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Effect of Tinospora Cordifolia on Gamma ray-induced Perturbations in Macrophages and Splenocytes

Lakshman SINGH¹, Sonia TYAGI¹, Moshahid Alam RIZVI^{2*} and Harish Chandra GOEL¹

Ionizing irradiation/Radioprotection/Splenocyte/Macrophage/Cytokine/Apoptosis/Tinospora cordifolia/Phagocytosis.

Tinospora cordifolia (RTc) has already been reported to protect whole-body lethally irradiated mice. This study has focussed on certain aspects of immuno-competence, which are adversely affected by irradiation. This study included estimation of spleen size, cell count, DNA fragmentation and apoptosis in splenocytes. The adherence, spreading and phagocytic activities of macrophages were also assessed. Cytokines in serum and anti-oxidants in plasma were also estimated. Administration of RTc (200 mg/ kg.b.wt.) one hour before irradiation showed recovery of spleen weight from 49% of control in irradiated group to 93%; apoptosis from 19% to 2.8%; DNA fragmentation from 43% to 20.4%; macrophage adherence form 75% of control to 120% and macrophage spread size from 8 μ m to 15 μ m. RTc also stimulated proliferation in splenocytes in a dose-dependent manner. RTc administration before irradiation also increased levels of IL-1 β and GM-CSF levels, from 56 pg/ml and 53 pg/ml respectively in irradiated group to 59 pg/ml and 63 pg/ml. Similarly, radiation-induced decrease of anti-oxidant potential of plasma (32 Fe²⁺ equiv.) as compared to control (132 Fe²⁺ equiv.) was countered by administration of RTc before irradiation (74.2 Fe²⁺ equiv.) RTc treatment thus reveals several radio-protective mechanisms.

INTRODUCTION

Ionizing radiations generate free radicals leading to lipid peroxidation, protein oxidation, base modifications, DNA strand breaks and genomic instability ultimately resulting in cell death.¹⁾ In the whole-body irradiated animal, the hemopoeitic system is the most susceptible system where cell loss occurs even at low doses of exposure (2 Gy). This causes perturbations in the immune system since loss of bone marrow stem cells affects hematopoeisis. At higher doses this effect is more pronounced leading to a total loss of immunocompetent cells and causing immunosupression that contributes to the emergence of opportunistic infections as observed during the hemopoeitic syndrome.²⁾ The effect of radiation on various cells of the immune system depends on the cell type and the dose of radiation. Total body irradiation is known to suppress lymphocyte proliferation and activity, a condition well exploited to control allograft rejections.³⁾ Macrophages and other phagocytes are more radioresistant than lymphocytes. Low dose radiation has been reported to increase activity of peritoneal macrophages.⁴⁾

Tinospora cordifolia (Family: Menispermaceae) has been widely used in the Indian System of Medicine (Ayurveda) as *Rasayana* for the treatment of jaundice, diabetes, rheumatoid arthritis, gout, general weakness, skin diseases, anaemia, emaciation and infections.^{5,6)} It is known to have hepatoprotective, hypolipidaemic and anti-neoplastic properties^{7–9)} and it also scavenges free radicals.¹⁰⁾

In our laboratory, aqua-alcoholic (50/50 v/v) extract of *Tinospora cordifolia* (RTc) was evaluated, for the first time, as a radioprotector. It showed promise as it rendered 76.3% survival (30 day) against lethal dose (10 Gy) of gamma radiation in mice.¹¹⁾ It suppressed radiation-induced micronuclei formation in bone marrow cells. Thus, its radioprotective efficacy could be attributed to protection of bone marrow cells, apart from other mechanisms like free-radical scavenging.¹⁰⁾ The cellular processes attributing to radioprotection could be mediated by cytokines that play an important role in induction of most cellular and inter-cellular responses. Natural levels of interleukin-1 beta (IL-1 β) and tumor necrosis factor- α (TNF- α) contribute to radioresistance of normal mice, since blocking these cytokines or their receptors by antibodies leads to increased mortality after irradia-

^{*}Corresponding author: Phone: +91-9911661657, Fax: +91-11-26980229, E-mail: rizvi_ma@yahoo.com

¹Department of Radiation Biology, Institute of Nuclear Medicine and Allied Sciences, Brig. S.K.Mazumdar Marg, Delhi - 110 054, India; ²Department of Biosciences, Faculty of Natural Sciences, Jamia Millia Islamia, New Delhi - 110 025, India doi:10.1269/jrr.07001

L. Singh et al.

tion.¹²⁾ Also, exogenous administration of rIL-1 β and rTNF- α provides radioprotection in mice.¹³⁾ In fact, IL-1 β and TNF- α influenced increase of hemopoeitic cytokines like GM-CSF to achieve enhanced proliferation of bone-marrow stem cells.¹⁴⁾ To understand the mechanism of action of RTc, we evaluated its effect on radiation-induced perturbations in splenocytes, peritoneal macrophages and serum levels of IL-1 β and TNF- α .

