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Article

Protection effect of sanguinarine on whole-body exposure of X radiation in BALB/c mice

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To investigate the effects of sanguinarine (SAN) on acute radiation induced injury in mice, 45 mice were randomly divided into control, 10 Gy and SAN+10 Gy groups. Mice in the 10 Gy and SAN+10 Gy groups were exposed to single X-ray radiation with an accumulated dose of 10 Gy. Mice in the SAN+10 Gy group were administered intraperitoneally with 2.5 mg/kg body weight of SAN before radiation. Five days after radiation exposure, 5 mice from each group were sacrificed and samples of the small intestine, lung, spleen and liver were fixed for histopathological examinations. Compared with the 10 Gy group, radiation sickness was obviously delayed or attenuated in the SAN+10 Gy group. Survival analysis showed a significant difference between 2 radiation groups ($P<0.05$) and mean survival time was 3 days longer in the SAN+10 Gy group than in the 10 Gy group (7.21±0.19 *vs.* 4.20±0.13, $P<0.001$). Radiation-induced organ damage, based on histopathological examinations, was decreased by SAN pretreatment. Chiu's pathology grading scores, which is an index of intestinal damage, was significantly lower in the SAN+10 Gy group than in the 10 Gy group (2.77±0.48 *vs.* 4.37±0.31, $P<0.01$). A similar result was obtained in the pathological score of lung (1.67±0.21 *vs.* 2.33±0.38, $P<0.01$). Our preliminary findings demonstrated that SAN protects animals against radiation-induced sickness and acute damage to organs and following animal death.

Uniterms: Sanguinarine/radioprotection. X-ray/radiation/effects. Radiation sickness. Radiation/survival analysis.

Para investigar os efeitos da sanguinarina (SAN) em lesões induzidas em ratos por radiação aguda, 45 ratos foram aleatoriamente divididos em grupo controle, grupo 10 Gy e grupo SAN+10 Gy. Os ratos dos grupos 10 Gy e SAN+10 Gy foram expostos à radiação de raio-X simples com uma dose acumulada de 10 Gy. Aos ratos do grupo SAN+10 Gy administraram-se, intraperitonealmente, 2.5 mg/kg de peso de SAN antes da radiação. Aos 5 dias de exposição à radiação, sacrificaram-se 5 ratos de cada grupo e retiraram-se amostras do intestino delgado, pulmões, baço e fígado para exames histopatológicos. Comparando com o grupo 10 Gy, a doença por radiação foi claramente atrasada e atenuada no grupo SAN+10 Gy. A análise de sobrevivência mostrou diferença significativa entre os dois grupos de radiação ($P<0.05$) e o tempo de sobrevivência média foi de mais 3 dias no grupo SAN+10 Gy do que no grupo 10 Gy (7.21±0.19 vs 4.20±0.13, $P<0.001$). Danos induzidos nos órgãos por radiação, baseados em exames histopatológicos, foram reduzidos pelo pré-tratamento com SAN. As pontuações de classificação da patologia Chiu, um índice para os danos intestinais, foi significativamente menor no grupo SAN+10 Gy do que no grupo 10 Gy (2.77±0.48 vs 4.37±0.31, $P<0.01$). Resultado semelhante foi obtido na pontuação patológica do pulmão (1.67±0.21 vs 2.33±0.38, $P<0.01$). As nossas descobertas preliminares mostram que SAN protege os animais contra doenças induzidas pela radiação e danos agudos nos órgãos seguidos de morte animal.

Unitermos: Sanguinarina/radioproteção. Raio X/radiação/efeitos. Doenças por radiação. Radiação/análise de sobrevivência.

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INTRODUCTION

Applications of ionizing radiation are rapidly increasing and the deleterious effect of human exposure is attracting increasing attention from researchers and the general public. Several drugs of synthetic and natural origin are found to mitigate injury caused by whole exposure in experimental models and human clinical trials. These radioprotective compounds include ethyl phosphorothioic acid (amifostine), vitamin E, vitamin C, carotenoids, plant polyphenols and aminothiol (Hosseinimehr, 2007). However, no ideal radioprotectors are available because of their toxicity at their optimal concentrations.

