Radioprotection of small intestinal injury by *Mentha piperita* in Swiss albino mice

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**ABSTRACT**

Naturally occurring dietary components like *Mentha piperita* offer opportunities for development as effective radioprotective agents because of their potential low toxicity and therapeutic actions. The present investigation is an attempt to evaluate the radioprotective effect of *Mentha piperita* (Linn.) leaves on small intestine of Swiss albino mice exposed to gamma radiation. Mice were administered the optimum dose (100mg/kg/day) of 50% alcoholic extract of *Mentha piperita* (ALM) for three consecutive days prior to 6,8, 10 Gy irradiation and autopsied at 1,3,7,14 and 30 day post irradiation to observe the histopathological changes in small intestine. The control group (irradiation alone), drug alone (ALM treatment at optimum dose) and normal group were run simultaneously. The results of histopathological studies did not show any changes in the normal histological structure of small intestine in the normal (Group I) and drug alone (Group II) at all the autopsy intervals. The control group (Group III) of animals exhibited marked histopathological lesions at all radiation doses (6, 8 and 10 Gy). The initial radiation damage was observed on day 1 and 3 which gradually recovered at later autopsy intervals. The histopathological lesions like sloughing and shortening of villi with shrunken stroma, suppressed mitosis, decreased number of normal epithelial cells and increased pyknotic cells were observed. The treatment of ALM prior to irradiation was found to be effective in suppressing the extent of radiation induced injury and promoted early recovery as compared to control as significantly higher number of mitotic figures, epithelial cells and goblet cell/crypt section was observed. The injury in terms of reduced villus height and pycknotic cell/crypt was also found to be significantly arrested in ALM treated group as compared to irradiation alone. It was evident that ALM pretreatment was able to reduce initial damage and stimulates early recovery in the post irradiation period in small intestine of swiss albino mice.

**Keywords:** *Mentha piperita*; Small Intestine; histopathological lesion; irradiation; mice

**INTRODUCTION**

Small intestine, because of its anatomic location, it often receives injurious doses of ionizing radiation even though it rarely needs therapeutic radiation itself. It has generally been claimed that the intestine constitute the most radiosensitive tissue in the gastrointestinal tract. A number of active chemicals have been found to reduce the intensity of radiation injury when administered before exposure.

Although synthetic radioprotectors such as aminothiols have yielded the highest protective factors; but typically they are considered to be more toxic (Rades, et al 2004) than naturally occurring radioprotectors (Weiss and Landauer, 2003). This has given impetus to screen herbs for their radioprotective ability. A large number of plants contain antioxidant phytochemicals reported to be radioprotective in various model systems. These include Ayurvedic preparations like Chyavanaprasha and Brahma rasayana (Rekha and Kuttan-Kuttan, 2000, Jagetia and Baliga, 2004) which was found to provide significant protection to irradiated mice. The radiomodulatory effect of Mentha oil on mice survival and its haematological parameters along with serum phosphatases have been reported previously (Samarth et al 2002, 2004). The optimum concentration of alcoholic extract of *Mentha piperita* leaves have proved to exhibit significant radioprotection in terms of survivability and hepatic injury at considerably low dose as compared to aqueous extract (Kaushik et al 2012, 2013).

The present investigation is an attempt to evaluate the radioprotective effect of alcoholic extract of *Mentha piperita* (Linn.) leaves on histopathological damage to small intestine of Swiss albino mice exposed to gamma radiation.
MATERIALS AND METHODS

Alcoholic extract of Mentha (ALM)

Fresh leaves (100 gm) of Mentha piperita (Linn.) were washed, air dried, powdered and extracted with 1500 ml of 50% ethanolic solution with double distilled water (DDW) and then refluxing for 36h (3x12½) at 60°C. The extract thus obtained was vacuum evaporated; powdered and known concentration was dissolved in DDW. Each mouse was administered 0.1 ml of ALM suspension (100mg/kg/day for three consecutive days prior to irradiation) by oral gavage (Kaushik et al 2012).

Animals

Six-eight week old Swiss albino mice (female) weighing 25±2 gm selected from an inbred colony (obtained from Hamdard University, Delhi). Animals were provided standard mice feed (procured from Hindustan Liver’s, Delhi) and water ad libitum.