MATERIAL AND METHODS

Materials for cell culture

Silicagel 60 F₂₅₄ TLC plates were obtained from Merck. Cultureware was obtained from Nunc, Denmark; slides and coverslips (12 mm diameter) were obtained from Bluestar, India. Dulbecco's Modified Eagle's Medium (DMEM) and Phosphate Bufferred Saline (PBS) were obtained from Himedia. Concanavalin A (Con A) was obtained from Calbiochem, USA. Fetal Bovine Serum (FBS) was obtained from Hyclone (U.S.A.), whereas MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Lipopolysaccharide (LPS), Propidium Iodide (PI), RNase A and Sodium Pyruvate were obtained from SIGMA (USA). Other reagents used in the study were of analytical grade and procured locally.

Animals

Six to eight week-old male Swiss albino Strain 'A' mice (average weight 28 g) were obtained from the Animal Breeding Facility of the Institute. Animals were kept under standard conditions (temperature of 25°C, humidity of ~70 \pm 5%, 12 hr-dark and light cycle) and fed *ad libitum*. All experiments were conducted in accordance with the recommendations of the Animal Ethics Committee of the Institute. Five animals were taken per group for all the studies and each experiment was repeated thrice.

Extract

Aqua-alcoholic (50/50'v/v) extract of *T. cordifolia* stem was procured from the Institute of Himalayan Biotechnology (IHBT), Palampur, Himachal Pradesh, India and coded as RTc. The extract was administered intra-peritoneally into mice one hour before irradiation.

Biochemical Analysis

Polyphenolic Content

Polyphenolic content of the extract was estimated following the Singleton method.¹⁵⁾ Briefly, 500 μ l of Folin Ciocalteu reagent (1N) was added to desired concentrations of RTc (taken in 100 μ l final volume). This was followed by addition of 400 μ l of 7.5% Na₂CO₃. The resulting solution was vortexed for 15 seconds and incubated at 30°C for 10 minutes. Optical density of the final reaction mixture was measured at 765 nm on a spectrophotometer using gallic acid as a standard and the result was expressed as gallic acid equivalents/mg of extract.

TLC (*Thin Layer Chromatography*)

The TLC was performed on a 10×10 cm sheet coated with 0.25 mm silica gel 60 F₂₅₄ (Merck) to find the active components present in RTc, as per the method described by Harborne for plant flavonoids.¹⁶⁾ The extract was resuspended in 50/50 aqua-alcoholic mixture and subjected to TLC. The first run was made in BAW (butanol: acetic acid: water; 4:1:5; upper phase) while the second run was made in 5% acetic acid. A master key was prepared for the chromatogram obtained and the components found were compared with available literature.

Irradiation

A cobalt-60 gamma-ray source (Atomic Energy Canada, Model-220) was used for irradiation of animals. Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's chemical dosimetry method. The irradiation chamber was connected to a pump that supplied fresh air to the chamber to avoid hypoxic conditions during exposure. Each animal was irradiated individually in perforated plastic bottles.

Experimental Groups

Animals were divided into four groups, viz; i) Control (saline-injected alone), ii) RTc-treated (200 mg/kg body weight, i.p.), iii) Radiation-exposed (2 Gy) and iv) RTc-treated (200 mg/kg body weight, i.p.) 1 hour before radiation exposure (2 Gy).

Spleen Weight

Spleen weight was estimated following the method of Goel *et al.*¹⁷⁾ Briefly, after various treatments, spleens excised from animals at different time intervals were transferred to pre-weighed 15 ml centrifuge tubes containing 2 ml DMEM. The tubes were weighed again and the difference between the two weights was taken as the relative spleen weight. This procedure of weighing was adopted to maintain sterility of spleen that was essential for further studies. In order to abolish individual differences in spleen weights within the groups, spleen weights (mg/g body weight of the animals) were considered.

Splenocyte Isolation & Count

Splenocytes were isolated by the method described elsewhere¹⁷⁾ with certain modifications. Briefly, aseptically removed spleens from mice were placed in 2 ml DMEM in a 35 mm culture plate. The spleens were crushed between chilled frosted slides to form a single cell suspension. Debris, containing extracellular tissue was separated from splenocytes by allowing the suspension to stand for 10 minutes on ice. Cell supernatant was carefully removed and

centrifuged at 1000 rpm for 10 minutes. The pellet so obtained contained lymphocytes, macrophages as well as RBCs. RBCs were removed by treating the pellet with RBC lysis buffer (0.15 mM NH₄Cl, 0.01M KHCO₃, 0.1mM EDTA, pH 7.2) for 2 minutes followed by centrifugation for 10 minutes. Splenocytes, thus obtained, were washed twice in PBS (pH 7.2) and re-suspended in DMEM containing 100 U/ml penicillin, 100 μ g / ml streptomycin and 100 μ g/ml gentamycin, 0.01% sodium pyruvate and 4.5 g/l glucose. Total splenocyte count was performed on a Neauber's Chamber.

