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Effects of X-Irradiation on N-Methyl-N-nitrosourea-induced Multi-organ Carcinogenesis in Rats

Yukio Morishita,[1,3] Takuji Tanaka,[1] Hideki Mori[1] and Shunsaku Sasaki[2]

[1]*First Department of Pathology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500 and* [2]*Division of Physiology and Pathology, National Institute of Radiological Sciences, 4-9-1 Anagawa, Chiba 260*

The effects of X-irradiation on N-methyl-N-nitrosourea (MNU)-induced multi-organ carcinogenesis were examined in both sexes of ACI/N rats. At 6 weeks of age, rats in groups 1 (25 males, 25 females) and 3 (24 males, 23 females) received a single i.p. injection of MNU (25 mg/kg body weight), while those in groups 2 (25 males, 26 females) and 4 (25 males, 25 females) were administered the carcinogen at a dose of 50 mg/kg body weight. At 10 weeks of age, groups 3 and 4 were X-irradiated at a dose of 3 Gy. Group 5 (24 males, 24 females) received X-irradiation alone. Group 6 (21 males, 21 females) served as an untreated control. As a result, neoplasms developed mainly in the digestive tract, kidney, uterus, and hematopoietic organ in groups 1–5. The incidences of adenocarcinoma in small and large intestines of male rats of group 4 (50 mg/kg MNU and X-irradiation) (small intestine: 48%, large intestine: 32%) were significantly higher than those of group 2 (50 mg/kg MNU) (small intestine: 17%, $P<0.05$; large intestine: 8%, $P<0.05$), and also the frequency of adenocarcinoma in the large intestine of males of group 3 (25 mg/kg MNU and X-irradiation) (22%) was significantly greater than that of group 1 (25 mg/kg MNU) (0%, $P<0.05$). These results indicated that X-irradiation enhanced the development of intestinal neoplasms induced by MNU in male ACI/N rats.

Key words: Enhancing effect — X-Irradiation — N-Methyl-N-nitrosourea — Multi-organ carcinogenesis

It is evident that human beings are exposed to low doses of a variety of hazardous chemicals in the environment. Although many chemical, physical, or biological agents with carcinogenic potential are known, the interactions of multiple agents have not yet been studied well. Since the occurrence of many human cancers may be related to environmental factors,[1–3] investigation of the combined effects of multiple carcinogenic agents is clearly important. It has been shown that concomitant exposure to multiple chemical carcinogens with similar organotropism can have an additive carcinogenic effect.[4]

It is well known that X-irradiation alone induces a variety of neoplasms in humans as well as experimental animals. In humans, epidemiological studies have indicated a high incidence of neoplasms in such organs as the hematopoietic system, digestive organs, respiratory system, breast, and uterus in atomic bomb survivors.[5] Similarly, exposure of animals to X-irradiation is reported to induce tumors preferentially in the bone marrow, mammary gland, stomach, and lung.[6] Since the first report of possible interactions between ionizing radiation and chemical carcinogens,[7] several studies have tried to explore the combined effects of two agents.[8–29] However, the results have been basically contradictory. Some authors have found a synergistic or summative effect,[8–21] whereas others have failed to find such an effect.[22–29] In addition, most of these data concerned only a few organs.

In the present study, the effects of whole-body X-irradiation on chemical carcinogenesis by N-methyl-N-nitrosourea (MNU), which has carcinogenic potential for multiple organs, were analyzed in ACI/N rats.

MATERIALS AND METHODS

Animals and diet Inbred ACI/N rats of both sexes, which had been maintained in our laboratory, were used. At 5 weeks of age, these rats were transferred to the holding room, and randomized into experimental and control groups. Rats were housed three to five to a wire cage. The holding room was maintained at 23±2°C and 50±10% humidity, with a 12 h light-dark cycle. Pellet CE-2 (Clea Japan, Inc., Tokyo) was used as a basal diet, and diet and tap water were freely available.

Chemical and X-irradiation MNU was purchased from Nacalai Tesque, Inc., Kyoto. Animals were given whole-body X-irradiation of 3 Gy. Exposure factors were as follows: 200 kVp, filter 0.5 mm Cu+0.5 mm Al, focus-to-animal distance 97.5 cm, tube current 20 mA, half-value thickness 1.2 mm Cu, and dose rate 0.254 Gy/min.

