National Center for Radiation Research and Technology (NCRRT)[1], Department of Biochemistry, Cairo and National Research Center (NRC)[2], Department of Pharmacology, Cairo, Egypt

Effect of *Ginkgo biloba* extract on carrageenan-induced acute local inflammation in gamma irradiated rats

I. Hedayat[1], O. M. E. Abdel Salam[2], A. R. Baiuomy[2]

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Omar M. E. Abdel Salam (MD, Ph.D.), Department of Pharmacology, National Research Centre, Tahrir St., Dokki, Cairo, Egypt

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The effect of low dose whole-body gamma irradiation on inflammation and its possible modulation by *Ginkgo biloba* extract (GbE) was studied in the carrageenan-induced paw oedema model. Rats were subjected to two doses of gamma radiation (2 Gy or intermittent radiation at 2 Gy increment delivered daily up to cumulative dose of 4 Gy), 4 h before unilateral subplantar injection of carrageenan. The effect of GbE (25 or 50 mg/kg) administered subcutaneously daily for 3 days was also studied. Local oedema (days 1–3), the content of gamma glutamyl transpeptidase (GTT), malondialdehyde (MDA) and glutathione (GSH) in paw (72 h), were determined. In rats subjected to 4 Gy fraction, paw oedema was significantly reduced 1–4 h post-carrageenan injection (−26.2 to −16.2% vs control group). Moreover, at 24, 48 and 72 h after carrageenan, paw oedema was much reduced in the 2 Gy (−33.6, −46.4, −40%) or 4 Gy (−55, −56, −71.8%) irradiated groups compared to carrageenan unirradiated control. In addition, in irradiated rats, the carrageenan oedema was further significantly reduced by the administration of GbE, the effect of the agent being more marked in those irradiated with 2 Gy fraction. Changes in paw oedema were matched by a reduction in GGT and MDA paw tissue levels, while GSH content decreased in inflamed paw tissue 72 h post-treatment. These results indicate that exposure to 4 Gy fraction decreased the carrageenan-induced paw oedema and that the administration of GbE further lessened the severity of this inflammatory response in irradiated rats. The effects observed may be related in part to the inhibition of GGT activity and MDA production, and partly to augmentation of GSH content in the inflamed paw tissue.

1. Introduction

Variable effects have been reported in literature as regards to the effect of radiation on inflammation. At high doses, radiation is generally pro-inflammatory. On the other hand, low dose radiation has a long history of use in the treatment of inflammatory disease. This suggests the involvement of multiple mechanisms that may operate differentially at different dose levels (Pojonk et al. 2001). The efficacy of low dose radiotherapy in the treatment of painful osteoarthitis is well known and is regarded as a very effective treatment for pain relief (Keilhoz et al. 1998). Since the total doses of treatment are in the order of 5–10% of curative radiotherapy doses given for cancer, it is very unlikely that the same radiobiological mechanisms are involved. Experimental investigations to detect possible mechanisms involved remain scarce (Trott 1994). High radiation doses have been shown to induce the expression of pro-inflammatory cytokines in endothelial cells and other cell lines (Eissner et al. 1996). Hanning et al. (2000) hypothesized that high doses of gamma radiation increased cell membrane permeability leading to osmotic swelling and vascular transudation. However, a dose dependent modulation of the nitric oxide (NO) pathway was observed with significant inhibition by the low radiation doses used in anti-inflammatory radiotherapy but with super stimulation by the high radiation doses used in cancer radiotherapy (Hildebrandt et al. 1998). Nitric oxide (NO), is produced also during the course of variety of rheumatic diseases (McCartney-Francis et al. 1993). In experimental animal models, inhibition of NO production attenuates rheumatoid arthritis (Clancy and Abramson 1995) and osteoarthritis (Pelletier et al. 1997).

Inflammation occurring during free radicals generation is associated with cyclooxygenase and lipoxygenase enzymes acting on arachidonic acid in cell membrane. These enzymes oxidize arachidonic acid and form potent pro-inflammatory metabolites, including prostaglandins, leukotriens and thromboxans. Many flavonoids exhibit inhibition of cyclooxygenase and lipooxygenase which seems to be related to their antioxidant activities (Rui 1991). Recent studies have revealed that extract of *Ginkgo biloba* contains many different flavones, glycosides and terpenoids, possessing free radical scavenging properties, a releasing effect on vascular walls, an antagonistic action on platelet-activating factor, an improving of microcirculation and inhibitory effect on cyclooxygenase 2 isoenzyme (Louajri et al. 2001).

