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The Protective Role of Ginkgo Biloba against Radiation Induced Injury on Rat Gastro-intestinal Tract

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GINKGO BILOBA extract (EGb 761) is an antioxidant substance exhibits a wide variety of biological activities. The present study was performed to evaluate oxidative stress and inflammatory parameters of gastrointestinal injury induced by exposing rats to acute doses of γ-rays and the potential value of EGb 761 in preventing changes in these parameters. Male albino rats were treated orally with the extract in a dose of 100 mg/ kg for 7 successive days before whole body exposure to acute radiation levels of 2 and 6 Gray (Gy). Control groups were run concurrently. The rats were sacrificed 3 days after irradiation. Various inflammatory mediators and biochemical parameters were determined in the stomach and intestine. Both tissues were also examined histopathologically. Exposure to radiation led to dose dependent changes in the level of oxidative stress biomarkers (elevation of thiobarbituric acid reactive substance (TBARS) and nitrite associated with a glutathione (GSH) decrease as well as in the level of inflammatory parameters (elevation of Tumour necrosis factor-ά (TNF-ά) and myeloperoxidase (MPO) associated with depletion of prostaglandin E_2 (PGE_2). Pre-treatment with EGb 761 protected against the changes in both oxidative stress biomarkers and inflammatory mediators. EGb 761 exerted a protective effect against the radiation induced gastrointestinal damage, possibly through its anti-inflammatory and anti-oxidant properties.

Keywords: γ-rays, oxidative stress, inflammatory mediators, stomach, intestine, Ginkgo biloba.

Radiation therapy is a widely used therapeutic measure in the management of a wide variety of tumours, but its immediate and delayed side effects on normal tissues limit the effectiveness of therapy. The rapidly proliferating cells of the

gastrointestinal tract are the most sensitive to radiation effects. Regarding the small intestine, acute morphological changes are associated with motor dysfunction **(**Frisby *et al.*, 2007). Acute radiation induced gastro-enteritis is manifested by appetite loss, nausea, vomiting, diarrhoea, salivation, lethargy, dehydration and sepsis (Vitolo *et al.*, 2004). The effects of radiation are caused mainly by the excessive generation of reactive oxygen species (ROS), which interact with biological molecules producing toxic free radicals leading to lipid peroxidation and DNA damage (Agrawal *et al.*, 2001 and Daly *et al.*, 1999). Radiation-induced lipid peroxidation is a free radical process (Sies, 1986) that involves oxidative conversion of polyunsaturated fatty acids to several products including malondialdehyde (MDA) and lipid peroxides (Bakan *et al.*, 2002). Radiation induces an inflammatory response in the target and surrounding normal tissues, and accumulation of leukocytes (Panés and Granger, 1998). TBARS and MPO play a fundamental role in oxidant production by neutrophils, and cause tissue damage (Kettle and Winterbourn, 1997). The tissues most susceptible to radiation are those involving rapidly proliferating cells, such as the bone marrow, skin, hair follicles and the gastrointestinal tract.

The search for safe and effective agents that could potentially be used to reduce the side-effects of radiation on these tissues has concentrated recently on the use of anti-oxidants and other radio protective agents. The present study explores the possible effectiveness of EGb 761 in protecting against radiation damage to the stomach and intestinal tissue. EGb 761 is known to contain about 24% ginkgo-flavone glycosides and 6% terpenoids which confer on it various biological and pharmacological properties, including antioxidant, anti-inflammatory and immuno-modulatory effects (Kusmic *et al*. 2004, Pehlivan *et al.*, 2002, Yirmibesoglu *et al*., 2012 and Zeybek *et al*., 2003). Various parameters that could play a role in the deleterious effects of gamma irradiation on both stomach and intestinal mucosa will be studied in order to evaluate the protective value of the extract.

Materials and methods

Chemicals

EGb 761 was obtained from Al-Ameryia pharmaceutical company, Cairo, Egypt. The extract contains 24% flavone glycosides.

All chemicals and reagents were purchased from Sigma Aldrich (Saint Louis, Missouri, (USA).