Splenocyte Proliferation Assay

Splenocyte proliferation was assessed by performing the MTT reduction assay.¹⁸⁾ Briefly, splenocytes isolated from untreated control mice were plated in U-bottom 96-well tissue culture plates (0.25×10^6 cells/200 µl/well). Two sets of experiments were performed. In the first, splenocytes were treated with different concentrations of RTc ($6.25-100 \mu g/ml$) while in the second RTc was compared with LPS ($10 \mu g/ml$) and Con A ($10 \mu g/ml$). After various treatments, cells were incubated at 37° C, 5% CO₂ for 48 h. Two hours before termination MTT was added (final concentration 500 µg/ml). Cells were separated by centrifugation at 6000 rpm for 10 minutes. The supernatant was carefully removed followed by addition of DMSO (200μ l/well). Plates were read at 570 nm with 630 nm as reference wavelength.

Assay for Apoptosis

In this assay, total DNA contents were measured flowcytometrically.¹⁹⁾ Briefly, samples prepared from aliquots (1×10^6 cells) obtained from splenocyte suspensions used for other parameters were washed and centrifuged once in PBS (pH 7.2). The pellet obtained was resuspended in 1 ml PBS. The sample was treated with 200 µg/ml RNase A for 30 minutes at 37°C followed by staining with propidium iodide (50 µg/ml) for another 30 minutes at 37°C. From each sample, data on 10,000 cells was acquired flowcytometrically and analysed using Cell-Quest software.

Assay for DNA Fragmentation

DNA fragmentation in splenocytes was measured spectrophotometrically by the DPA method.²⁰⁾ This method is based on the separation of chromosomal, intact DNA from fragmented DNA by centrifugal sedimentation followed by its precipitation and quantification using DPA (diphenylamine). Briefly, splenocytes $(1 \times 10^6 \text{ in 1 ml PBS})$ were taken in a 1.5 ml centrifuge tube (tube B) and centrifuged (200 g, 4°C, 10 minutes) to obtain a cell pellet. The supernatants were transferred to fresh tubes (tube S). The obtained pellet (tube B) was suspended in 1 ml TTE (Tris Triton EDTA) buffer, pH 7.4 (TE buffer with 0.2% Triton X-100), vortexed vigorously and centrifuged at 20,000g (4°C, 10 minutes). The supernatant obtained was transferred to fresh tubes (tube T) and the pellets obtained were again suspended in TTE buffer. TCA (tri-chloro acetic acid, 1 ml of 25%) was added to tubes T, B and S and vortexed vigorously. Tubes were kept overnight at 4°C followed by centrifugation at 20,000g (4°C, 10 minutes). The supernatant was discarded and the pellet was hydrolysed by the addition of 160 μ l of 5% TCA followed by heating at 90°C for 15 minutes. This was followed by addition of 320 μ l of freshly prepared DPA. The colour was developed by incubation at 37°C for 4 hours. Optical density of the solution was read at 600 nm in an ELISA reader (Biotek Instruments).

Percentage DNA Fragmentation was calculated using the following formula –

% Fragmented DNA =
$$\frac{S + T}{S + T + B} \times 100$$

Peritoneal Cell Isolation

Peritoneal cells were isolated following the protocol described earlier.²¹⁾ Briefly, mouse peritoneum was washed with 10 ml of ice-cold DMEM and the aspirate was collected in a 15 ml centrifuge tube. Cells were obtained by centrifugation at 1000 rpm for 10 minutes. Cell pellet was washed twice in PBS (pH 7.2) followed by resuspending cells in DMEM. Cells were counted on a hemocytometer using 0.05% trypan blue.

Macrophage Function Assays

Adherence Capacity

For this assay, a previously described standard protocol was applied.²²⁾ However, coverslips were used instead of Leighton tubes in the original protocol. Briefly, 0.1×10^6 peritoneal cells were plated in DMEM containing 10% FBS on coverslips that were, placed in 35mm culture plates. The plates were incubated for 2 hours at 37°C and 5% CO₂ after which the medium was aspirated and collected in 1.5 ml centrifuge tubes. PBS from two consecutive washes was also collected in these tubes and cells were counted using a Neauber's Chamber.