In a previous study, we have shown that GbE exerted marked anti-inflammatory effects on the carrageenan-induced paw oedema. The agent also increased the anti-oedema effect of indomethacin, celecoxib, rofecoxib, dexamethasone or melatonin, suggesting that it may be of clinical value as an anti-inflammatory and analgesic drug alone or in conjugation with NAISDs (Abdel-Salam et al. 2004).

The present experiments aimed to (1): study the effect of low doses of gamma radiation on the course of inflammation evoked in rat by sub-plantar carrageenan injection and; (2) whether treatment with *Ginkgo biloba* extract (GBE) will reduce the inflammatory response to carrageenan in irradiated rats.

2. Investigations and results

2.1. Carrageenan-induced paw oedema

2.1.1. Effect of radiation

The intraplanter injection of carrageenan elicited an inflammatory response that was characterized in paw oedema. The maximal increase in paw volume was observed 4 h after injection (142.8% vs pre-carrageenan basal value) (Fig. 1). The carrageenan-induced paw oedema was decreased by prior irradiation with 2 or 4 Gy fractions. The effect of radiation was both dose and time-related. During the 1–4 h period following carrageenan injection and corresponding to the time of development of the inflammatory response to the agent, a 16.9% and 26.2% ($P < 0.05$) reduction of paw oedema was observed at 1 h post-carrageenan in rats subjected to 2 or 4 Gy fraction, respectively. Over the 24–72 h after carrageenan, oedema in irradiated rats were substantially decreased compared to the unirradiated carrageenan control group (maximal effect being −33.6, −46.4 and −40% for the 2 Gy treated group and −55.1, −56, −71.8 for the 4 Gy treated group, at 24, 48 and 72 h time points after carrageenan injection, respectively) (two-way ANOVA: treatment effect: $F_{2, 123} = 65.1$; $P < 0.0001$; time effect: $F_{6, 123} = 75.3$; $P < 0.0001$; treatment × time interaction: $F_{12, 123} = 3.8$; $P < 0.001$). Post-hoc comparisons showed significant inhibition of oedema formation by 2 or 4 Gy fraction at 24, 48 and 72 h time points, with those treated with 4 Gy fraction showing significantly less oedema than rats subjected to 2 Gy fraction at these time points (Fig. 1).

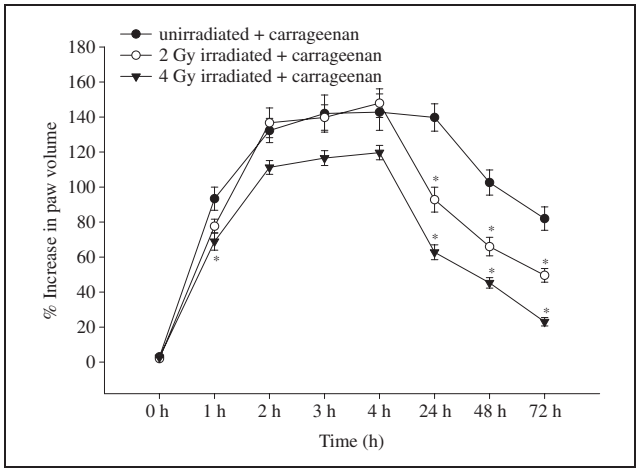

Fig. 1: Effect of low dose gamma radiation on the course of oedema formation in carrageenan injected paw in the rat. Rats were subjected to two doses of gamma radiation (2 Gy or intermittent radiation at 2 Gy increment delivered daily up to cumulative dose of 4 Gy), 4 h before unilateral subplantar injection of carrageenan. Results are expressed as a percentage change from control (pre-carrageenan) values, each point represents mean ± SE of 7 rats per group. Asterisks indicate significant change from corresponding control value at respective time points

2.1.2. Effect of GbE

GbE administered to 2 Gy irradiated rats at time of carrageenan injection at doses of 25 or 50 mg/kg suppressed the paw oedema response (Fig. 2A) (two-way ANOVA: treatment effect: $F_{2, 123} = 107$; $P < 0.0001$; time effect: $F_{6, 123} = 76.6$; $P < 0.0001$; drug × time interaction: $F_{12, 123} = 2.3$; $P < 0.05$). The inhibition of oedema formation by both doses of GbE was most marked at 1–2 h post-carrageenan and maintained throughout the study (−47.6%, −47.4%, −37.4%, −36.3%, −34.2%, −50% for 25 mg/kg GbE and −52.2%, −45%, −37.2%, −36.7%, −33.1%, −35.3, −51% for 50 mg/kg GbE vs control values at 1, 2, 3, 4, 24, 48 and 72 h time points, receptively). Post-hoc comparisons showed significant inhibition of oedema formation by either dose of GbE at all time points.