Animals and radiation facilities

Male Wistar rats, each weighing 150-180g, were purchased from the animal breeding unit of the National Research Centre (NRC), Giza, Egypt. Rats were housed under appropriate conditions of controlled humidity, temperature and light. The animals were allowed free access to water and were fed a standard pellet rat diet. The rats were acclimatised in the animal facility of the NCRRT, NASR City, Egypt for at least one week before subjecting them to experimentation. Whole body irradiation of animals was carried out at the NCRRT using the Gamma Cell-40 biological irradiator furnished with a Caesium^{137} source, Atomic Energy of Canada Limited; Sheridan Science and Technology Park, Mississauga, Ontario, Canada. The radiation dose rate used was 0.42 Gy/ min.

Study design

Rats were divided into 5 groups (each of 8 rats) allocated as follows; a negative control group (normal non-irradiated animals), two positive control groups irradiated with either 2 or 6 Gy, and two treated groups given EGb 761extract orally (100 mg/ kg body wt) for 7 consecutive days before exposure to 2 Gy and 6 Gy, respectively. Animals were sacrificed 3 days after radiation exposure and the stomach and a segment of the small intestine were dissected out. A small section from the fore-stomach and jejunum was taken for histological examination by transmission electron microscopy while in another section the mucosa of both tissues was scraped off and used to prepare homogenates (10% w/v) for assay of biochemical parameters and inflammatory mediators. GSH was measured according to Beutler *et al.* (1963). The method depends on the reduction of 5,5`-dithio bis (2-nitrobenzoic acid) [Ellman's reagent] by sulfhydryl (SH) moiety of GSH while TBARS measured according to Uchiyama and Mihara (1978). The method depends on colorimetric determination of a pink coloured product, resulting from the reaction of TBARS with thiobarbituric acid in acidic medium, at high temperature. Nitrite content was measured according to Miranda *et al.* (2001) The assay determines total nitrite /nitrate level based on the reduction of any nitrate to nitrite by vanadium

followed by the detection of total nitrite (intrinsic +nitrite obtained from reduction of nitrate) while, MPO content was measured according to Bradley *et al.* (1982), The assay method is based on measuring the hydrogen peroxide-dependent oxidation of electron donor (e.g. halides), catalyzed by MPO. TNF-α and PGE_2 immunoassay measured using ELISA kits**.**

Statistical analysis

Quantitative data were expressed as mean± standard error (S. E.) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. Graph pad software instant (version 2) was used to carry out these statistical tests. The level of statistical significance was taken at $P \leq 0.05$.

Results

Biochemical and inflammatory markers

Exposure to gamma irradiation led to variable changes in the different parameters of oxidative stress measured in the stomach mucosal tissue. GSH levels were markedly reduced from normal by 46% and 77% after exposure to 2 Gy and 6 Gy, respectively.

The reduction in GSH was associated with an increase in level of the other oxidative stress parameters, namely TBARS and nitrite. The former was raised from normal by 27-70% while the latter was raised by 26-56% after exposure to 2 and 6 Gy, respectively (Fig. 1).

Pre-treatment with Gingko extract almost completely protected against the changes induced by 2 Gy but was not as effective in preventing the changes induced by 6 Gy. Nevertheless, the extract reduced the extent of damage induced by the high radiation dose (Fig.1).

The inflammatory markers, MPO and TNF-α, were not significantly affected by exposure to 2 Gy but were raised by 61% and 72% respectively under the higher radiation exposure. The level of PGE_2, on the other hand, was markedly depressed by 46% but only after exposure to 6Gy. Pre-treatment with the extract tended to protect against the changes induced by the high radiation dose in these parameters (Fig.2).