Irradiation

The cobalt teletherapy unit (ATC-C9) in the Cancer Treatment Center, S.M.S. Medical College and hospital, Jaipur was used for irradiation. Unanaesthetized animals were restrained in well-ventilated Perspex boxes and the whole body exposed to different doses (6, 8, 10 Gy) of gamma radiation at a distance (SSD) of 77.5 from the source to deliver the dose rate of 1.64Gy/min.

Experimental Design

The animals selected for this study were divided into four groups

Group I (normal): Animals (n=10) of this group were sham-irradiated.

Group II (Drug alone): Animals (n=10) of this group were given orally 100 mg/kg b.wt./day of ALM for 3 consecutive days.

Group III (Control/Radiation alone)

These animals were given DDW for 3 consecutive days equal to the volume of ALM and then exposed to different doses of Gamma radiation as follows: (10 animals were taken in each subgroup)

Subgroup IIIa : DDW + 6 Gy
Subgroup IIIb : DDW + 8 Gy
Subgroup IIIc : DDW + 10 Gy

Group IV (Experimental/ALM + Radiation treatment)

Animals of this group were given the optimum dose of 100 mg/kg b.wt./day of ALM orally for 3 consecutive days and then exposed to different doses of gamma radiation as follows: (10 animals were taken in each subgroup)

Subgroup IVa : ALM + 6 Gy
Subgroup IVb : ALM + 8 Gy
Subgroup IVc : ALM + 10 Gy

 Autopsy Intervals

A minimum of five animals from all the above groups were autopsied by cervical dislocation at 1, 3, 7, 14 and 30 day post-treatment/irradiation and their intestine were taken out for histopathological studies. The Intestine was removed and flushed with normal saline after the autopsies, fixed in Bouin's fixative. Following 16-24 hours of fixation, tissues were washed, dehydrated, cleared and embedded in paraffin wax (58-60°C) followed by their microtome sectioning at 5μ. The staining techniques employed for histological studies involved haematoxylin-eosin.

Histopathological study of Small Intestine

The following parameters were studied to assess the modulation of radiation damage in Swiss albino mice by alcoholic extract of Mentha (ALM).

(i) Qualitative studies

The haematoxylin-eosin stained sections of intestine were observed for qualitative histopathological damage like shrinking and slaughing of villi, paneth cell stain, oedema, lymphocytic infiltration, cytoplasmic vacuolization, necrosis and enucleation in villus and crypt region.

(ii) Quantitative studies

Crypts: The crypts which had been cut longitudinally and centrally which were evidenced by a continuous lumen from the base of villus to paneth cells at the bottom, and which had a neck, were considered for the counting. Cells were counted on either side of the crypts starting from the base at a point where lumen axis touches the epithelium upto crypt villus junction. The latter was identified from the base of the crypt, the nucleus of each succeeding cell gradually gets centrally placed. The epithelial cells with a typically placed nucleus were thus considered to be the crypt-villus junction.

Only those cells were counted, the nucleus of which was visible in the section and the cytoplasm was in contact with the basement membrane. Atleast 3 sections in each animal was examined and approximately 3-4 ideal crypts from each section were considered for counting cells.

Parameters quantitated were

(a) Number of pyknotic cells/crypt section: All the cells showing pyknosis and karyorrhexis were counted. The average was presented in the form of histogram.

(b) Number of mitotic figures/crypt section: All identifiable stages of mitosis from prophase to telophase observed in the crypt section were considered and counted. Abortive mitotic cells were not included. The average was presented by histogram.

(c) Number of Goblet cells/crypt section: These cells are found scattered throughout the crypt. A typical
goblet cell has a definite and crescentric nucleus attached to the goblet vacuole and is characterized by globules of mucus. The cells were identified by PAS \textsuperscript{\textregistered} staining and average values were represented in the form of histogram.

(d) Number of epithelial cells/crypt section: The rest of the cells which do not contain any type of secretory granules and paneth cells were counted. Cells which had a definite centrally placed nuclei were considered for counting.

(e) Number of goblet cells/villus section: Goblet cells identified as above were counted in villus.

(f) Villus height: Villus height was calculated in \( \mu \text{m} \) with calibrated oculometer. The measurement was done from the base of villus to the tip in at least 3 animals from each group and 3-4 sections in each. At least 10 ideal villi from each section were selected for measurement.

Statistical Analysis

The results obtained from the present study were expressed as mean \( \pm \) S.E. Student’s ‘t’ test was used to make a statistical comparison between the groups. A statistical comparison was completed with the irradiation alone group vs. normal, normal vs. drug alone and irradiation alone vs. radiation and ALM treated combined group. The significance levels were set at \( p < 0.05 \), \( p < 0.01 \) and \( p < 0.001 \).