In 4 Gy irradiated rats, the effect GbE was less than that observed in rats irradiated with 2 Gy fraction, with a max-

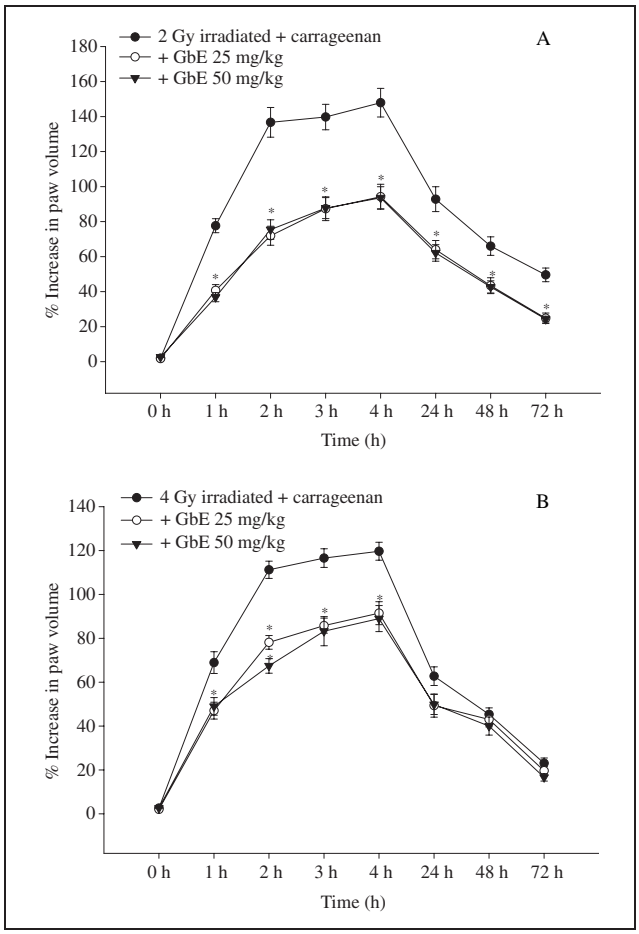

Fig. 2: The suppressive effect of *Ginkgo biloba* extract (GbE) on carrageenan-induced paw oedema formation in rats treated with 2 Gy gamma radiation (A) or 4 Gy gamma radiation (B). GbE was administered s.c., at 25 or 50 mg/kg daily for 3 days, starting at time of carrageenan injection. Results are expressed as a percentage change from control (pre-drug) values, each point represents mean ± SE of 7 rats per group. Asterisks indicate significant change from corresponding control value at respective time points