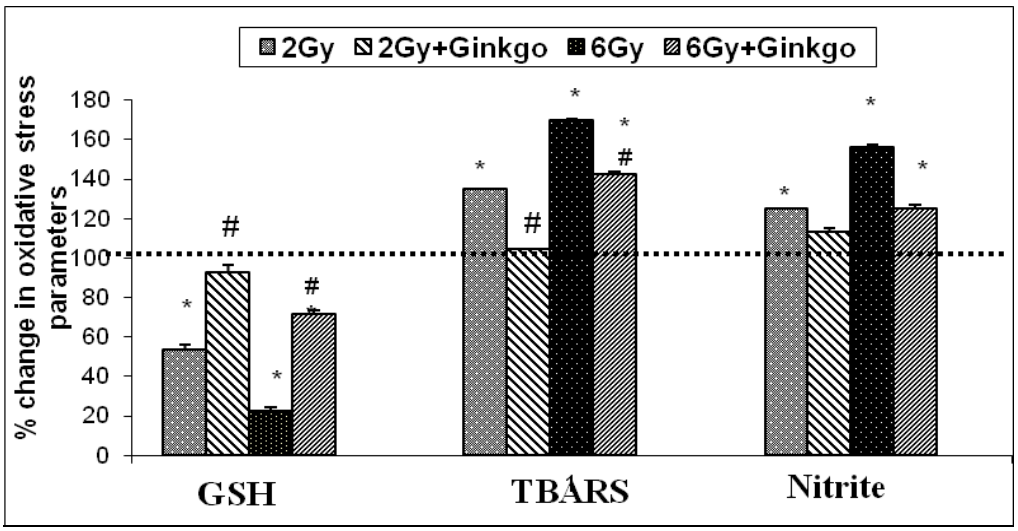


Fig. 1. Per cent change from control (horizontal dotted line) in oxidative stress parameters in stomach mucosa of rats acutely exposed to 2Gy and 6Gy irradiation before and after treatment for 7 successive days with Ginkgo biloba (100mg/ kg body wt).

Values are expressed as means, error bars indicate the standard error of the mean (SEM) for n=8. Data was analysed by one way ANOVA followed by Tukey-Kramer's test. *Significant difference from control group $P< 0.05$. #Significant difference from respective irradiated group $P< 0.05$.

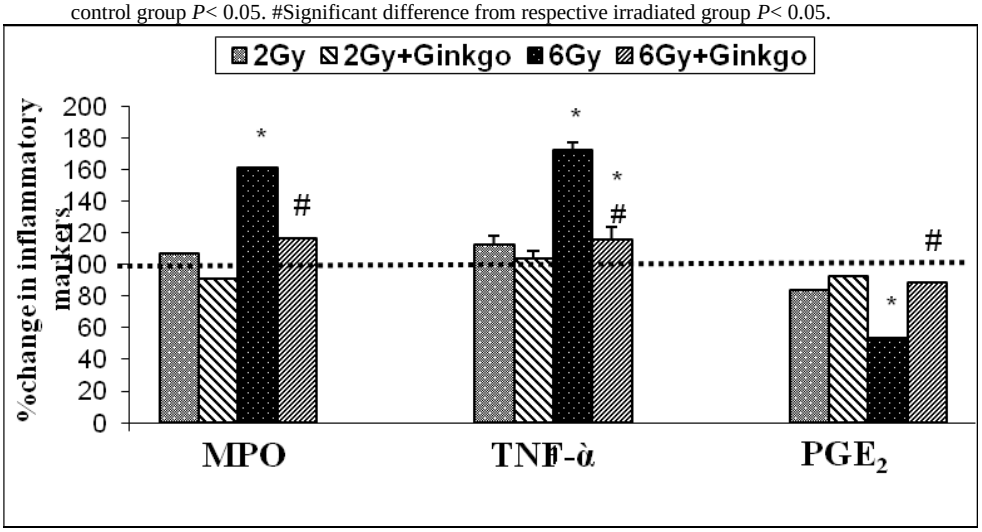


Fig. 2. Per cent change from control (horizontal dotted line) in inflammatory markers in stomach mucosa of rats exposed to acute dose levels of 2Gy and 6Gy irradiation before and after treatment for 7 successive days with EGb761 (100mg/ kg).

Legends as in Fig. 1.

The changes in oxidative stress parameters in the intestinal mucosa ran almost a similar pattern to those in the stomach. Thus, GSH levels were reduced from normal by 37% and 56% while TBARS levels were increased by 28-62% and the nitrite raised by 40-66% after exposure to 2Gy and 6Gy, respectively**.**

Pre-treatment with EGb761 extract protected to different extents against the radiation induced changes. The extract afforded nearly full protection against changes induced by 2 Gy but was not as effective in reversing fully the changes induced by 6 Gy (Fig.3).