Treatments of animals A total of 288 ACI/N rats (144 males and 144 females) were divided into six groups. At 6 weeks of age, the rats of groups 1 (25 males and 25

[3] To whom all correspondence should be addressed.

females) and 3 (24 males and 23 females) were given a single i.p. injection of MNU at a dose of 25 mg/kg body weight, and those in groups 2 (25 males and 26 females) and 4 (25 males and 25 females) were given 50 mg/kg MNU. At 10 weeks of age, groups 3 and 4 were X-irradiated. Group 5 (24 males and 24 females) received X-irradiation as for groups 3 and 4, but this group was not given the carcinogen. Group 6 (21 males and 21 females) served as an untreated control. All rats were carefully observed and at intervals of four weeks during the experiment they were weighed. The experiment was terminated 400 days after the start. The animals were autopsied at death or after being killed under ether anesthesia when they became moribund or at the end of the experiment. After complete autopsy, all removed organs were fixed in 10% neutral buffered formalin, and blocks were taken from regions suspected of bearing neoplastic lesions and all of the following organs: heart, lung, tongue, thyroid gland, trachea, esophagus, forestomach, glandular stomach, small intestine, large intestine, liver, spleen, pancreas, kidney, adrenal gland, testis or ovary, prostate or uterus, urinary bladder, breast, brain, spinal cord, and vertebra. They were routinely processed for histology: that is, embedded in paraffin, sliced at a thickness of 3 μm and stained with hematoxylin and eosin. All preparations were microscopically observed and detected lesions were histopathologically diagnosed.

Table I. Mean Body and Organ Weights at the End of the Experiment

Male

Group no.	Treatment	Body weight (g)	Heart (g)	Liver (g)	Spleen (g)	Kidney (g)	Adrenal (g)	Testis (g)	Brain (g)
1	25 mg/kg MNU	281	1.31	11.3	0.52	1.79	0.05	0.95	1.44
2	50 mg/kg MNU	279	1.50	10.9	0.50	1.46	0.04	0.91	1.56
3	25 mg/kg MNU +Radiation	269	1.35	11.3	0.54	1.36	0.04	0.35[a]	1.57
4	50 mg/kg MNU +Radiation	255[b]	1.23[c]	11.4	0.62	1.38	0.03	0.37[d]	1.46
5	Radiation	301	1.22	12.3	0.49	1.51	0.03	0.40[e]	1.51
6	Non-treatment	297	1.26	12.3	0.53	1.39	0.03	1.14	1.64

Female

Group no.	Treatment	Body weight (g)	Heart (g)	Liver (g)	Spleen (g)	Kidney (g)	Adrenal (g)	Ovary (g)	Brain (g)
1	25 mg/kg MNU	204	1.05[f]	8.20	0.63[g]	1.15	0.05	0.07	1.38
2	50 mg/kg MNU	189[h]	0.95	6.73[i]	0.87[j]	1.12	0.04	0.08	1.56
3	25 mg/kg MNU +Radiation	182[k]	0.92[l]	7.01[m]	0.70	1.06	0.04	0.04[n]	1.49
4	50 mg/kg MNU +Radiation	181	0.96	6.51	0.75	1.11	0.04	0.04[o]	1.34
5	Radiation	188[p]	0.86	7.60[q]	0.49	1.01	0.04	0.05	1.36
6	Non-treatment	208	0.91	8.91	0.41	1.03	0.04	0.07	1.43

a, k–n) Significantly different from group 1 (*m*, $P<0.05$; *l, n*, $P<0.0005$; *a, k*, $P<0.0001$).
b–d, o) Significantly different from group 2 (*b, c*, $P<0.05$; *o*, $P<0.01$; *d*, $P<0.0001$).
e–j, p, q) Significantly different from group 6 (*g*, $P<0.01$; *f*, $P<0.005$; *q*, $P<0.0005$, *e, h–j, p*, $P<0.0001$).

One female rat in group 4 that was dead on the 171st day of the study had squamous cell carcinoma of the forestomach, leukemia and hemangiosarcoma of subcutaneous tissue. Therefore, the rats surviving longer than 171 days were counted as effective animals.

Statistics For the statistical analysis, Welch's test was used for body and organ weights and the chi-squared test for incidences of neoplasms and preneoplastic lesions.

RESULTS

Body and organ weights Table I shows body and organ weights of animals in each group at the end of the study.