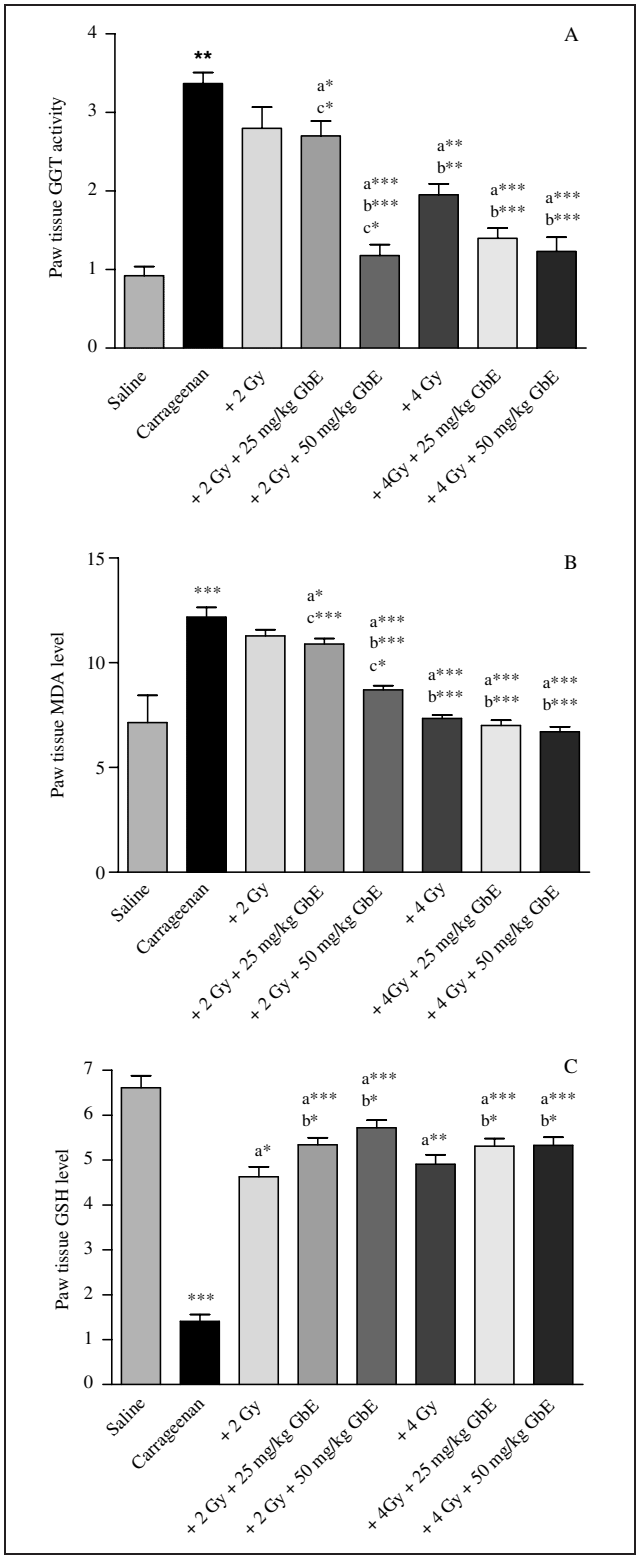

Fig. 3: Effect of radiation and GbE on paw tissue gamma glutamyl transpeptidase (GTT) activity (nmol/min/mg protein) (A), malondialdehyde (MDA) (μmol/g wet weight) (B) and glutathione (GSH) (μmol/g wet weight) (C) levels at 72 h post-irradiation and carrageenan administration. Values are mean ± SE of 7 animals. Values in brackets indicate percentage change from corresponding carrageenan group. Asterisks alone indicate significantly difference between carrageenan group and corresponding saline group ($^{*}P < 0.001$; $^{***}P < 0.001$, Student's t test). Other statistical differences are indicated by the letters [a], [b] and [c] and asterisks beside these letters indicate the level of significance ($^{*}P < 0.05$, $^{**}P < 0.001$; $^{***}P < 0.001$). [a] significantly different from corresponding carrageenan group. [b] significantly different from corresponding carrageenan + 2 Gy group. [c] significantly different from corresponding carrageenan + 4 Gy group. (One way ANOVA and Bonferroni post hoc test for multiple comparisons)

imal reduction of paw oedema of −31.9 to −31.6% and 28.9 to 41% by 25 and 50 mg/kg observed 1–2 h post-carrageenan (Fig. 2B). Two-way ANOVA showed: treatment effect: $F_{2, 123} = 66.2$; $P < 0.0001$; time effect: $F_{6, 123} = 165.1$; $P < 0.0001$; drug × time interaction: $F_{12, 123} = 3.6$; $P < 0.01$). Post-hoc comparisons showed significant inhibition of oedema formation by either dose of GbE at 1, 2, 3 and 4 h time points.

2.2. Biochemical markers of inflammation

At 72 h post-irradiation and injection of carrageenan and/or GBE administration, paw tissues were analysis for the biochemical markers.

2.2.1. Gamma glutamyl transpeptidase

The corresponding paw tissue GGT activities were shown in Fig. 3A. As shown, GGT activity in rats exposed to 4 Gy before carrageenan administration decreased significantly ($P < 0.001$), however there is no significant ($P > 0.05$) change in rats exposed to 2 Gy gamma radiation comparing to rats injected only by carrageenan. Groups treated with 25 mg or 50 mg/kg GbE in parallel with 4 Gy radiation and also rats treated with 50 mg/kg GbE in parallel with 2 Gy radiation showed a significant ($P < 0.001$) decrease in GGT activity when comparing to rats receiving carrageenan only or to rats exposed to 2 Gy gamma radiation. Significant ($P < 0.05$) changes were also recorded in the activity levels of GGT in groups of rats exposed to 2 Gy gamma radiation and treated with 25 mg/kg GbE when comparing to rats injected by carrageenan or to rats exposed to 4 Gy gamma radiation.

