy of the NR combination may be attributed to its dual protective effects (cytogenetic and immunological enhancement) against damage caused by γ-irradiation.

INTRODUCTION

Though radiotherapy is one of the main therapeutic ways against malignant tumor progression, its damaging effects even acute or chronic toxicities habitually will be appeared. So serious studies were carried on to provide evidence that, using free radical scavengers immediately before radiation exposure is a necessity to alleviate DNA damage and prevent cell death. Further research has led to a large number of experimental studies defining suitable radioprotectors (1).

Natural compounds are non-toxic over wide dose ranges and are inexpensive and effective. Additionally, pharmacological strategies have been developed that use radioprotectors to inhibit radiation-induced toxicities (2). Nicotiflorin, a phenolic compound contains flavonoids moiety O-glycosidically linked to carbohydrate moiety at the C3-position. It is found in many plants as ginkgo nuts and tea (3). Moreover, nicotiflorin was recorded as a potent compound against severe immunological and chemical injuries; this might be attributed to its antioxidant and immunoregulatory prospect (4). Rutin also is a plant pigment citrus flavonoid formed from glycoside combining the flavonol quercetin and the disaccharide rutinose (5). It is found in a wide variety of plants such as buckwheat, Japanese pagoda tree, lime tree flowers, and Eucalyptus. Rutin is phytochemical with multiple pharmacological activities, anticancer effects on different cell lines, and decreases DNA damage (6). The combination of various flavonoids showed high scavenging activity on hydroxyl radicals either due to its direct potential in scavenging free radicals or modulating the antioxidant defence system. In parallel, flavonoids have anti-inflammatory potency via different pathways as the inhibition of NO release, and the suppressing the TNF-α and IL-6 (7).

There are various methods used for the recognition of early DNA damage due to environmental and occupational exposure to ionizing radiation (8). Comet assay was introduced for the detection of double-strand breaks, single-strand breaks, alkali labile sites, DNA cross-linking, and incomplete excision repair sites (9). Supporting comet assay, the MN assay has been developed for genotoxicity and mutagenicity that can modify chromosome structure and induce segregation error (10). As so far the application of genotoxicity testing suggests that no single assay can fully detect all genotoxic aspects [11].

Cytokines are glycoproteins produced by a variety of cells and are secreted into the extracellular space to participate in the immune response and inflammatory regulation [12]. Many studies report that inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, all induced by ionizing radiation, significantly contribute to the disorders associated with radiotherapy in the blood [13]. Moreover, the increase in TNF- α is counterbalanced by simultaneous synthesis of an anti-inflammatory cytokine IL-10, which suppresses the production of many activating and regulatory mediators [14].

NR combination generates synergetic and dual power action to protecting animals from ionizing radiation drastic hazards. The present novel combination NR made it strongly capable of being an antioxidant, free radical scavenger, and immunoregulatory agent.

MATERIAL AND METHODS

Animals and management

Twenty-four healthy adult male rats weighing 160 -190 gm aged 8±2weeks were acquired from the animal house of the NCRRT. They were accommodated in polypropylene cages regarding typical laboratory circumstances and regulated temperature (24±4°C) throughout the experiment. All the study's protocols, animal precautions, and treatment were in agreement with the guiding principles allocated by the Research Ethics Committee (REC-NCRRT) with No. (25A/20).

γ- irradiation

The γ-irradiation was done by the Canadian γ-cell-40 for biological irradiation (Cesium-137) in the NCRRT, Nasr city, Cairo, Egypt, giving a dose rate of 0.43Gy/minutes at the time of the experiment. It provides a constant exposure to the whole body of the animals (the total dose delivered to animals was 6Gy) [15], whereas regarding the whole shielding for the working staff.

Preparation of NR and its administration

A mixture of nicotiflorin and rutin prepared in a ratio of 1:1and was dissolved in DMSO as solvent. The mixture was diluted by ratio 1solvent:9 distilled water. The mixture was freshly prepared before the administration. Dose at a concentration of 20 mg/kg body weight/ day was applied orally for 2 weeks before gamma radiation exposure [16, 17].

Experimental design

The rats were separated into four groups, six animals per group, and were managed as follows: Control group administered the solvent diluted in distilled water, NR group received 20 mg/kg/day of NR orally for two weeks, Irradiation group which exposed to 6Gy γ-rays and finally, NR+ irradiation group which received NR 20 mg/kg/day orally for 2 weeks before 6Gy γ-irradiation. After the end of treatments, rats were sacrificed under light ether anaesthesia.