In both male and female rats, changes in body weight were similar in all groups. In female rats, the body weights in group 2 given 50 mg/kg body weight MNU alone and group 5 X-irradiated alone were significantly decreased compared to those in group 6 as a control ($P<0.0001$). The body weights of rats of both sexes in groups 3 and 4 treated with MNU and X-irradiation were considerably reduced compared to other groups (male: 4–15% reduction, group 2 vs. 4, $P<0.05$, female: 4–13% reduction, group 1 vs. 3, $P<0.0001$).

In female rats, MNU treatment increased the weights of heart (group 1 vs. 6, $P<0.005$) and spleen (group 1 vs. 6, $P<0.01$, group 2 vs. 6, $P<0.0001$), but decreased liver weight (group 2 vs. 6, $P<0.0001$). X-Irradiation reduced the weights of heart (male: group 2 vs. 4, $P<0.05$, female: group 1 vs. 3, $P<0.0005$), liver (female: group 1 vs. 3, $P<0.05$, group 5 vs. 6, $P<0.0005$), testis (group 1 vs. 3, $P<0.0001$, group 2 vs. 4, $P<0.0001$, group 5 vs. 6, $P<0.0001$), and ovary (group 1 vs. 3, $P<0.0005$, group 2 vs. 4, $P<0.01$).

Histopathology and incidences of induced neoplastic and preneoplastic lesions Neoplasms were found in almost all organs of rats in all groups, with higher incidences in forestomach, small and large intestines, kidney, uterus, and hematopoietic organ in rats treated with MNU or X-irradiation. Histology and incidences of tumors are indicated in Tables II and III. In the forestomach, tumors were histologically papilloma or squamous cell carcinoma. Incidence of papilloma in male rats of group 4 treated with 50 mg/kg MNU and X-irradiation (16%) was significantly lower than that in group 2 given 50 mg/kg MNU alone (46%, $P<0.05$). No forestomach neoplasms were present in group 5 given X-irradiation alone. Most neoplasms in the intestine were histologically adenoma or adenocarcinoma. The number of small intestinal adenocarcinomas in male rats of group 4 given 50 mg/kg MNU and X-rays (48%) was significantly increased compared to group 2 administered 50 mg/kg MNU alone (17%, $P<0.05$). Also, the incidences of adenocarcinoma in large intestine of males which received both treatments were 22% in group 3 and 32% in group 4, and those values were significantly higher than those of males in groups 1 (0%, $P<0.05$) and 2 (8%, $P<0.05$) given MNU alone. There were no intestinal neoplasms in group 5 (X-irradiation alone). Most tumors in the uterus were hemangioma. The incidences of uterine hemangioma were 78% in group 3, 50% in group 4, and 61% in group 5, and were significantly higher than those in rats of groups 1 (0%, $P<0.00001$) and 2 (4%, $P<0.0005$) given MNU alone or group 6 as a control (0%, $P<0.00005$). In group 6, only one male had an intestinal tumor (leiomyoma).

Besides tumors, preneoplastic lesions were also observed in heart, thyroid gland, forestomach, glandular stomach, liver, pancreas, adrenal gland, and urinary bladder (Tables IV and V). No altered liver cell foci were seen in male rats treated with MNU alone, while a few were found in X-irradiated rats (group 3: 30%, $P<0.005$, group 4: 16%, $P<0.05$, group 5: 14%). The incidence of proliferative foci of adrenal gland in males of group 4 given 50 mg/kg MNU and X-rays (48%) was significantly higher than that in group 2 treated with 50 mg/kg MNU alone (21%, $P<0.05$). In group 5 given X-irradiation alone, these lesions were found in 14% of males. In group 6, one male had a preneoplastic lesion (spindle cell hyperplasia) in the heart.

DISCUSSION

In the present study, the incidences of adenocarcinoma of the small and large intestines of males given both treatments (MNU and X-irradiation) were significantly greater than those of the groups given MNU alone. This result clearly indicates an enhancing effect of X-irradiation on the intestinal carcinogenesis by MNU. The enhancing effect was more remarkable in animals given a high dose of MNU. Similarly, the incidence of uterine hemangioma of the groups with both treatments was significantly higher than that of the groups given the carcinogen alone. Also, X-irradiation increased the incidences of preneoplastic lesions induced by MNU in the liver (liver cell foci) and adrenal gland (proliferative foci) of males. However, these neoplastic and preneoplastic lesions were present in rats treated with MNU or radiation alone with relatively high incidences. Therefore, it is considered that X-irradiation did not enhance the occurrence of these lesions, but merely added its own effect to the MNU effect. The finding of increased incidence of intestinal neoplasms by X-irradiation could have important implications in humans, since X-rays have been used frequently for various clinical purposes. The results, accordingly, may provide some indications for the risk assessment of X-irradiation in human intestinal carcinogenesis.