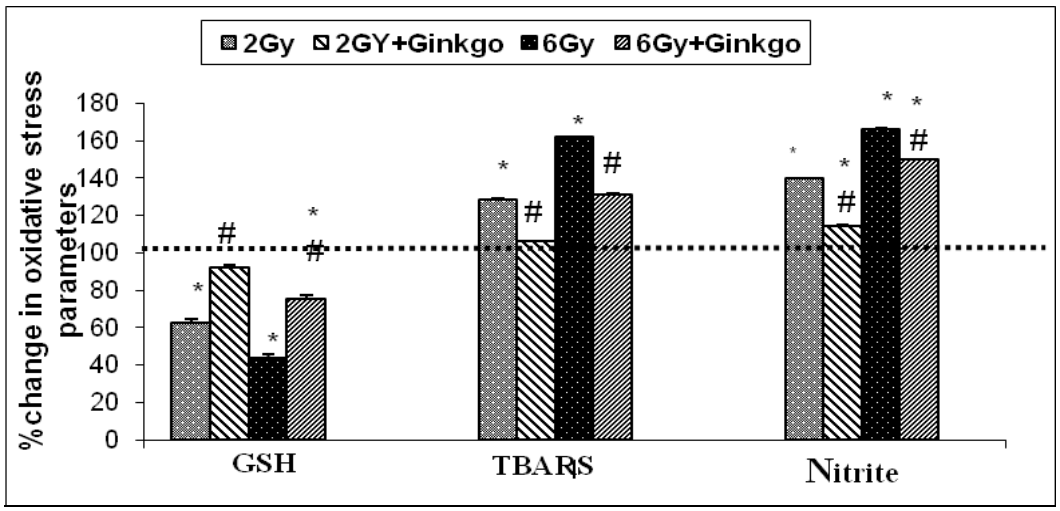


Fig. 3. per cent change from control (horizontal dotted line) in oxidative stress parameters in intestinal mucosa of rats acutely exposed to 2Gy and 6Gy irradiation before and after treatment for 7 successive days with EGb (100mg/ kg).

Legends as in Fig. 1.

Exposure to 2 Gy led to a 52 % increase in MPO activity but did not affect the other inflammatory parameters, while exposure to 6 Gy led to an even higher rise in MPO activity together with a dramatic 2-fold increase in TNF-α and a 50% drop in PGE_2. Pre-treatment of irradiated groups with EGb 761 led to protection against the elevation in the MPO activity, but provided only moderate protection against the changes in both TNF-α and PGE2 (Fig.4)

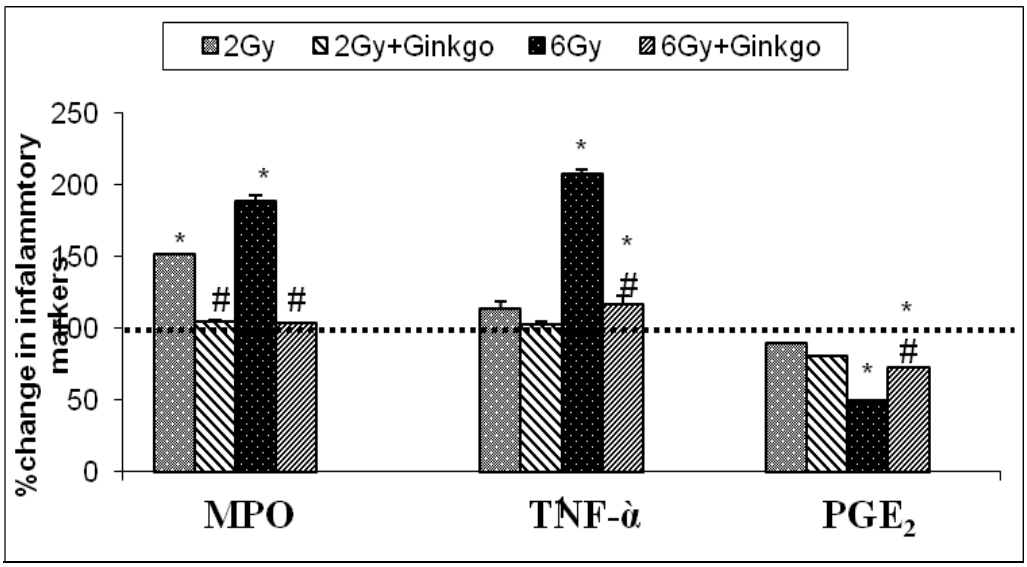
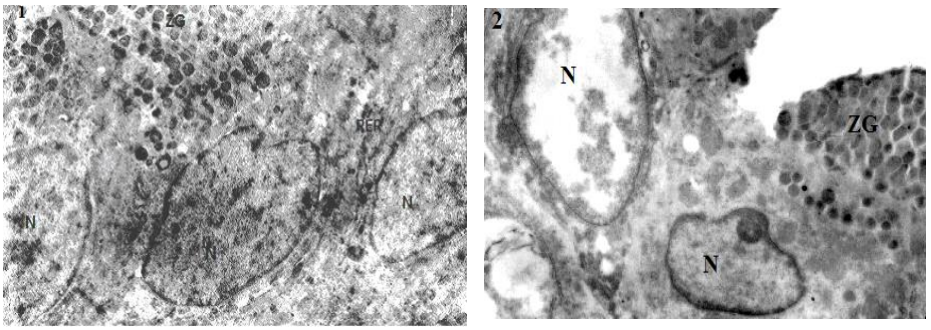