Spreading Capacity

Approximately, 0.1×10^6 cells in 100 µl were plated on coverslip and kept in a 35 mm culture dish and incubated for 2 hrs at 37°C and 5% CO₂. Following adherence the plates were washed once with PBS (pH 7.2) and 2 ml DMEM containing 10% FBS was added. The plates were incubated again. At intervals of 24 and 48 hours media was aspirated and the coverslips washed twice in PBS. Cells were fixed in methanol for 30 minutes, stained with Giemsa and mounted in glycerol. Cell size was estimated using Lieca software that measured cell size in micrometers. Cell size was recorded for 200 cells/slide and the average size was calculated.

Phagocytic Activity

Phagocytosis of heat-killed yeast was performed in peritoneal macrophages following the procedure of Mondal and Rai²³⁾ with some modifications. Yeast suspension was prepared by dissolving 30 mg baker's yeast in 15 ml PBS (pH 7.2) and heating the suspension at 85°C for 15 minutes. For complement activation, 500 μ l of yeast suspension (1 × 10⁶/ ml in PBS) was incubated with an equal volume of freshly isolated autologous serum for 20 minutes. Peritoneal cells (0.1×10^6) were plated on round, glass cover slips (12 mm diameter) kept in a 24-well cell culture plate and this plate was incubated for 2 hours at 37°C, 5% CO₂ for macrophage adherence. After incubation, coverslips were washed twice with PBS (pH 7.2) and 200 μ l yeast suspension (1 × 10⁶/ml in PBS) was added and the plates were again incubated for 1 hour at 37°C and 5% CO2. This was followed by washing twice with PBS (pH 7.2), fixing in methanol, staining with Giemsa and mounting on slides in glycerol. Phagocytic index was estimated by calculating the number of yeasts phagocytosed/number of macrophages undergoing phagocytosis in a population of 300 cells per slide. Five slides were prepared for each group.

Cytokine levels

Serum levels of IL-1 β and GM-CSF were estimated using ELISA kits (BD OptEIA) following the manufacturer's protocol. Briefly, diluted (4 ×) sera samples were analysed on capture antibody-coated 96-well plates. After recommended washing followed by incubation with detection antibodies, plates were developed with O-phenyl diamine (OPD) substrate for horse-radish peroxidase activity. Plates were read at 490 nm on a spectrophotometer. The results were compared with recombinant protein standard curves of both cytokines.

FRAP (ferric reducing ability of plasma)

Anti-oxidant capacity of RTc was evaluated by FRAP assay as described by Benzie *et al.*²⁴⁾ Briefly, blood drawn through heart puncture from mice was heparinised and centrifuged at 2000 × g for 10 min at 4°C to separate the plasma and stored at -80°C. The assay was performed on thawed samples within a week. Ten microlitres (µl) plasma and 40 µl of double-distilled water were added to 200 µl of FRAP reagent, freshly prepared by mixing 25 ml acetate buffer (3.1 g sodium acetate and 16 ml acetic acid per litre buffer, pH 3.6), 2.5 ml TPTZ (0.01 M in 0.04 M HCl) and 2.5 ml ferric chloride hexahydrate (0.02 M). After 10 min of incubation, optical density was read at 593 nm. A standard curve was plotted using 100 to 1000 µM FeSO₄.7H₂O.

Statistical Analysis

The data obtained from triplicate set of each experiment were pooled and subjected to student's *t*-test ($p \le 0.05$). Data analysis was performed using Microsoft Excel.

L. Singh et al.

RESULTS

Polyphenolic Content

Total polyphenolic contents of RTc were estimated to be $37 \pm 2.7 \ \mu g$ gallic acid equivalents/mg of extract.

TLC Analysis

The TLC chromatogram of RTc revealed a total of 7 components under the given conditions (Fig. 1). The active components were mainly flavones and its derivatives (3-glycosides, flavonones, and flavonols). A quercitin related compound was found with an R_f value of 64 (table 1).



Fig. 1. Master key obtained from two-dimensional TLC chromatogram of RTc developed in BAW (butanol: acetic acid: water; 4:1:5) and 5% acetic acid. The run was performed on a 0.25 mm silica gel-60 coated TLC sheet (10×10 cm).

Table 1. Components of RTc found after TLC separation. Extract was dissolved in 50/50 (v/v) aqua-alcohol and chromatographed on 0.25 mm silica gel-60 coated TLC sheet for 10×10 cm length. R_f values obtained were compared with available literature.