Thus, herbal drugs have become candidates in offering an alternative to the synthetic compounds as they are considered either non-toxic or less toxic than their synthetic counterparts. Sanguinarine (SAN, 13-Methyl[1,3]benzodioxolo[5,6-c][1,3]dioxolo[4,5-i] phenanthridin-13-ium), which is a benzophenanthridine alkaloid derived from the root of *Sanguinaria canadensis* and other poppy species, is widely used in toothpaste and mouthwash to suppress dental plaque formation and to reduce gingival inflammation (Mackraj *et al*., 2008). Previous studies have indicated that SAN inhibits the growth of cancer cells at micromolar concentration. One of the underlying mechanisms is the scavenging of free radicals induced by ionizing radiation (Ahsan *et al*., 2007; Chang *et al*., 2007; Choi *et al*., 2008). In the present study, a reasonable hypothesis was first put forward to evaluate protective effects of SAN on radiation injury. We observed radiation sickness incidence, survival rates and organ histopathological changes after an acute whole body exposure of X-ray radiation in Balb/c mice.

MATERIAL AND METHODS

Animals and treatments

A total of 45 male Balb/c mice were obtained at 8-10 weeks of age from Shanghai Laboratory Animal Center (Chinese Academy of Science, Shanghai, China). All mice were housed in polycarbonate cage under controlled environmental condition (22±2 °C, 60±5% relative humidity, 12 h light/dark cycle) and were provided standard mouse feed and water *ad libitum* throughout the experiment. The care and use of laboratory animals followed the guidelines for Animal Experiments of Soochow University.

After one week of acclimation, all animals were assigned randomly to three groups with different treatments: *i.e.* (1) control group, (2) 10 Gy group, and (3) SAN+10 Gy group. For 10 Gy group, each mouse was placed in a separate plastic container and received whole body X-ray radiation using a PRIMUS accelerator (SIEMENS Medical Solutions, Erlangen, Germany). The absorbed dose rate was 2.0 Gy/min at a 100 cm distance. The mice were exposed to single radiation and the accumulated dose reached 10 Gy, which was determined using Fricke's chemical dosimeter. Fresh air was continuously circulated in the radiation chamber to avoid the generation of hypoxic conditions. About 30 min before radiation, the mice in SAN+10 Gy group were administered intraperitoneally with SAN. SAN chloride hydrate (98% pure, Sigma-Chemical Co., St. Louis, MO) was dissolved in ethanol as stock solutions (3.32 mg/mL) and was previously configured to the desired concentration sing saline. The amount of 2.5 mg/kg body weight was selected based on our previous study (Xu *et al*., 2012). On the other hand, the same volume of saline solution was administered in control group and radiation group.

All animals were observed daily for any signs of radiation sickness, morbidity and mortality. Body weight and death number of mice were recorded daily. The percentage of surviving animals each day was used for survival analysis. Before treatment, daily consumption of chow and water was measured by subtracting from those that were not consumed after 24 h. The daily consumption was measured again between day 4 and day 5, where the day of radiation was indicated as day 0. The experiment did not end until the last mouse in the 10 Gy group or SAN+10 Gy group died.

Autopsy and histopathological examinations

At day 5, five survived mice from each group were randomly selected and sacrificed by cervical dislocation under anesthesia. These mice were excluded in the final calculation of body weight change and survival rate. After weighing some organs, samples of the small intestine (1 cm segments at 5 cm proximal to the terminal ileum), lung, spleen and liver were fixed in 10 % buffered formaldehyde-saline solution. 5 μm sections of paraffin-embedded samples were stained with hematoxylin and eosin (Sigma-Aldrich) and were examined by a pathologist under blinded conditions. Furthermore, histopathological changes of the small intestine was evaluated using Chiu's pathology grading system, which is classified into six grades based on changes in villi and glands of the intestinal mucosa from normal mucosal villi (grade 0) to disintegration of the lamina propria and hemorrhage (grade 5) (Chiu *et al*., 1970). The lung was evaluated

using Lee's pathology grading system based on pulmonary consolidation, parenchymal collapse, hemorrhage and alveolar from normal (score 0) to extensive damage (score 3) (Lee, Rhee, 2008).

Statistical analysis

The results obtained in the present experiment were expressed as mean±standard deviation (SD). Data were analyzed using SPSS version 17.0 for Windows (SPSS Inc., USA). One-way analysis of variance (ANOVA) was used to determine differences among groups. Survival rate was analyzed by the log-rank test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

General status of mice

All animals exposed to radiation exhibited some signs of radiation sickness within 2 days. The main symptoms included decreased food and water intake, irritability, watery eyes, lethargy, hair ruffling, diarrhea, emaciation and epilation. Facial edema, paralysis and difficulty of breathing were also observed in a few animals. SAN pretreatment (SAN+10 Gy group) delayed the appearance or attenuated these symptoms induced by radiation.