Table II. Incidence of Neoplasms in Each Organ of Male Rats

Group no.	1	2	3	4	5	6
Treatment	25 mg/kg MNU	50 mg/kg MNU	25 mg/kg MNU +Radiation	50 mg/kg MNU +Radiation	Radiation	Non-treatment
Effective number of animals	25	24	23	25	22	21
Heart						
Fibrosarcoma	0	1 (4)[a]	3 (13)	1 (4)	0	0
Tongue						
Papilloma	0	3 (13)	3 (13)	3 (12)	0	0
Thyroid gland						
Adenoma	0	1 (4)	0	2 (8)	1 (5)	0
Adenocarcinoma	0	0	0	2 (8)	0	0
Forestomach						
Papilloma	0	11 (46)	2 (9)	4 (16)[b]	0	0
Squamous cell carcinoma	0	4 (17)	0	10 (40)	0	0
Glandular stomach						
Adenoma	0	1 (4)	0	0	0	0
Adenocarcinoma	1 (4)	0	0	2 (8)	0	0
Leiomyoma	0	1 (4)	0	0	0	0
Small intestine						
Adenoma	1 (4)	1 (4)	1 (4)	3 (12)	0	0
Adenocarcinoma	1 (4)	4 (17)	1 (4)	12 (48)[c]	0	0
Leiomyoma	0	0	0	0	0	1 (5)
Hemangiosarcoma	0	0	0	1 (4)	0	0
Large intestine						
Adenoma	0	0	0	2 (8)	0	0
Adenocarcinoma	0	2 (8)	5 (22)[d]	8 (32)[e]	0	0
Kidney						
Nephroblastoma	0	2 (8)	1 (4)	0	2 (9)	0
Hemangiosarcoma	0	0	1 (4)	2 (8)	0	0
Adrenal gland						
Adenocarcinoma	0	1 (4)	1 (4)	0	0	0
Urinary bladder						
Papilloma	0	0	1 (4)	1 (4)	0	0
Seminal vesicle						
Adenocarcinoma	0	1 (4)	0	0	0	0
Ear duct						
Squamous cell carcinoma	0	1 (4)	1 (4)	3 (12)	0	0
Brain						
Astrocytoma	0	1 (4)	0	2 (8)	0	0
Skin						
Squamous cell carcinoma	0	0	0	1 (4)	0	0
Subcutaneous tissue						
Neurinoma	0	2 (8)	0	0	0	0
Hemangiosarcoma	0	1 (4)	0	0	0	0
Bone						
Osteosarcoma	0	1 (4)	0	0	0	0
Hematopoietic organ						
Leukemia	0	1 (4)	1 (4)	3 (12)	0	0

a) Numbers in parentheses are % of tumor-bearing animals.
b, c, e) Significantly different from group 2 ($P<0.05$).
d) Significantly different from group 1 ($P<0.05$).