Colour properties of components in UV light				
Component No.	R _f ×100 (BAW)	Colour in UV light		
		Alone	With Ammonia	Indication
1	40	Pale yellow	Bright yellow	Flavonol-3-glucoside
2	49	Bright yellow	Pale yellow	Flavonone
3	49^*	Violet	Bright violet	Glycoflavonol
4	50	Pale yellow	Bright yellow	Flavonol-3-glucoside
5	64#	Pale yellow	Bright yellow	Flavonol-3-glucoside (quercetin related derivative)
6	69	Bright yellow	No change	Flavone.flavonol
7	71	Pale yellow	No change	Flavonol-3-glucoside

• = UV (254 nm)

unmarked = UV (365 nm)

appears both at 254 nm and 365 nm

Radioprotection by Tinospora Cordifolia

Spleen Weight

As described in material and methods section, spleen weight was expressed as mean spleen weight/g body weight of the animal. Data obtained was normalised against respective untreated control values and expressed as percentage (Fig. 2). Administration of RTc alone resulted in 31.13% increase of spleen weight than untreated control within 24 hours. Radiation exposure alone decreased spleen weight significantly (p < 0.01) to $49.2 \pm 10.3\%$ of untreated control values by 24 hours. By 48 hours after irradiation, however, the spleen weight recovered to 62.7% of untreated controls. RTc administration (200 mg/kg body weight), one hour prior to irradiation did not manifest any significantly observable changes in spleen weight for the first 24 hours. However, by 48th hour after irradiation, spleen weight recovered significantly (p < 0.01) to 92.8 \pm 12.1% of values for untreated control.



Fig. 2. Effect of RTc administration (200 mg/kg body weight, -1 hr) on spleen weight in γ -irradiated mice. Spleens were excised 24 or 48 hours post-treatments and relative weights (mg spleen wt./g body weight) were recorded. Data is normalized against control values and expressed as percentage of control. Values from three independent experiments having five animals per group are expressed as mean \pm SD. * = p < 0.01 is a representation of the difference between RTc + 2 Gy and 2 Gy group at 48 h..

Splenocyte Count

The total mean number of splenocytes obtained was normalized against the control data and was expressed as percent of control (Fig. 3). Administration of RTc alone led to an increase in splenocyte count (20% higher than untreated control). In irradiated mice, the count significantly (p < 0.01) decreased to 39.7 ± 4.3% of values for untreated control in the first 24 hours after exposure. This decreased count improved significantly (p < 0.01) by 48 hours after irradia-



309

Fig. 3. Effect of RTc administration (200 mg/kg body weight, - 1 hr) on total splenocyte count in γ -irradiated mice. Spleens were excised 24 or 48 hours after various treatments. Cell counting was performed on a Neauber's chamber. Data is presented as mean ± SD of percentage of control from three independent experiments having five animals per group. Differences was compared between untreated group and 2 Gy irradiated group (a) as well as between RTc + 2 Gy treated group and 2 Gy irradiated group (b) using student's test * = p < 0.01, ** = p < 0.001.

tion (51.1%). Administration of the RTc prior to irradiation retarded the fall in splenocyte count and by the 48th hour, the count was $62.7 \pm 3.1\%$ of control, much higher (p < 0.001) than the irradiated group.

Splenocyte Proliferation

Proliferation was assessed using MTT reduction assay. In the first experiment (Fig. 4a) RTc induced significant proliferation (p < 0.01) in splenocytes in a dose-dependent manner, which was highest at 50 µg/ml (9.5 fold). In the second experiment (Fig. 4b), a comparison between the maximum proliferative concentration (50 µg/ml) of RTc and known mitogens (LPS and Con A) was performed. In this experiment RTc showed a significantly (p < 0.005) higher mitogenic potential as compared to Con A, but lower than LPS (Con A < RTc < LPS).

DNA Fragmentation

In animals with RTc treatment alone (Fig. 5), fragmented portion of DNA after 24 hours was 7.2%. Radiation exposure led to a significant rise (p < 0.01) in DNA fragmentation (42.8%), whereas administration of RTc before irradiation reduced DNA fragmentation significantly (p < 0.05) to 20.4%. No significant difference in fragmented portion of DNA was found in the 48 hour samples.



Fig. 4. Effect of RTc on splenocyte proliferation. Cells from untreated control mice were used and proliferation was measured using MTT reduction assay, 48 hours after various treatments. a. Effect of different concentrations of RTc on proliferation of normal mouse splenocytes. Differences between proliferation levels of untreated control wells and RTc treated wells were compared using student's t-test. * = p < 0.01, b. Comparison of mitogenic potentials of RTc with known mitogens (10 µg/ml Con A and 10 µg/ml LPS) in splenocytes irradiated with 2 Gy gamma radiation. Data is expressed as mean ± S.D from three wells per sample. Three experiments were performed independently. Difference in proliferation between Con A and RTc treated groups was performed using student's test. # # = p < 0.005.

Apoptosis

Analysis for apoptosis in splenocytes showed 19.03% rise (p < 0.01) in irradiated group 24 hours after exposure (Fig. 6). RTc treatment prior to irradiation decreased apoptosis significantly (p < 0.05) to 2.79%. RTc itself did not cause any measurable apoptosis. Apoptosis in all samples had subsided by 48 hours.