Cytogenetic study

Micronucleus test: Bone marrow samples were collected from the rat's femur at the sacrificing time, according to Schmid [18], three sample slides were prepared for each animal for the micronucleus assay. The slides were stained with 5% Giemsa stain diluted in phosphate buffer (Na_2HPO_4 0.06 M and KH_2PO_4 0.06 M, pH 6.8). According to the study of Albanese and Middleton [19], they concluded that the scoring MNi in the PCEs is more accurate in the bone marrow due to the considerations of staining and sample size per animal. So, for each animal 1500 polychromatic erythrocytes (PCEs) were counted. The slides were scored blindly according to the conventional criteria [20, 21].

Comet assay: Single-cell suspension preparation: Bone marrow from the femur of rats was washed three times with phosphate buffer solution (PBS: NaCl 8.0 g, KCl 0.2 g, $Na_2HPO_4{\cdot}12H_2O$ 2.8 g, KH_2PO_4 0.2 g, pH 7.4), homogenized and resuspended with PBS. The assay was performed according to Singh *et al.* [22]. For each group, 1000 cells were analysed (original magnification ×200) under a fluorescent microscope (BX51, Olympus) equipped with a green light excitation and 590-nm barrier filter. The comet parameters were calculated and photographed by TriTek Comet Score v1.5 software. The recorded comet parameters to characterize the DNA damage are the percentage of DNA in the comet tail (TDNA %), tail length (TL), tail moment(TM), and olive moment (OTM).

Immunological study

Determination of IL-1β, IL-6, IL-10, and TNF-α: Its principles according to Catalog No. (MB5825017, MBS355410, MBS355232, and MBS355371) respectively, MyBioSource (China).

Sandwich Enzyme-Linked Immunosorbent Assay for quantitative detection of rat IL-1 beta, IL-6, IL-10, and TNF-α concentrations in cell culture supernatants, serum, plasma, tissue homogenates. An antibody specific for IL-1β, IL-6, IL-10, and TNF-α well-coated plate was used. Standards and samples (plasma) are pipetted into the wells, each one present in a sample is bound to the immobilized antibody specific for it. Then were washed and biotinylated anti-interleukins were added. After washing away the unbound biotinylated antibody, conjugated streptavidin is pipetted to the wells. Then were washed, finally, a substrate solution was added so

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color developed in proportion to the amount of interleukin bound. The Stop Solution changes the color from blue to yellow, and the intensity of the colour is measured at 450 nm.

All previous immunological parameters were measured by Eliza reader: TECAE, A 5082 made in AUSTRIA with serial No: 9000308 type spectra classic.

Statistical analysis

Obtained data were expressed as mean ± standard error. Statistical analysis was performed using (Statistical Package for Social Science) (SPSS) software version 20 for windows by one-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests (P values < 0.05 were considered as significant [23].

RESULTS

The effect of NR treatment (20 mg/kg body weight) once daily for two weeks on the MN frequencies induced by γ-irradiation in rats' bone marrow is represented in table 1. The data revealed that NR had no significant effect on the induction of MNi and aberrant cells as compared with that of the control group. On the other hand, radiation exposure provoked a significant increase in both (total number of aberrant cells and MNi frequencies) by ≈20 and ≈22 folds respectively when compared with control. Table 1 also revealed that the micronucleated cells with 1MN and 2MNi were increased significantly in the irradiated group with ≈18 and ≈29 folds, respectively when compared with the control group. In addition, the first and only appearance of cells with 3MNi was detected in the irradiated group. Meanwhile, treatment with NR before the irradiation showed a significant reduction of total aberrant cells and total frequencies of MN by ≈0.6 and ≈0.5 fold respectively when compared with the irradiated group, but still significantly higher than that of control values.

Table 1. Bone marrow MNi frequencies of rats treated with NR and/or 6 Gy γ-irradiation.

Groups	Normal cells	No. of MNi/ 1000 Cells			Total MNi	Total No. of aberrant cell
		Cell+ 1MN	Cell+ 2MN	Cell+ 3MN		
Control	997.00± 0.63	2.67± 0.61	0.33± 0.21	00.00	3.33± 0.71	3.00± 0.63
NR	997.17± 0.48	2.50± 0.34	0.33± 0.21	00.00	3.17± 0.65	2.83± 0.48
Irrad	938.83± 3.96^{ab}	48.33± 4.22^{ab}	9.67± 0.99^{ab}	2.33± 0.21ab	74.67± 4.67ab	61.17± 3.96^{ab}
NR+ Irrad	965.00± 2.89^{abc}	31.33± 2.29^{abc}	2.83± 0.79^{abc}	0.83± 0.31abc	39.50± 3.84abc	35.00± 2.89^{abc}

Data expressed as mean ± standard error. a: Significant difference with the control group, b: Significant difference with NR group.c: Significant difference with Irrad group.*p<0.05.