Body weight of mice decreased from day 0 in both radiation groups. Mice were significantly heavier in SAN+10 Gy group than in 10 Gy group from day 3 until one animal remained alive (Figure 1). The average intakes of food and water in baseline were similar among 3 groups. At day 4, food intake significantly decreased in 2 groups exposed to radiation, with more remarkable decrease in the 10 Gy group. For water intake, there was little change in control group and SAN+10 Gy group. However, the mice in the 10 Gy group drank more water after radiation (Table I).

Survival analysis

All mice in the control group were alive at the end of experiment. Eight mice died at day 5, and the others died next day in the 10 Gy group. The survival duration was between day 4 and day 12 in SAN+10 Gy group (Figure 2). Survival analysis showed statistical significance between two radiation groups and the control group (for 10 Gy group $P = 0.005$; for SAN+10 Gy group $P = 0.016$). Significant difference was also found between 10 Gy group and SAN + 10 Gy group ($P = 0.037$). Mean survival

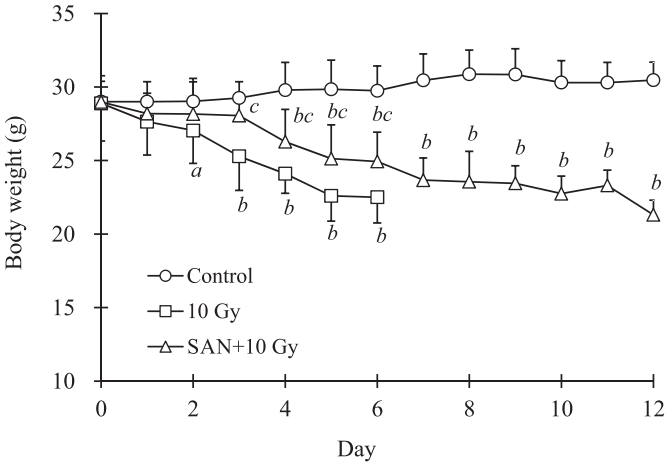

FIGURE 1 - The body weight of mice during the experiment (n=10). *a* P<0.05 and *b* P<0.01, compared with control group; *c* P<0.01 compared with 10 Gy group.

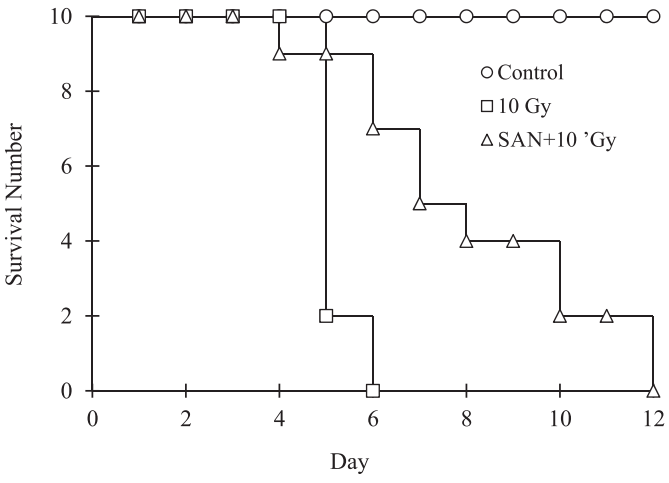

FIGURE 2 - The survival curve of the three groups during the experiment (n=10).

TABLE I - Food and water intake at day 4 (n=10)

Intake (g)	Control group	10 Gy group	SAN+10 Gy group
Food	6.22±1.03	1.13±0.98 [b]	3.26±1.18 [b c]
Water	6.72±0.61	11.34±1.12 [b]	6.86±0.45 [c]

[b]P<0.01, compared with control group; [c]P<0.01 compared with 10 Gy group

time was 3 days longer in SAN+10 Gy group than in 10 Gy group (7.21 ± 0.19 *vs* 4.20 ± 0.13, *P*<0.001) and the protection factor for SAN was 1.72 (7.21/4.20).