Macrophage Functions Adherence

RTc administration (alone) marginally increased adherence of macrophages to 103% of control group by 24 hours (Fig. 7a). Radiation exposure significantly decreased adhered cell percentage (75.5% of the control value; (p < 0.001). Adherence further decreased in radiation group to 65.65% of control value by the 48th hour after irradiation (p < 0.01). Administration of RTc significantly increased (p < 0.001) adherence to 120% of control by 24 hours, although

L. Singh et al.



Fig. 5. Effect of RTc administration (200 mg/kg body weight, - 1 hr) on DNA fragmentation in splenocytes. Spleens were excised 24 hours after various treatments. DNA fragmentation was assessed by DPA method (see material and methods sections for details). Values are expressed as mean \pm S.D of five independent experiments each having five animals per group. Differences in % DNA fragmentation were compared between untreated group and 2 Gy irradiated group (a) as well as between RTc + 2 Gy treated group and 2 Gy irradiated group (b) using student's test. * = p < 0.01., # = 0.05.



Fig. 6. Effect of RTc administration (200 mg/kg body weight, - 1 hr) on apoptosis in splenocytes. Spleens were excised 24 hours and 48 hours after various treatments and apoptosis was analysed using flow cytometer (BD Facscan). a). Analysis of total DNA content using Cell Quest software. b). Bar diagram presentation of apoptosis at 24 hour. Differences in apoptosis level were compared between untreated group and 2 Gy irradiated group (I) as well as between RTc + 2 Gy treated group and 2 Gy irradiated group (II) using student's test.* = p < 0.01., # = p < 0.05.

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Fig. 7. Effect of RTc treatment (200 mg/kg body weight, - 1 hr) on adherence and spreading of resident peritoneal macrophages (see material and methods for details). a). Adherence expressed as percentage of control. b). Spreading expressed as percentage of control. Data presented is mean \pm SD of five independent experiments with five animals per group. Differences in both adherence and spreading were compared between untreated group and 2 Gy irradiated group (I) as well as between RTc + 2 Gy treated group and 2 Gy irradiated group (II) using student's test.* = p < 0.001., ** = p < 0.001., # = p < 0.05.

adherence decreased slightly in this group by the 48th hour, it still remained higher than control values.

Spreading

Radiation exposure decreased the average size of macrophages significantly (p < 0.05) to 8.27 ± 0.5 µm equivalent to 64.3% of control values by the 24th hour after irradiation (Fig. 7b). Spreading further decreased to 7.94 ± 1.17 µm by the 48th hour, which corresponded to 57.3% of control values. RTc administration before irradiation increased the size of macrophages to 15.16 ± 2.7 µm (109% of control) by 24 hours, which was significantly higher (p < 0.001) than radiation group. Spreading was marginally higher in this group at 48th hour (15.4 µm, 111.7% of control). Administration of RTc alone caused a marginal decrease in spreading at 24 hours (11.35 \pm 2.43 µm, 81.8% of control). At 48 hours, however, there was a significant (p < .0.05) decrease in spread size (8.5 \pm 1.935 µm, 61.6% of control).

Phagocytosis

RTc administration decreased phagocytic index (20.5 \pm 1.75) significantly as compared to control values (p < 0.01) at both 24 and 48 hour intervals after treatment (Fig. 8). Irradiation increased phagocytic index significantly to 67.7 \pm 2.41 (p < 0.05) by 48 hours as compared to control values (43.7 \pm 0.17). Administration of RTc before irradiation led to increased phagocytic index (92.6 \pm 0.16) at 24 hours (p < 0.001).



Fig. 8. Effect of RTc treatment (200 mg/kg body weight, - 1 hr) on phagocytic capacity of resident peritoneal macrophages. Macrophages were isolated 24 and 48 hours after various treatments. Phagocytosis was performed using yeast as substrate. Ingestion index was calculated as percentage of the ratio of the number of yeast phagocytosed to the number of cells undergoing phagocytosis. Values are expressed as mean \pm SD of percentage of control from five independent experiments each having five animals per group. Differences in phagocytic index were compared between untreated group and 2 Gy irradiated group (a) as well as between RTc + 2 Gy treated group and 2 Gy irradiated group (b) using student's test ** = p < 0.001., # = p < 0.05.

L. Singh et al.

Serum Cytokine Level $IL-1\beta$

By 24 hours irradiation (10 Gy) increased IL-1 β levels to 59.5 ± 1.7 pg/ml, that was significantly higher (p < 0.05) as compared to untreated control (30.1 ± 2.7). At 48 hours there was a decline that recovered to 56 ± 1.7 by 72 hours (Fig. 9a). A single dose of RTc alone, increased the levels of IL-1 β progressively from 24 h to 72 h. Just as in radiation group, in RTc pre-administered and irradiated group, the levels of IL-1 β decreased at 48 hours, but recovered to some extent by 72 hours (59 ± 1.8). The values, however, were also significantly higher than irradiated group (p < 0.01) at all intervals.