Fig. 4. Per cent change from control (horizontal dotted line) in inflammatory markers in intestinal mucosa of rats exposed to acute dose levels of 2Gy and 6Gy irradiation before and after treatment for 7 successive days with EGb761 (100mg/kg).

Legends as in Fig. 1.

Histological Findings

Normal control sections of the stomach showed regularly arranged cells with basal arrangement of rough endoplasmic reticulum (RER) and membrane bound secretory vesicles crowded in the apical cytoplasm. Irradiation of rats led to degeneration of secretory granules and microvilli. Pre-treatment with EGb761 led to protection against the irradiation induce damage (Fig. 5).



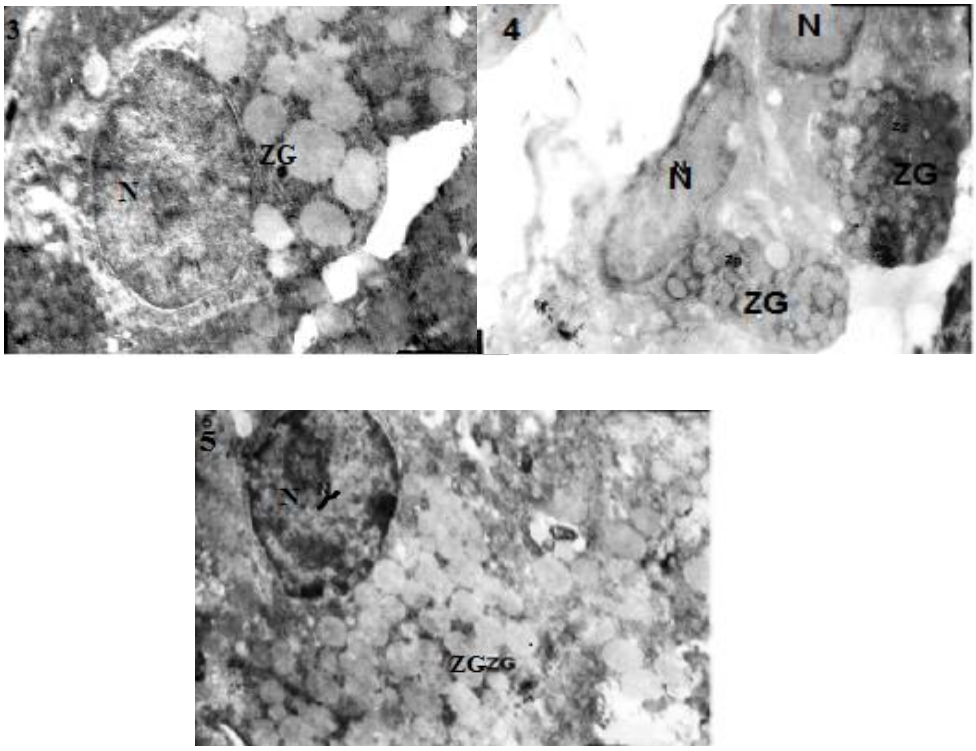


Fig. 5. Electron micrographs of stomach mucosa of: (1) Control rats showing normal structure. (2) Irradiated rats with 2Gy showing degenerative changes in cells, some having an irregular nucleus with peripheral nucleolus (N) or a kyroletic nucleus (N), as well as erosion of microvilli (Arrow) and electron dense secretory granules (ZG). (3) Rats treated with EGb761 prior to irradiation with 2Gy showing regeneration of cells having euchromatic nucleus (N) and apical secretory granules (ZG) and recovery of cisternae of RER. (4) Irradiated rats with 6Gy showing highly degeneration of two cells with necrotic nucleus (N), ill-defined cytoplasmic organelles, secretory granules increased in number with melted structure (ZG), degeneration of microvilli (MV). (5) Rats treated with Gingko prior to irradiation with 6Gy showing regeneration of cells with normal nucleus (N), normal structure of RER and secretory granules (ZG). (X-2800)

The mucosa of the jejunum from normal control animals showed epithelial cells with regularly arranged columnar cells and oval nuculei with euchromatin, goblet cells with normal structure. Mitochondria have a typical cylindrical shape, regular cisternae of RER.