Observation at autopsy

Five mice from each group were sacrificed at day 5. Radiation significantly decreased the ratios of liver and spleen to body weight, and increased the ratio of lung to body weight. SAN pretreatment improved these ratios, with significant liver/body weight ratio (data not shown). In general, the stomach was enlarged and the small intestine became thin and pale in the 10 Gy group, possibly because of gastric retention. In addition, the liver and lung changed to dark red, which suggests sign of haemorrhage. However, such adverse changes were alleviated or not observed in the SAN+10 Gy group (Figure 3).

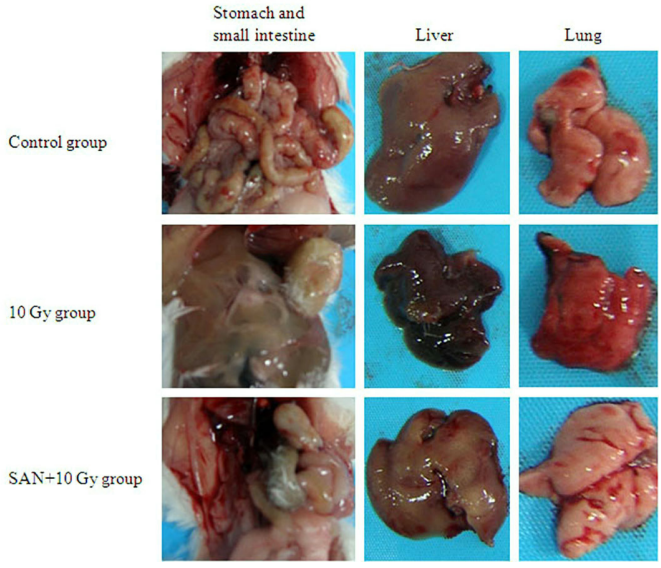

FIGURE 3 - Appearance of the stomach, small intestine, liver and lung at day 5 (n=5).

Histopathological changes

The microstructure of the small intestine, lung, spleen and liver is shown in Figure 4. Small intestinal tissues of mice in the 10 Gy group showed the destruction of villi structure and denudation of epithelium surfaces. The changes in the SAN+10 Gy group was only observed in short and widened villi structure, swollen epithelial cells and vacuolated cytoplasm, suggesting the part restore of structure by SAN pretreatment. Chiu's pathology grading scores were 0.06±0.06 in the control group and this score remarkably increased in both radiation groups, with significantly lower score in SAN+10 Gy group than in 10 Gy group (2.77 ± 0.48 vs. 4.37 ± 0.31, P<0.01). In lung tissues, radiation exposure caused hemorrhage in alveolar space and perivascular edema, resulting in parenchymal consolidation and alveolar epithelial edema. Pretreatment with SAN apparently decreased these damage. The pathological score of the lung was 0.03±0.05, 2.33±0.32 and 1.67±0.21 in control group, 10 Gy group and SAN + 10 Gy group, respectively. Pretreatment with SAN significantly decreased the score compared with radiation alone (P<0.01). Lymphocytes in the spleen were decreased by radiation. Poor germinative centers and marginal zones were seen in lymphadens and periarterial lymphoid heads. This damage reached about 90% in 10 Gy group and about 50-70% in SAN+10 Gy group. Mice exposed to 10 Gy showed dilatation and congestion of liver blood vessels with prominent hemorrhage. Small hemorrhagic areas with mononuclear cell infiltration were seen in the portal tracts. Similarly, SAN pretreatment provided protection against the liver damage from radiation.

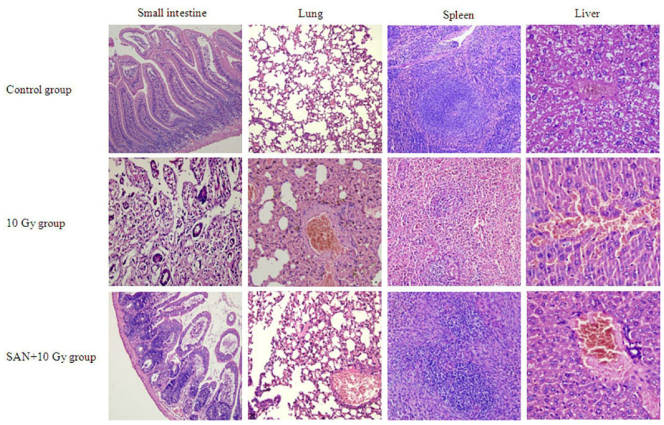

FIGURE 4 - Histopathological examinations of the small intestine, lung, spleen and liver at day 5 (n=5).