Likewise for comet assay results which are represented in figure 1 and table 2, the data revealed that NR had no significant effect on the induction of DNA damage as indicated by comet parameters when compared with the control group. On the other hand, radiation caused a significant increase in the TL, TDNA%, TM, and OTM by ≈19 folds,≈12 folds, ≈29 folds, and ≈17 folds, respectively when compared with the control group. Meanwhile, NR treatment before irradiation showed a significant reduction of the TL, TDNA%, and TM by ≈0.4 fold, and reduction of OTM by ≈0.2 fold when compared with the irradiated group, but still significantly different from control values.

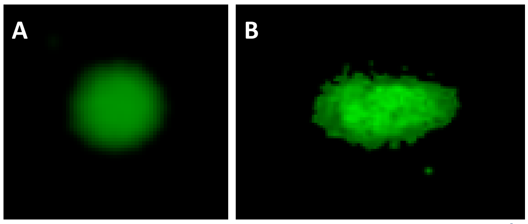

Figure 1. Demonstrative photomicrograph showing, **(A):** Typical nuclei of undamaged cells of the control group and **(B):** DNA damage observed as comets.

Table 2. The effect of NR administration with or without 6 Gy γ-irradiation on the comet assay parameters

Groups	TL	TDNA%	TM	OTM
Control	3.90±0.61	5.60±1.48	1.20±0.45	1.38±0.19
NR	4.28±0.75	7.25±1.49	0.93±0.56	1.63±0.17
Irrad	72.60±2.60^{ab}	67.85±5.71^{ab}	34.68±3.66^{ab}	23.33±3.32^{ab}
NR+Irrad	27.85±1.57^{abc}	23.75±1.81^{abc}	12.58±0.52^{abc}	5.08±0.92^{c}

Legends as in Table (1). TDNA %: The percentage of DNA in the comet tail. TL: Tail length. TM: Tail moment. OTM: Olive moment.

Figure 2 symbolized different responses of interleukins. First of all, NR treated group recorded non-significant changes when compared with the control group. Exposure to γ-irradiation causes a significant increase in pro-inflammatory interleukins (IL-1β, IL-6, and TNF-α), while regulatory interleukin (IL-10) recorded drastic inhibition. On the other hand, the treatment with NR before irradiation could inhibit the inflammatory effect and re-enhance the regulatory effect of IL-10. These levels didn't reach normal values but recorded considerable enhancements.

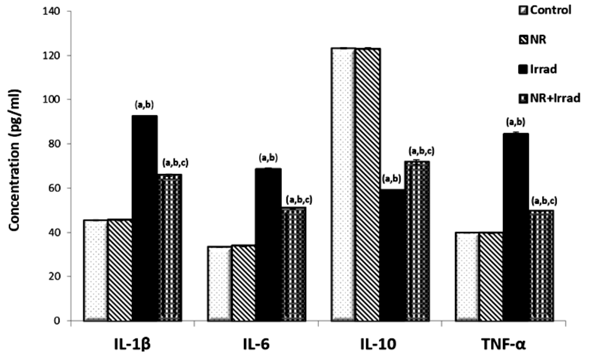

Figure 2. The Effect of NR treatment with or without 6 Gy γ-irradiation on rats serum pro-inflammatory and regulatory cytokines IL-1β, IL-6, IL-10, and TNF-α.

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DISCUSSION

Ionizing radiation could directly affect sensitive cellular constituent causing excitation and initiation of sequence events that end with biological cell injury, whichever through killing the cell or altering the DNA [24]. As well, this may happen indirectly due to the radiolysis of water molecules producing reactive chemical species which by its role lead to cellular macromolecules damage. Excessive production of these radicals leads to oxidative injury and induces chromosomal damage coupled with MNi manifestation [25]. The induced DNA damage either single or double-strand breaks can be detected by the alkaline comet assay [26]. In the present work, the group of animals which was exposed to 6Gy γ-irradiation shows a significant increase in the DNA damage detected by MN test and comet assay. In a like manner, our results were in agreement with the study of Azzam *et al.* [27] which denoted the deleterious effect of free radicals on DNA.

Recently, a strong association was discovered between MNi formation and increased inflammation markers [28]. MNi are formed by lagging acentric fragments or whole chromosome that straggled through mitosis telophase [29]. However, evolving novel information reveals that chromosomes enclosed in MNi may go through a high fragmentation rate. Additional track of latest studies revealed that leaked DNA and chromatin material from ruptured MNi activate the instinctive immune process through cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP–AMP) synthase (cGAS) and its downstream signalling effectors stimulator of interferon genes (STING) [cGAS-STING] which stimulates inflammation reaction [28]. This stimulation is done by variable pathways and streaming consequences, including the progress and transcription of the pro-inflammatory cytokines as IL-1β and IL-6 respectively [30]. Not only free radicals production but also inflammatory reactions are induced by ionizing radiation mediated by many inflammation-related cytokine genes [31]. Given these points, the massive increase of the IL-1β and IL-6 levels after exposing the experimental animals to 6Gy, in the present study, could be explained as a cumulative and progressive consequence of either γ-irradiation effectors disrupted MN implication.