2.2.2. Malondialdehyde

The effect of GbE treatment and/or irradiation on the lipid peroxididation measured in terms of MDA in paw tissue is shown in Fig. 3B. A non-significant decrease ($P > 0.05$) is shown in rats exposed to 2 Gy. This decrease was more pronounced ($P < 0.05$) in rats exposed to 4 Gy. Groups treated with 25 mg or 50 mg/kg GbE in parallel with 4 Gy radiation and also rats treated with 50 mg/kg GbE in parallel with 2 Gy radiation showed a significant ($P < 0.001$) decrease in MDA level when comparing to rats receiving carrageenan only or to rats exposed to 2 Gy gamma radiation. Significant ($P < 0.05$) changes were also recorded in the MDA levels in groups of rats exposed to 2 Gy gamma radiation and treated with 25 mg/kg GbE when comparing to rats injected by carrageenan and a highly significant ($P < 0.001$) decrease is also shown when comparing to rats exposed to 4 Gy gamma radiation.

2.2.3. Glutathione

The concentration of GSH was evaluated to estimate endogenous defenses against hydrogen peroxide formation. Fig. 3C show the changes in GSH content evaluated in the paw tissue. A marked decrease in GSH concentration was found in paw tissue of rats injected by carrageenan. Treatment with the two dose levels of gamma radiation and the two concentrations of administrated GbE significantly ($P < 0.001$) inhibited the decrease in GSH levels when comparing to rats injected by carrageenan. This inhibition decreases when rats are exposed only to each dose of radiation without GbE administration.

3. Discussion

The carrageenan induced paw oedema assay is a widely used model for the investigation of anti-inflammatory agents. In this study, the effect of low doses of gamma irradiation as well as GbE administration were assessed on the basis of physical parameter (paw oedema) and biochemical markers in the site of inflammation. The obtained data provided evidence that low doses of gamma irradiation protect against the carrageenan-induced development of acute inflammation for 72 h post-treatment. The study in addition demonstrates a beneficial effect for the administration of GbE on the course of inflammation in irradiated rats.

Variable effects have been reported in the literature as regards to the effect of radiation on inflammation. The effect of radiation on inflammation is dose-dependent, with higher doses given in cancer therapy being associated with exacerbation of the inflammatory response. The pro-inflammatory response is mediated by several mechanisms namely the release of histamine and/or serotonin from mast cells, activation of histamine H_1 receptor, production of nitric oxide and the involvement of kinins and tachykinin (Calixto et al. 2003). On contrary, low dose radiation has an anti-inflammatory effect in acute and chronic inflammatory processes (Hildebrandt et al. 1998). It has been shown that a single external dose of 6 Gy did not induce histological alteration in the synovium of rabbits and decreases general inflammation and possibly also immunological reactions (Steffen et al. 1982). Irradiation reduced the inflammatory proliferation of synovial lining cells, the production of synovial fluid and the swelling of the joint which in turn may have resulted in pain relief (Budras et al. 1986). Also, irradiation with five daily doses of 1 Gy (5×1 Gy) reduced the clinical signs of inflammation and pronounced decreases in the thickness of the synovial membrane in arthritic rabbits (Fischer et al. 1998).

The activities of macrophages are central to the initiation and maintenance of the effector phases of acute and chronic inflammation as well as resolution (Adams and Hamilton 1984). There is evidence that the metabolic activity and proliferation of macrophages are suppressed in a dose dependent fashion by radiation (Van Furth 1979). Reduced adhesion of peripheral blood mononuclear cells to endothelial cells after low doses of irradiation was observed suggesting that this treatment affects the initial step of inflammatory response (Roedel et al. 2002). Hildelrandt et al. (1998) suggested that the suppressive interference of low doses of radiation with the NO pathway may be one of the radiobiological mechanisms that underlies the clinically documented efficacy of anti-inflammatory radiation therapy. Nitric oxide contributes to oedema formation and increase of the vascular permeability (Mulligam et al. 1991) and is also involved in inflammatory pain (Holthusen 1997).