Table III. Incidence of Neoplasms in Each Organ of Female Rats

Group no.	1	2	3	4	5	6
Treatment	25 mg/kg MNU	50 mg/kg MNU	25 mg/kg MNU + Radiation	50 mg/kg MNU + Radiation	Radiation	Non-treatment
Effective number of animals	25	25	23	24	23	21
Heart						
Fibroma	0	1 (4)[a]	0	0	0	0
Fibrosarcoma	1 (4)	2 (8)	0	0	0	0
Tongue						
Papilloma	1 (4)	2 (8)	1 (4)	0	0	0
Squamous cell carcinoma	0	1 (4)	0	0	0	0
Thyroid gland						
Adenoma	2 (8)	0	0	0	0	0
Adenocarcinoma	0	0	0	1 (4)	0	0
Forestomach						
Papilloma	2 (8)	3 (12)	1 (4)	7 (29)	0	0
Squamous cell carcinoma	0	1 (4)	0	2 (8)	0	0
Small intestine						
Adenoma	0	0	1 (4)	2 (8)	0	0
Adenocarcinoma	0	1 (4)	0	0	0	0
Large intestine						
Adenoma	0	1 (4)	0	0	0	0
Adenocarcinoma	1 (4)	2 (8)	0	2 (8)	0	0
Pancreas						
Islet cell adenoma	0	0	0	0	1 (4)	0
Kidney						
Transitional cell carcinoma	0	0	0	1 (4)	0	0
Nephroblastoma	0	5 (20)	2 (9)	2 (8)	0	0
Hemangiosarcoma	0	0	1 (4)	0	0	0
Urinary bladder						
Transitional cell carcinoma	1 (4)	0	0	1 (4)	1 (4)	0
Uterus						
Papilloma	0	0	1 (4)	1 (4)	0	0
Hemangioma	0	1 (4)	18 (78)[b]	12 (50)[c]	14 (61)[d]	0
Mammary gland						
Adenocarcinoma	0	2 (8)	0	0	0	0
Ear duct						
Papilloma	0	1 (4)	0	2 (8)	0	0
Squamous cell carcinoma	1 (4)	0	0	1 (4)	0	0
Salivary gland						
Adenosquamous carcinoma	0	1 (4)	0	0	0	0
Brain						
Astrocytoma	0	1 (4)	0	0	0	0
Skin						
Trichoepithelioma	0	0	0	0	1 (4)	0
Squamous cell carcinoma	0	0	0	0	1 (4)	0
Subcutaneous tissue						
Hemangiosarcoma	0	0	0	2 (8)	0	0
Fibrosarcoma	0	0	0	1 (4)	0	0
Bone						
Malignant fibrous histiocytoma	0	0	0	1 (4)	0	0
Skeletal muscle						
Rhabdomyosarcoma	0	0	0	1 (4)	0	0
Hematopoietic organ						
Leukemia	6 (24)	15 (60)	7 (30)	10 (42)	1 (4)	0

a) Numbers in parentheses are % of tumor-bearing animals.
b–d) Significantly different from group 1 (*b*, $P<0.00001$), group 2 (*c*, $P<0.0005$) or group 6 (*d*, $P<0.00005$).

Table IV. Incidence of Preneoplastic Lesions in Each Organ of Male Rats

Group no.	1	2	3	4	5	6
Treatment	25 mg/kg MNU	50 mg/kg MNU	25 mg/kg MNU +Radiation	50 mg/kg MNU +Radiation	Radiation	Non-treatment
Effective number of animals	25	24	23	25	22	21
Heart						
Spindle cell hyperplasia	2 (8)[a]	8 (33)	4 (17)	10 (40)	0	1 (5)
Thyroid gland						
C-cell hyperplasia	0	1 (4)	0	0	0	0
Forestomach						
Papillary hyperplasia	0	3 (13)	2 (9)	5 (20)	3 (14)	0
Glandular stomach						
Surface cell hyperplasia	0	1 (4)	0	2 (8)	0	0
Liver						
Altered liver cell foci	0	0	7 (30)[b]	4 (16)[c]	3 (14)	0
Pancreas						
Atypical acinar cell foci	4 (16)	10 (42)	5 (22)	10 (40)	1 (5)	0
Adrenal gland						
Proliferative foci	7 (28)	5 (21)	11 (48)	12 (48)[d]	3 (14)	0
Urinary bladder						
Papillary hyperplasia	0	1 (4)	0	1 (4)	0	0

a) Numbers in parentheses are % of lesion-bearing animals.
b) Significantly different from group 1 ($P<0.005$).
c, d) Significantly different from group 2 ($P<0.05$).

Table V. Incidence of Preneoplastic Lesions in Each Organ of Female Rats

Group no.	1	2	3	4	5	6
Treatment	25 mg/kg MNU	50 mg/kg MNU	25 mg/kg MNU +Radiation	50 mg/kg MNU +Radiation	Radiation	Non-treatment
Effective number of animals	25	25	23	24	23	21
Heart						
Spindle cell hyperplasia	6 (24)[a]	6 (24)	2 (9)	4 (17)	0	0
Thyroid gland						
C-cell hyperplasia	0	1 (4)	1 (4)	0	0	0
Forestomach						
Papillary hyperplasia	0	5 (20)	1 (4)	2 (8)	1 (4)	0
Liver						
Altered liver cell foci	1 (4)	0	3 (13)	1 (4)	3 (13)	0
Pancreas						
Atypical acinar cell foci	5 (20)	2 (8)	2 (9)	3 (13)	0	0
Adrenal gland						
Proliferative foci	3 (12)	2 (8)	3 (13)	4 (17)	3 (13)	0
Urinary bladder						
Papillary hyperplasia	0	0	1 (4)	1 (4)	0	0

a) Numbers in parentheses are % of lesion-bearing animals.