DISCUSSION

A number of studies have demonstrated that SAN is a novel agent against some cancer cells. The present study is the first report to show the potential radioprotection of SAN in mice exposed to lethal dose of whole-body X-ray radiation.

The occurrence time of organ damage induced by radiation depends on the cell kinetics and radiation tolerance of the tissues, where proliferating cells are highly sensitive to radiation exposure. Gastrointestinal (GI) epithelium is crucial for life maintenance and any damage to this organ drastically impairs the normal physiological processes in the body. Because GI epithelium has relatively short cell transit time, early symptom induced

by acute radiation appeared in the GI system (Nunia *et al*., 2007; Patil *et al*., 2012). In the present study, exposed mice showed signs of GI damage including reduced food intake, diarrhea, and even bloody diarrhea. Thus, water drink increased because of reduced food intake and diarrhea induced by radiation exposure. Consistently, histopathological findings indicated corresponding lesions of epithelium surface and villi structure in the small intestine. Decreased body weight in exposed mice also supported the GI damage by radiation. Despite the relatively high sensitivity of bone marrow stem cells to radiation, the peripheral blood cells have longer transit time than the intestinal cells. It is well-known that death within 10 days after radiation is due to GI damage and death from 11 to 30 days caused by hemopoietic damage (Jagetia *et al*., 2003; Patil *et al*., 2012). Since all mice in the 10 Gy group died before day 6 and all mice in the SAN + 10 Gy group died before day 12, GI damage is the main cause of death induced by lethal dose of 10 Gy.

In the present study, we found that the damage induced by radiation (10 Gy group) was decreased by SAN pretreatment (SAN+10 Gy group). Although the exact mechanism is unclear, the antioxidant activity is found to be involved in this process. The free radicals generated during the radiolysis of water have important function in the direct biological damage induced by radiation. Radiation protection by chemicals, such as SAN, at cellular and subcellular level likely reflects the effects of scavenging of radiation-induced free radicals and the repair of damaged targets and molecules. Exposure to ultraviolet B (UVB) radiation induces the generation of hydrogen peroxide (H_2O_2), which is also triggered by ionizing radiation. Ahsan *et al*. (2007) found that pre- and post-treatments of SAN markedly inhibited UVB exposure-mediated generation of H_2O_2 in the skin of SKH-1 hairless mice, suggesting SAN protection against UVB-mediated damage to the skin partly through its antioxidative effects. Anti-oxidative effects of SAN were also observed using DMSO-differentiated HL-60 cells, where SAN inhibited both *N*-formyl-Met-Leu-Phe (fMLP) and phorbol 12-myristate 13-acetate (PMA) induced oxidative bursts, during which large quantities of reactive oxygen species (ROS) are generated (Vrba *et al*., 2004).

The other mechanism of radioprotection offered by SAN was due to the protection of hematopoietic system. SAN may have therapeutic potential for the treatment of diseases with related platelet dysfunction, through the mechanisms including activation of adenylate cyclase, inhibition of platelet Ca^{2+} mobilization and TXB2 production (Jeng *et al*., 2007). Our study provided the direct evidence that radiation-induced hemorrhage, which was observed in histopathological samples of the lung, liver and small intestine, was improved by SAN pretreatment.

In addition to GI and hematopoietic syndromes, bacteremia and inhibition of immune system may be the outcome of radiation damage. In the present study, pretreatment with SAN obviously decreased the radiation-induced poor germinative centers in the spleen, an important part of immune system. Studies have reported the antibacterial and anti-inflammatory activities of SAN (Chaturvedi *et al*., 1997; Miao *et al*., 2011; Niu *et al*., 2012). Nuclear factor kappa B (NF-κB) activation, which plays an important role in processes of cell immunity and inflammation, was induced by ionizing radiation (Lee *et al*., 1998). In Chaturvedi *et al*. (1997) study, for example, SAN potentially inhibited the activation of NF-κB, suggesting one of possible mechanisms by which SAN reduced the radiation-induced inflammatory response.

In conclusion, our preliminary study provided the evidence that SAN protects animals against radiation-induced sickness and acute damage to organs and following animal death. This study suggested that SAN is a candidate for radioprotective agent. Further studies are needed to investigate the exact mechanism of action and feasibility of this agent in clinical practice.

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