Fig. 9. Effect of RTc on cytokine levels in serum of mice. Serum was obtained from blood sample of treated animals. Two cytokines were analysed by ELISA. (i) IL-1 β levels (ii) GM-CSF levels. Data are mean \pm SD from five animals per group, the experiments being performed thrice (n = 15). Differences in cytokine expression were compared between untreated group and RTc (alone) treated group (a), between untreated group and 2 Gy irradiated group (b) and between RTc + 2 Gy treated group and 2 Gy irradiated group (c) using student's test * = p < 0.01., ** = p < 0.001., #= p < 0.05.

GM-CSF

GM-CSF levels in irradiated mice increased significantly (p < 0.05) by 24 hours (58.2 \pm 5.3 pg/ml) as compared to untreated controls (23.8 \pm 4.9; Fig. 9b). The levels, however, declined significantly thereafter upto 72 hours (53.1 \pm 2.6; p < 0.005). In RTc (alone) treated group GM-CSF level was significantly higher (63.31 \pm 1.94; p < 0.05) at 24 hour interval as compared to irradiated group and it remained significantly higher than this group at all time intervals (24, 48 and 72 hours). Administration of RTc before irradiation increased GM-CSF levels significantly (p < 0.05) by 24 hours (65.5 \pm 1.8 pg/ml) and it remained significantly higher in this group as compared to irradiated group.

FRAP

Radiation led to a significant (p < 0.01) decrease in antioxidant potential of plasma by 8 hours ($32.4 \pm 2.7 \text{ Fe}^{2+}$ equiv.), though it recovered completely by 48 hours (Fig. 10). In RTc alone treated group, there was a decline in antioxidant potential at 8 hours but it was insignificant as compared to untreated control ($132 \pm 3.7 \text{ Fe}^{2+}$ equiv.). However, 24 hours and thereafter the values increased significantly (p < 0.01). RTc administration before irradiation significantly (p < 0.01) countered radiation induced decrease by 8 hours



Fig. 10. Effect of RTc administration on the anti-oxidant activity of blood plasma. FRAP assay was performed in plasma obtained from heparinised blood of variously treated animals sacrificed at each time point. Data represents mean \pm SD from five animals per group. Experiments were performed three times. Differences in FRAP values measured as μ M FeSO₄ equivalents were compared between untreated group and 2 Gy irradiated group (a) as well as between RTc + 2 Gy treated group and 2 Gy irradiated group (b) using student's test * = p < 0.01, ** = p < 0.001.

 $(74.2 \pm 4.2 \text{ Fe}^{2+} \text{ equiv.})$. At later time intervals there was complete recovery and it rather exceeded the control values.

DISCUSSION

Irradiation damages many crucial systems in an organism simultaneously. Successful treatment of these systems is, therefore, required for radioprotection. Aqua-alcoholic extract of T.cordifolia (RTc) has been reported to provide significant radioprotection in lethally irradiated mice, thus conferring protection at the organism level.¹¹⁾ The present study was undertaken to evaluate its anti-oxidative potential in mitigation of radiation-induced oxidative damage. Effects of RTc on splenocytes and peritoneal macrophages along with cytokine expression in mice were also studied to examine the changes in immuno-competence after RTc treatments. Spleen weight and splenocyte counts are known to decrease in whole-body irradiated (2 Gy) mice.²⁵⁾ This immunosuppressive action of radiation is exploited in host conditioning for allografts.³⁾ In order to understand the effect of RTc on splenocytes and peritoneal macrophages, gamma radiation at a dose of 2 Gy was used. Radiation caused a significant (p < 0.01) decrease in spleen weight in the first 24 hours (47.6% of control), which recovered close to untreated control values by 72 hours (Fig. 2). Administration of RTc before irradiation countered radiation-induced loss of spleen weight (Fig. 2); that could be correlated with inhibition of radiation-induced depletion of splenocyte count (Fig. 3). Administration of RTc alone increased the splenocyte count significantly (p < 0.001). This increase can be attributed to increased proliferation of these cells as seen by results from MTT reduction based proliferation assays of splenocytes isolated from untreated control mice (Fig. 4). We observed a dose-dependent increase of splenocytes in RTc treated group, indicating its mitogenic potential. In fact, molecules like arabinogalactan, extracted from T.cordifolia, have been shown to exhibit mitogenic activity.²⁶⁾ Radiation is known to cause apoptotic cell death in spleen.²⁷⁾ Our observations corroborated the radiosensitivity of splenocytes; 19.03% of splenocytes isolated from animals 24 hours post-irradiation underwent apoptosis (Fig. 6), which was also shown by similar observations in DNA fragmentation (42.8%, Fig. 5). Administration of RTc before irradiation reduced apoptosis and DNA fragmentation substantially (2.79% and 20.4% respectively). Thus RTc protected splenocytes in two ways, by stimulating their proliferation and by decreasing apoptosis during cell repair from irradiation-induced damage.