RESULTS AND DISCUSSION

The normal (Group I) and drug alone (Group II) did not show any changes in the normal histological structure of intestine (Figure 1).

In the present investigation, whole body irradiation (control group) of mice with 6, 8 and 10 Gy gamma radiation resulted in plethora of radiation damage to intestine. Marked damage to intestine was observed on day 1 as significantly shortened villi, reduced number of normal epithelial cells/crypt section, almost ceased mitosis, and increased number of pyknotic cells in crypts as well as decline in goblet cell number in crypt and villus section. On day 3 the damage became more pronounced, thereafter trend towards recovery started. Upto day 30, considerable recovery from radiation insult was observed but these values were not similar to normal. Maximum damage was observed in animals exposed to 10 Gy gamma radiation followed by 8 and 6 Gy (Figure 6). There were no survivors at 10 Gy irradiation group and ALM treated group irradiation after 7 days and 14 days respectively.

Figure 2: 6 Gy control (irradiation alone) at day 1 showing sloughing of villi (100X).

Figure 3: 6 Gy experimental (ALM+irradiation) at day 1 showing mild sloughing of villi (100X).

Figure 4: 8 Gy control (irradiation alone) at day 3 showing many pyknotic nuclei and cytoplasmic vacuolization in crypt region and damaged mucosa (400X)
The quantitative changes produced after whole body external irradiations were in agreement with the reports of many workers (Uma Devi and Veena, 1993; Brennan et al., 1998; Potten, 2004, Patel et al., 2012). The intestinal tract is highly sensitive to ionizing radiation (IR), which results in denuding of the intestinal mucosa and ultimately to death. Immediately after irradiation, intestinal crypt epithelial cells undergo apoptosis (Potten, 1990).

At irradiation doses greater than 8 Gy, the stem cells located at the base of the crypt of Lieberkuhn are also targeted, resulting in severe GI damage (Potten, 1991). Although mitotic arrest occurs immediately after IR treatment, cell migration continues to occur, resulting in shrinkage of the crypts. Release from mitotic arrest occurs at ~36 hrs, and the surviving stem cells divide and restore normal intestinal architecture by 2 weeks (Potten, 1990). Thus the gradual increase in villus height after day 3 may be mediated through stimulation of stem cell compartment, it may promote intestinal healing.
ALM pretreatment prior to irradiation in mice showed considerable protection of all the intestinal components in such a way that survival of animals from gastrointestinal syndrome increases. This amelioration of radiation damage to small intestine was exhibited in terms of increased villus height (Figure 8), higher number of mitotic figures/crypt section (Figure 10), reduction in pyknotic cells, elevation in number of normal epithelial cells per crypt section and increased number of goblet cells in crypt and villus regions (Figure 11, 12).

In the ALM treated group a few mitotic figures were seen on day 1 post irradiation, when there was almost complete suppression of mitosis in control (irradiation alone) group, this suggests that ALM protects the intestine during early intervals. Consequently the damage to villi is not as severe as in control group. This may also explain lesser shortening of villi of experimental (ALM + irradiation) group as compared to irradiation alone group and day 3 onwards the mitotic figures exhibited a trend towards recovery with increased mitotic figures.

It is likely that ALM, at least in part, act by protecting the chromosomes in the cells which are about to divide, thus restoring their normal structure. Once the division resumes there is rise in mitotic index and hence more mitotic figures in drug treated group. Again it is well known that DNA is the major target of ionizing radiation, significant protection of DNA molecule does provide protection to mammalian cells.

These results are in good agreement with Uma Devi et al (1979) who studied the radioprotective effect of 2-mercaptopyrropropionyl glycine on the intestinal crypts of Swiss albino mice after Cobalt-60 irradiation and observed that MPG afforded considerable protection to the intestinal crypts by causing early recovery from mitotic inhibition followed by an early regeneration of crypt epithelium.

The radiation-induced chromosomal damage has been found to be significantly reduced in bone marrow of mice by administration of aqueous extract of Mentha piperita (Samarth and Kumar, 2003).

Sloughing of villi may subject the intestine to Bacteremia. ALM may provide considerable protection against Bacteremia due to antimicrobial and antioxidant (menthone and isomenthone) property of Mentha piperita (Iscan et al 2002; Mimica-Dukic et al 2003).