In the present study, 72 h after injection of carrageenan to rats subjected to gamma irradiation and/or administration of GbE, results revealed a parallelism between the decrease observed in the hind paw volume and the two tested biochemical markers, GGT activity and MDA level detected at the site of inflammation. Several authors found that the increase in GGT is associated with an increase in MDA of lipid peroxide content in the exudate of inflamed rat paw and that a number of drugs were able to lower them (Sadique et al. 1998). Gamma-Glutamyl Transpeptidase (GGT) is an enzyme located at the external surface of epithelial cells. It initiates intracellular glutathione breakdown, provides cells with a local cysteine supply and contributes to maintain intracellular glutathione levels. Chikhi et al. (1999) showed that GGT expression is highly sensitive to oxidative stress and that the transcription of the GGT gene from several promoters offers multiple DNA and RNA targets for various oxidative stimuli and contributes to a broad antioxidant cell mechanism defense through GGT induction. Hammarstom et al. (1979) indicated that the GGT activity increases at the site of inflammation because of its role in amino acid transport and protein biosynthesis and also for its role in the formation of leukotrienes which are lipoxygenase metabolites of arachidonic acids. Singh et al. (1986) reported that GGT increased greatly in the site of inflammation *in vivo*, the level of this enzyme increased about 4 fold, 4 h after induction carrageenan acute inflammation. The authors suggested also that the increase in GGT activity is a feature common to all the inflammatory conditions and that the *in vivo* lowering in enzyme activity relates to the anti-inflammatory activity of drugs rather than the direct effect of drugs on GGT activity. Further the activity did not distinguish between steroidal and non-steroidal drugs which explains the decreases in our results of GGT activity in all cases of treatment either by gamma irradiation or by administration of GbE. Results obtained in the present work further suggest that inhibition of GGT *in vivo* may be a valuable biochemical marker in screening and predicting the anti-inflammatory potencies of any treatment. The simplicity and ease of measurement, the stability of the enzyme at high temperature and the low cost of the assay offer additional advantages of the procedure for routine primary screening of newer anti-inflammatory agents.

Lipid peroxidation is considered a critical mechanism of the injury that occurs during inflammation. The evidence supporting these biochemical changes is based on the analysis of the large number of intermediate products. An indicative method, extensively used in evaluating lipid peroxidation is analysis of tissue malonaldehyde. Campo et al. (2003) demonstrated a large amount of malonaldehyde in the articular joints of knee and paw of arthritic rats which is reduced by treatment with glycosaminoglycans. Bilici et al. (2002) observed a significant increase in MDA and NO in the paw tissue of carrageenan treated rats and also a decreased level in glutathione. The authors suggested that in the delayed phase of carrageenan induced oedema, the inflammatory response was linked to the neutrophil infiltration, production of neutrophil-derived free radicals such as hydrogen peroxide, superoxide and OH radicals as well as the release of other neutrophil-derived mediators. When all membrane was attacked by oxygen free radicals in inflammatory tissue during pathological changes of inflammation, peroxidation took place and lipid peroxidation material was produced which regarded to stable MDA which can cross-link to lipids and proteins in cell membrane to make it malfunction (Zhang et al. 2002). Results recorded by Leduc et al. (2002) on MDA content are in accordance with the evolution of paw oedema width during time which supports that MDA reflects the intensity of inflammation. Same results were recorded by Costantino et al. (1998), showing that maximal increase of MDA was observed in association with maximal increase in inflamed paw volume of rats. MDA content markedly lessened in inflamed paw after administration of antioxidative drugs (Li et al. 2001).

Standardized extracts from ginkgo biloba leaves are gaining much interest and are widely used especially in improving cognitive functions in patients with Alzheimer's disease (Oken et al. 1998). GbE contains many flavone

glycosides and terpenoids (van Beek 2002). Besides its direct scavenging action on free radicals, GbE exerts an anti-inflammatory effect (Abdel-Salam et al. 2004; Kim et al. 1999; Kwak et al. 2002). Ginkgetin, a biflavone isolated from *Ginkgo biloba* leaves reduced arthritic inflammation in animal model of adjuvant-induced arthritis (Kim et al. 1999). GBE inhibited the carrageenan-induced paw oedema, suggesting that it may be of clinical value as an anti-inflammatory and analgesic drug alone or in conjugation with NAISDs (Abdel-Salam et al. 2004). Kwak et al. (2002) showed that ginkgetin inhibited the production of prostaglandin E2 by down-regulation of COX-2 expression *in vivo* and exerted an anti-inflammatory activity against skin inflammatory responses. Nishida and Satoh (2003) demonstrated vasodilating actions of GbE and bilobalide, a major constituent of GbE and suggested that this action is due to the inhibition of Ca^{2+} influx through calcium channels and the activation of NO release. Recent studies conducted by Yang et al. (2003) showed a decrease of MDA and NO levels after treatment with GbE in several tissues, supporting that GbE is an active antioxidant and NO inhibitor, protecting cells from injury by inhibiting membrane lipid peroxidation. Horakova et al. (2003) observed also that GbE intensively decreased the content of MDA in rat pheochromocytoma cells treated with hydrogen peroxide.