Up to the present, several studies of the modifying effect of X-irradiation on chemical carcinogenesis have been done using different animal models. In these studies, synergistic effects of X-irradiation were shown in such organs as the liver,[14, 15, 19, 21] mammary gland,[12, 16] colon,[20] skin,[9, 13, 17] and cheek pouch,[18] while inhibitory effects were also found in brain,[23, 24, 26, 27, 29] glandular stomach,[25, 28] and lung.[22] Thus, the effects of X-irradiation

may vary with the organs or the carcinogens used. In this study using MNU, which has carcinogenic potential to multiple organs, an enhancing effect of X-irradiation was demonstrated in the intestine, but not in other organs. Such organ-specificity of the combined effect of X-irradiation and chemicals has not previously been reported.

Previously, Maekawa *et al.*[30] reported that oral administration of MNU for 42 weeks in ACI/N rats induced neoplasms in the nervous system, digestive tract, urinary tract, and heart. In the present study, incidences of neoplasms in the nervous system and urinary tract were low, although those in the gastrointestinal tract and heart were relatively high. This discrepancy is probably due to the difference of administration route or dose of the carcinogen.

The present result that X-irradiation increased the incidence of colon adenocarcinoma is basically in agreement with the report of Sharp *et al.*,[20] showing a synergism between radiation and 1,2-dimethylhydrazine (DMH) in the induction of colonic tumors in rats. However, they administered radiation 3.5 days prior to the DMH exposure, differing from the protocol in the present experiment (MNU followed 4 weeks later by X-irradiation). Moreover, radiation in their study was given within the abdominal region only, not over the whole body, and with a much higher dose of 9 Gy.

In this study, the number of preneoplastic lesions (including liver cell foci) was increased by X-irradiation. The effect appeared to be additive. The result is compatible with our previous report[21] of a synergistic effect of X-irradiation on N-2-fluorenylacetamide-induced liver tumors and hepatocellular foci. In the previous study, X-irradiation alone did not induce hepatocellular foci, whereas in the present study, X-irradiation alone produced a few foci. This difference may have been caused by the difference of experimental duration.

In our previous study, X-irradiation acted as a progressor, which usually generates genetic changes and plays a role in tumor progression.[21] Papilloma and squamous cell carcinoma in the forestomach were found in 46% and 17%, respectively, of males in group 2 treated with 50 mg/kg MNU alone, while the corresponding values were 16% and 40% in males in group 4 treated with 50 mg/kg MNU and X-irradiation. These results may suggest that X-irradiation led to conversion of papilloma into carcinoma in the forestomach. In addition, some squamous cell carcinomas in group 4 tended to be poorly differentiated compared to group 2 (data not shown). Thus, X-rays might induce conversion and progression.

X-Irradiation has been thought to contribute to carcinogenesis through DNA damage caused by strand-breakage[31] or base change.[32] Therefore, candidate mechanisms for the interaction of MNU and X-irradiation shown here are expected to be as follows: (i) genetic changes mediated by X-irradiation leading to enhanced expression of genes mutated by MNU-mediated base alkylation, and (ii) inhibitory effects of X-irradiation on the repair of DNA damage produced by MNU treatment. Radiation, on the other hand, has been shown to induce some enzymes related to cell proliferation and growth.[33, 34] Fujiki *et al.*[33] reported that beta-ray irradiation of mouse skin elevated the activity of ornithine decarboxylase, a rate-limiting enzyme in the biosynthesis of polyamines, which are intracellular mediators of cell proliferation.[35, 36] Woloschak *et al.*[34] found that low linear energy of transfer radiations such as X-rays or γ-rays are able to induce an increased expression of mRNA for protein kinase C, playing an important role in tumor promotion and regulation of cell growth, in confluent Syrian hamster embryo cells. Such enzyme induction by radiation may be related to the enhancement of MNU-induced intestinal carcinogenesis observed in the present study.

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