Irradiation results in decrease in number of epithelial and goblet cells in experimental and control groups, especially on day 3 (Figure 11, 13). Here it can be suggested that ALM reduces abnormal mitosis by protecting the chromosomes (Samarth and Kumar, 2003), by which normal cells will enter into division when mitosis is resumed after inhibition. During the inhibition sufficient number of cells are not replenished by crypt to compensate the continuous loss of cells from villus tip.

Intestinal radiation protection involves the protection of surviving stem cells. Anant et al (2004) described a mechanism of radiation protection by Apobec-1 (RNA binding protein) through post-transcriptional regulation of cyclooxygenase-2 expression. Prostaglandins (PGs), generated through the action of cyclooxygenase-1 and cyclooxygenase-2 protects intestinal stem cells from ionizing irradiation. The mechanism of radioprotection involves the binding of apobec-1 to AU-rich sequences in the first 60 nucleotides of the 3' untranslated region of cyclooxygenase-2. Upon binding to the
AU rich sequence, apobec-1 stabilizes cyclooxygenase 2 messenger RNA. Wild type of mice treated with lipopolysaccharide before gamma irradiation were protected by marked increase in prostaglandin E_{2} mediated by cyclooxygenase-2. Similarly intestinal protection by ALM may be mediated by stabilization of cyclooxygenase-2 through Apobec mediation.

According to Linard et al (2004) the pathologic changes within the intestinal muscle layer may be at the origin of the cytokines that account for acute radiation-induced inflammation. He concluded that irradiation induced a cascade of inflammatory responses that involved the transcription factor NF-Kappa \beta and observed the reduction in inflammation by Caffeic acid phenyl ethyl ether (CAPE). Limonene a constituent of Mentha piperita (Mahmoud et al 2004) has also shown anti-inflammatory property (Keinan et al 2005). Thus the intestinal protection afforded by ALM can be attributed to the anti-inflammatory property due to the presence of limonene.

The extract of Mentha, being a plant product, is a natural combination of hundreds of compounds. It has been reported to contain a number of antioxidants and antiperoxidants like \alpha-tocopherol, caffeic acid, eugenol, rosmarinic acid, menthone and isomenthone, (Rastogi and Mehrtra, 1991; Krishnaswamy and Raghuramulu, 1998; Al-Sereiti et al 1999; Mimirica-Dukic et al, 2003; Ortmann et al 2004). Rosmarinic acid present in the Mentha oil inhibits the activation of complement system, thus preventing cellular death (Al-Sereiti et al 1999). \alpha-tocopherol has also been reported to reduce the amount of radiation-induced apoptosis (Ortmann et al 2004). Weiss et al (1995) reported that \alpha-tocopherol protects intestine from physiological damage, although it does not protect against high radiation doses resulting in Gastro-intestinal death.

The antioxidants and antiperoxidants properties of Mentha would have resulted in reduction in radiation mediated generation of free radicals, inhibition of lipid peroxidation and elevation of GSH (Kaushik et al 2013) and thus protection of crypt stem cells against radiation injury.

The reduction in apoptosis due to pre-irradiation treatment with ALM could be mediated through the reduced cellular injury caused by free radicals (antioxidant and antiperoxidant property) and thus reduced release of cytochrome-c by mitochondria. It could also be achieved by blocking the calcium channels in the outer membrane of the mitochondria by antia apoptotic protein Bcl-2 (Samali et al 1998). As use of peppermint is implicated in relaxing smooth muscles by reducing the availability of calcium (Beesley et al 1996) Mentha piperita have also been studied to provide protection against radiation induced chromosomal damage in bone marrow of mice (Samarth and Kumar, 2003). The Mentha tea extract have also been reported to show relaxation of gastrointestinal tissue (Mckay et al 2006). The peppermint oil exhibits dose related anti spasmodic effect on smooth musculature as it interferes with movement of calcium across the cell which inhibits the hypercontractility of intestinal smooth muscles (Grigoleit and Geigoleit, 2005)

Thus pre-irradiation administration of ALM with its free radical scavenging action and active interaction with DNA would have reduced apoptosis and thus protect the clonogenic cells in crypts against Radiation induced damage.

CONCLUSION

The results of the present study suggest that the gamma radiation induced early damage to small intestine and recovery is possible by prior administration of ALM. This is evident in the increased number of mitotic figure, normal epithelial cell, goblet cells and villus height in the treated group as compared to irradiation alone.

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