In conclusion, findings in the present study indicate that exposure to low doses of gamma irradiation decreased paw inflammation caused by carrageenan in the rat. The administration of GbE further reduced the inflammatory response in irradiated rats. It is suggested that GbE may find utility as a pharmacological agent in treating inflammatory conditions in conjunction with low dose irradiation.

4. Experimental

4.1. Animals

Sprague-Dawley strain rats weighing 130–150 g of body weight were used throughout the experiments. Animals were maintained under controlled environmental conditions and received standard diet and tap water *ad libitum*. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

4.2. Irradiation

Whole body Gamma irradiation has been conducted by Cesium-137 ventilated Gamma Cell-40 belonging to the National Center for Radiation Research and Technology (NCRRT), at a dose rate 0.64 Gy min^{-1} at the time of the experiment. Animals were irradiated either with a single dose 2 Gy or received intermittent radiation dose level at 2 Gy increment delivered daily up to cumulative dose of 4 Gy.

4.3. Carrageenan-induced paw oedema

Paw oedema was induced by a single sub-plantar injection of 100 µl of 1% sterile carrageenan lambda in saline into the right hind paw (Winter et al. 1962). Contralateral paw received an equal volume of saline. Paw volume was determined immediately before carrageenan injection and at selected times thereafter with a plethysmometer (Ugo Basile, Milan, Italy). The rats received saline (0.5 ml/rat) or GbE (25 or 50 mg/kg, s.c., 0.5 ml/rat) at time of carrageenan injection (0 time). The oedema component of inflammation was quantified by measuring the increase in paw volume (ml) at 1, 2, 3, 4, 24, 48 and 72 h after carrageenan injection with respect to the pre-injection value for each animal. Oedema was expressed as a percentage of change from control (pre-drug) values.

The animals were categorized in 7 groups each of 7 rats:

Group (1): Rats received carrageenan injection. Group (2): Rats exposed to a single dose of gamma irradiation and received carrageenan injection. Group (3): Rats exposed to intermittent gamma radiation doses and received carrageenan injection. Group (4): Rats exposed to a single dose of gamma irradiation, received carrageenan injection and GbE (25 mg/kg). Group (5): Rats exposed to a single dose of gamma irradiation, received carrageenan injection and GbE (50 mg/kg). Group (6): Rats exposed to intermittent gamma radiation doses received carrageenan injection and GbE (25 mg/kg). Group (7): Rats exposed to intermittent gamma radiation doses received carrageenan injection and GbE (50 mg/kg)

4.4. Biochemical studies

At 72 h post-irradiation and carrageenan-induced inflammation, the animals were scarified. Oedematous tissue of the inflamed paw was taken for biochemical analysis. Paw tissue was rinsed immediately in ice cold distilled water and homogenized in 1.15% KCl containing 0.2% Triton-X. Then the homogenate was centrifuged at $8000 \times g$ for 10 min and the supernatant was obtained. The activity level of gamma glutamyl transpeptidase was measured colorimetrically as described by Boelsterly and Zbinden (1980), malondialdehide level according to Yoshioka et al. (1979) and glutathione content according to the method of Griffith (1980).

4.5. Drugs

Carrageenan was purchased from Sigma, USA. GbE was purchased from EMA Pharm. Co., Cairo, A.R.E. The extract used is a preparation widely used clinically mainly for cerebral and peripheral circulatory disorders. It is a standardized GbE that is similar in composition to that of EGb 761 used in European countries.

4.6. Statistical analysis

The data were expressed as mean ± SEM. The results of carrageenan-induced paw oedema experiments are expressed as a percentage of change from control (pre-drug) values. Statistical analysis of data was performed by using one and two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons. When there were only two groups, Student's t test was used. A probability value less than 0.05 was considered statistically significant.

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