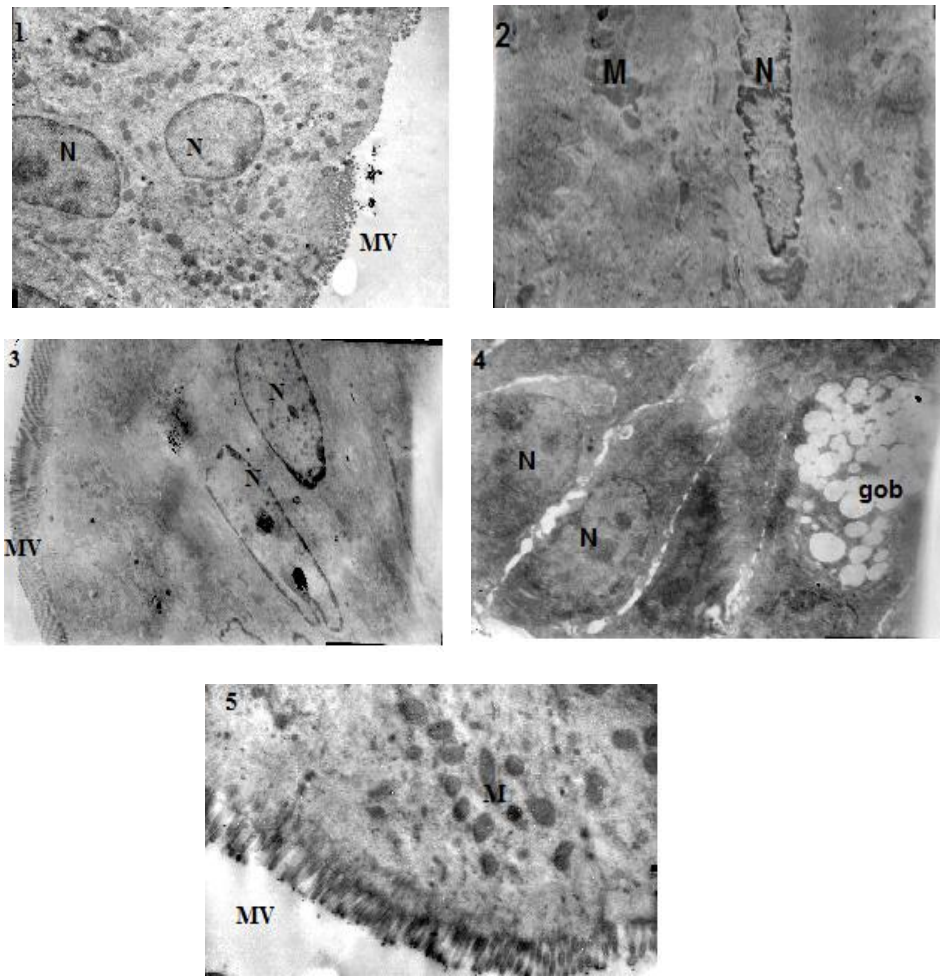


Fig. 6. Electron micrographs of intestinal mucosa of: (1) Control rats showing regular arranged columnar cells with oval nuclei (N), normal structure of microvilli (MV), RER and mitochondria (M). (2) Irradiated rats with 2Gy showing disorganization of columnar cells with clumped chromatin material and abnormal nucleus (N) with irregular nuclear membrane (thick arrow). (3) Rats treated with Ginkgo biloba prior to irradiation with 2 Gy showing normal arrangement of columnar cells with healthy nuclei (N) and euchromatin, normal shape of microvilli (MV). (4) Irradiated rats with 6Gy showing degenerated columnar cells, disorganization of cytoplasmic organelles and active goblet cell (gob). (5) Rats treated with Ginkgo biloba prior to irradiation with 6Gy showing regeneration of cytoplasmic organelles, recovery of mitochondria (M) and microvilli with regular arrangement (arrow). (X-5300)