Studies on the effects of radiation on resident peritoneal macrophages have revealed their radio-resistant nature.²⁸⁾ However, in addition to radio-resistance the functional capacity of macrophages has also been reported to alter by radiation.^{29,30)} Our studies demonstrated that adherence, spreading and phagocytic capacities, which are known parameters used to evaluate monocyte/macrophage func-

tion,²²⁾ were altered by irradiation. Radiation exposure decreased the ability of macrophages to adhere and spread on cover slips, an effect that was significantly reduced (p > 0.001) in RTc administered group (Fig. 7a & b). Adherence and spreading of macrophages requires the cytoskeleton components like actin and tubulin to be intact, and their alterations could influence the cell function adversely.³¹⁾ RTc treatment retarded the radiation-induced adverse effects on these components and could therefore increase adherence and spreading.

Our results on phagocytic activity of peritoneal macrophages showed that radiation exposure increased phagocytosis of macrophages (Fig. 8). RTc treatment before irradiation further enhanced this activity. This effect of RTc may be efficient and hasten clearance of dead cells and stimulate replenishment by new ones, thus leading to faster hemopoeitic recovery. The mechanism of such stimulation of phagocytic activity needs to be investigated further. Administration of RTc alone, however, decreased macrophage activation. According to Kapil and Sharma,²²⁾ administration of fractions isolated from T.cordifolia caused a decrease in the in vitro complement-dependent haemolysis of SRBCs (sheep RBCs) by inhibiting the C3 convertase enzyme of the classical complement pathway. RTc may possibly decrease this enzyme and thus reduce phagocytosis per se. In fact, we have observed decreased phagocytic activity (Fig. 8) that may be associated with decreased C3 convertase due to RTc treatment.

The results of cytokine activities (Fig. 9a & b) revealed that administration of a single dose of RTc before irradiation increased endogenous production of IL-1ß and GM-CSF in the serum of lethally irradiated mice. IL-1ß has been reported to induce radioprotection in mice against hemopoeitic depression.^{13,14)} Antibodies administered against this cytokine or its receptor has also resulted in decreased survival in irradiated animals.¹²⁾ Irradiation is known to cause neutrophilia which results in increased phagocytosis for several hours post-irradiation.³³⁾ This increased phagocytic activity was associated with elevated levels of IL-1ß at 24 hours (Fig. 9a). At 48 hours and thereafter, IL-1 β levels declined and then, recovered to normal levels. RTc administration before irradiation increased the basal level of IL-1B to higher values than in irradiated group. This elevated state of IL-1ß increased phagocytic activity (Fig. 8) could help faster clearance of dead cells and microorganisms. Thus the replenishment of lost bone marrow cells may be achieved faster. RTc also enhanced GM-CSF levels in serum that may promote faster recovery of hemopoeitic tissue. Also, a consistent increase in GM-CSF levels (Fig. 9b) could result in faster hemopoeitic recovery over a longer period. RTc has been shown to decrease micro-nuclei formation in bone marrow cells.¹¹⁾ This property of RTc to increase GM-CSF levels assured proper hemopoeitic recovery. Enhanced levels of hemopoeisis by RTc administered before irradiation in mice,

L. Singh et al.

in terms of endogenous CFU assay in spleen, have also been reported earlier.¹¹⁾ This increased cytokine levels, thus, unravel the mode of radioprotective action of RTc to some extent.

In order to assess the total anti-oxidant status of blood plasma after irradiation we adopted the FRAP assay. RTc administration countered the radiation-induced decrease in the anti-oxidative activity by 8 hours and restored the level to control values by 24 hours. Under *in vitro* conditions, RTc is reported to act as a free-radicals scavenger.¹⁰⁾ Probably its ability to enhance anti-oxidative activities in plasma is related to its free-radical scavenging property. Our study showed that RTc imparted radioprotective effects at cellular and molecular levels. It has been shown earlier that *Tinospora cordifolia* scavenged free-radical generation under *in vitro* conditions.¹⁰⁾ Our results on FRAP assay also corroborated these findings (Fig. 10).

Diverse modes of action of *Tinospora cordifolia* like activation of macrophages (attenuation of radiation-induced decrease in adherence and spreading), reduced apoptosis and increased cell proliferation and increased IL-1 β and GM-CSF levels may be responsible for impressive radioprotective efficacy. Further studies are required to unfold other intriguing intricacies regulating the process of radioprotection.

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