The elevation of serum IL-1β, IL-6, IL-8, and TNF-α levels after exposure to ionizing radiation was confirmed by many studies in various human or mammalian cells [31]. In Linard *et al.* [32] study, abdominal irradiation (10Gy) induced a cascade of inflammatory events characterized by an early (6 h after exposure) increase in IL-1β, TNF-α, and IL-6 mRNA levels in the rat ilealmuscularis layer, while IL-10 (an anti-inflammatory cytokine) expression vanished completely. In the present study, the serum IL-1β, IL-6, and TNF-α levels were elevated after 6Gy γ-irradiation, while, IL-10 recorded an inhibitory manner. The elevated levels of IL-1 β, IL-6, TNF-α are associated with radiation oxidative stress [14]. A significant elevation of IL-6 was recorded after irradiation which is considered as a multifunctional cytokine involved in cell proliferation and differentiation, maintaining immune homeostasis, macrophage function, and other key functions [32]. The equilibrium between TNF-α and IL-10 is a vital process to sustain immune homeostasis [33]. Necrosis is a result of enormous damage to DNA. It causes alterations in the immune system response which are allied with the development of oxidative stress and inflammation. The manifestation of inflammatory and pro-oxidant factors depends on time and tissue type [1].

The immunomodulation, anti-inflammatory, and anti-mutagenic properties are the most important characters must be found in applied medicinal plants. Many studies focused on these effects in different ways [34-36].

In the same fashion, our results show that NR has an anti-mutagenic effect against γ-irradiation which was detected by reducing MNi frequencies and comet measurements. This may be ascribed to either a reduced authentic number of injuries or to increase repair effectiveness [37]. Anderson *et al.* [38] and Huang *et al.* [39] studies revealed that protective anti-genotoxic effects of nicotiflorin and rutin may be attributed to their antioxidant properties as they cause reduction of DNA damage detected by comet assay. However, nicotiflorin was more efficient than rutin. This powerful free radical scavenging activity of nicotiflorin intensely is ascribed to the existence of free hydroxyl groups at C-3 and C-5 [40]. As mutagenesis inhibition is frequently a complex process and done by several pathways [41]. So, the present study preferred to use rutin in a combination with nicotiflorin which is a common dietary flavonoid and has numerous pharmacological antioxidant and anti-inflammatory effects [42]. Rutin protects cells against oxidative stress due to the presence of the phenolic groups which donate hydrogen to scavenge free radicals induced by radiation [43]. In agreement with our results, nicotiflorin has a strong ability to enhance all inflammatory IL-6, IL-1β, and IL-10 as its structure with many hydrogen bonds leads to free radicals scavenging. In addition to reducing oxidative stress and consequently reduce inflammation [44]. Our gained results exude a novel combination NR, where nicotiflorin's unique distinct structure (many-ranched hydrogen bonds and phenolic rings) made it strongly capable of being an antioxidant, free radical scavenger, and immunoregulatory agent [4]. Also, combined with rutin a strong antioxidant protector which can scavenge free radicals [40] via polyhydroxylated substitutions on rings A and B, a 2, 3-double bond, a free 3-hydroxyl substitution, and a 4 -keto moiety [45]. This combination, generates synergetic power action protecting animals from

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ionizing radiation drastic hazards. A group of treated animals injected with NR mixture before irradiation merrily proofed a significant enhancement of the antimutagenic (MNi frequencies and comet measurements decline) and anti-inflammatory responses (adjusting IL-10) protecting animals body from escalating inflammatory responses (decreasing IL-1β, IL-6, and TNF-α) when compared with irradiated group measurements.

In conclusion, the present study on NR mixture as a radioprotector revealed that NR can protect DNA from damage induced by γ-irradiation and decrease the incidence of MNi frequency by scavenging free radicals. Also, it can improve the inflammatory cascade process which aggressively happened after ionizing irradiation. So, NR may play a radio-protective role due to the cytogenetic and immunological efficacy of its components. We recommend further studies on the ability of NR in protection against radiation exposure hazards.

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Conflicts of interest: Declared none.

Ethical considerations: All the study protocols, animal precautions, and treatment were in agreement with the guiding principles allocated by the Research Ethics Committee (REC-NCRRT) with No. (25A/20).

Author contributions: All authors were involved in the research, data generation and preparation of the manuscript.

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