Irradiation led to disorganization of columnar cells with abnormal nuclei having irregular membrane and clumped chromatin material, dilatation of cisternae of the rough endoplasmic reticulum. It also led to a reduction in the height of the villi, poor differentiation of cytoplasmic organelles and activation of the goblet cells in which the mucus granules showed an irregular honeycomb structure. Prior treatment of the rats with EGb 761 conferred a large degree of protection against the changes in the columnar cells and cytoplasmic organelles induced by 2 Gy, but not as much in animals exposed to 6 Gy. (Fig. 6).

Discussion

The present findings showed that exposure of rats to ionizing radiation led to a dose dependent change in various biochemical and histological parameters in both stomach and small intestine. Similar findings were reported by Driák *et al.* (2008). Many of the damaging effects of ionizing radiation are due to the generation of reactive oxygen and nitrogen species leading to oxidative stress often represented by an increase in level of TBARS (Del Rio *et al.*, 2005) and nitrite (Freeman and Macnaughton, 2000) associated with a decrease in GSH (Navarro *et al.*, 1997 and Ross, 1988). Such changes were indeed evidenced in the present study in both gastric and intestinal mucosal tissue under the influence of ionizing radiation. Pre-administration of EGb 761 afforded protection against the radiation-induced changes in the above parameters. EGb 761 markedly attenuated the GSH depletion and reduced the elevation of nitrite and TBARS levels in both stomach and intestinal mucosa, an effect which may have been due to its ability to enhance the activity of the antioxidant enzymes, superoxide dismutase and glutathione peroxidase (Lin and Chang, 1997). These effects are closely related to the ability of ginkgo-flavone glycosides to capture oxygen-derived free radicals, such as superoxide anions, hydroxyl, peroxyl radicals and nitric oxide (Bridi *et al.*, 2001, Marccoci *et al.*, 1994 and Yirmibesoglu *et al*., 2012), and act as a donor of hydrogen to terminate pathological processes induced by free radicals (Bridi *et al*., 2001 and DeFeudis, 2003).

The changes in oxidative stress parameters were accompanied by a rise in TNF-α, one of the key cytokines involved in inflammatory processes. Many inflammatory responses, particularly in the gut, are mediated by the activation of transcription factors such as nuclear transcription factor kappa B (NF-κB) which in turn plays a pivotal role in the expression of many cytokines involved in gut immune and inflammatory responses. The sequential relation that was observed between radiation-induced activation of NF-κB and increased expression of TNF-

α (Linard *et al.,* 2003) suggest that this activation could play a role in the induction of this cytokine shown in this work. The elevation in the level of TNF-α may have also contributed to the rise in nitrite level (Pall, 2008) observed in the present study.

MPO is an essential enzyme for normal neutrophil function, and when neutrophils are stimulated by irradiation, MPO, as well as other tissue-damaging substances are released from the cells. The enzyme is therefore considered as a quantitative marker of neutrophil infiltration in gastrointestinal tissues (Kettle and Winterbourn, 1997). MPO activity was markedly increased following exposure to whole body γ-radiation, a finding which was previously reported by other authors (Erbil *et al.,* 1998, Sener *et al.,* 2006 and Swantek *et al.,* 2000). Furthermore, the rise in oxidative stress and increase in expression of free radicals has been shown to affect the activity of the constitutive cyclooxygenase enzyme (Gal *et al.,* 1984 and Hemmler and Lands, 1980). This would ultimately lead to a reduction in the level of PGE_2, as was shown in this study, and cause loss of its protective role on the mucosa of both stomach and intestine. Similar findings were reported by Legeza *et al.,* (1994). The fact that EGb761 suppresses the production of reactive oxygen and nitrogen species and suppresses the release of a variety of pro-inflammatory mediators produced by leukocytes and macrophages (Mustafa *et al.,* 2006, Okumus *et al.,* 2011, Yoshikawa *et al.,* 1999 and Zeybek *et al*. 2006) has led to the observed protection afforded by pre-treatment with the extract on the gastric and mucosal tissues. The protective effect of the extract was evidenced in part by the prevention in the rise of MPO activity following irradiation, a sign of reduction of neutrophil infiltration. Similar results had been reported by Otamiri and Tagesson (1989). Further evidence of the observed protective effect of EGb761 may be related its inhibitory influence on TNF-α as well as its protection against the decrease in level of PGE_2. The latter effect is probably related to the flavonoid content of the extract (Chao *et al.,* 2004), which could act by stimulating PGE_2 production by gastric mucosal cells (Beil *et al.,* 1995).

The changes in the different inflammatory mediators measured were associated with various histological changes indicating degenerative processes in both stomach and small intestine. Irregular shaped nuclei with chromatin condensation have been reported by Thomson *et al.,* (1998), epithelial cells frequently losing contact with one other and possessing many lateral and basal projections were documented by Carr (1981) and Fatemi *et al.* (1985), while disruption of intestinal mucosa after 4 days irradiation was reported by Akpolat *et*

al. (2009) and Somosy (2000) deformed villi similar to those seen by electron microscope in this study were also observed by Valk and Hornstra (2000) The observed histological changes could at least partly be attributed to the generation of free radicals causing cell damage and disruption of cell membranes (Valk and Hornstra, 2000). Pre-treatment with EGb 761 resulted in attenuation of tissue damage and reduction in cell infiltration.

The findings of the present study suggest that the extract of EGb 761 exerted beneficial effects against the radiation induced gastrointestinal damage in rats. The protective effect was evidenced both on the biochemical parameters as well as histologically. The extract possibly exerts its action by one or more of several mechanisms, including scavanging oxidative free radicals and downregulating some of the inflammatory mediators involved in the gastrointestinal immune response, such as TNF-α and PGE_2.

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الـدور الوقـائى للجنكـو بيلوبـا ضـد التـأثير الضـار المحـدث بالتعرض للاشعاع الجامى فى الجهاز الهضمى للجرذان

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قسم البحوث الدوائيه الأشعاعيه ، المركز القومى لبحوث و تكنولوجيا الأشعاع ، ص. ب. ٢٩ مدينة نصر ، و *كلية الصيدله ، جامعة القاهره ، مصر.

يشتمل هذا البحث على دراسة تأثير مستخلص الجنكو بيلوبا ضد بعض التغيرات الحيوية نتيجة تعرض ذكور الجرذان لأشعة جاما. و دراسة تأثيره في تقليل او الحماية من التأثير الضار للاشعاع فى الجهاز الهضمى للجرذان المشعة. و قد تم اعطاء ذكور الجرذان مستخلص الجنكو بيلوبا بجرعة ١٠٠ مجم/ كجم لمدة ٧ ايام متتالية قبل التعرض الكلى للجرذان بجرعتى إشعاع حادة من ٢ و ٦ جراي و بعد مرور ٧٢ ساعة تم فصل كلا من المعدة و جزء من الامعاء الدقيقة (الصائم) و فحصهم لقياس محتوى مالوندای الدهايد و جلوتاثيون المختزل و محتوى النيتريت (كدليل على التوتر الأوكسيدي) و كذلك انزيم ميلوبروكسيداز كما تم قياس محتوى طبقة الغشاء المخاطي للمعدة و الامعاء من البروستاجلاندين-هـ٢. و كذلك محتوي سيتوكينات الإلتهاب و هي: عامل الورم النخرى-ألفا و كذلك تم فحص الانسجة باستخدام الميكروسكوب الالكترونى. و لوحظ ان التأثير الضار للتعرض للاشعاع بجرعة ٦ جراى اعلى بطريقة ملحوظة من ذلك المحدث نتيجة التعرض لجرعة ٢ جراى. و قد أدى التعرض للاشعاع إلى زيادة التوتر التأكسدى نتيجة انتاج كلا من شقائق الاكسجين و النيتروجين الحرة و ذلك يشمل زيادة ملحوظة فى محتوى مالون داي الدهايد و النيتريت و قلة فى مستوى جلوتاثيون المختزل و كذلك زيادة ملحوظة فى مستوى ميلوبروكسيداز و كذلك زيادة ملحوظة فى سيتوكينات الإلتهاب و تحفيز للعامل النووي-كابا-بي و تقليل محتوى بروستاجلاندين-هـ٢ فى انسجة الجهاز الهضمى للجرذان المشعة و قد لوحظ فرق ملحوظ في القياسات بين المجموعات المشعة و المجموعات المعالجة قبل التشعيع.