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RADIATION–INDUCED APOPTOSIS IN THE SMALL INTESTINE OF RATS

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Abstract

Introduction. According to the literature data, it is known that persons exposed to ionizing radiation, together with a different of damaging effects, particular importance is also attached to the gastrointestinal tract. The dominant role of neutron−activated radionuclide Manganese−56 (^{56}Mn) was noted in the treatises of Japanese scientists who studied the A−bomb effects of Hiroshima and Nagasaki, deserving the interest today.

The research purpose. Investigate the microscopic changes in the small intestine of rats exposed to γ– and neutron radiation.

Materials and methods. In experiment, both sexes «Wistar» rats in amount of 36, weighting approximately 220−330 g. Four groups were identified: 1) ^{56}Mn which obtained by neutron activation of 100 mg MnO2 powder using the «Baikal-1» atomic reactor with a neutrons fluence of 4×10^{14} n/cm²; 2) nonradioactive MnO_2; 3) ^{60}Co γ−rays; 4) control group. Necropsy of the animals were on the 3rd, 14th and 60th days after irradiation, then the small intestine removed, after which it was fixed in 10 % formalin. Tissues fragments embedded in paraffin, then sections are manufactured serial transverse 4 μm thickness, which were subsequently stained by hematoxylin and eosin (H&E). Specific painting on apoptosis was performed by ApopTag. The difference between samples was examined using the Student's t–test.

Results. Increasing the number of mitotic cells in the small intestine of experimental animals observed on the 3rd day after exposure γ− and neutron radiation. Histological analysis of neutron−activated ^{56}Mn showed the high level of apoptosis in the investigated organ. Apoptosis as DNA strand breakage, correlated with cell damage observed on the 14th day after irradiation.

Conclusion. Thus, ^{56}Mn effect on the small intestine of rats showed a high level of risk exposure, which is confirmed by the apoptosis presence.

Keywords: radioactive ^{56}Mn, gastrointestinal syndrome, intestinal crypts, apoptosis.

Резюме

РАДИАЦИОННО–ИНДУЦИРОВАННЫЙ АПОПТОЗ В ТОНКОМ КИШЕЧНИКЕ КРЫС

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Введение. По данным литературных источников известно, что у лиц, подвергавшихся воздействию ионизирующего излучения наряду с различными повреждающими эффектами особое место отводится и желудочно–кишечному тракту. Доминирующая роль нейтронно–активированного радионуклида – Марганца–56 (^{56}Mn) отмечалась в трудах японских ученых, изучавших последствия атомной бомбардировки в Хиросима и Нагасаки, заслуживающий интерес по сей день.

Цель исследования. Изучить микроскопические изменения в тонком кишечнике крыс, подвергавшихся воздействию γ– и нейтронного излучения.

Материалы и методы. В эксперименте использованы крысы обоих полов линии «Вистар» в количестве 36, массой 220–330 гр. Были выделены 4 группы: 1) ^{56}Mn, полученный путём нейтронной активации 100 мг порошка MnO_2 на атомном реакторе «Байкал–1» при флюенсе нейтронов 4×10^{14} н/см²; 2) нерадиоактивный MnO_2; 3) ^{60}Co γ–лучи; 4) контрольная группа. Животных подвергали некропсии через 3, 14 и 60 дней после облучения, затем извлекали тонкий кишечник, после чего фиксировали его в 10 % формалине. Фрагменты тканей заливали в парафин, затем изготовливали поперечные серийные срезы толщиной 4 мкм, которые в дальнейшем окрашивали гематоксилином и эозином (H&E). Специфическую покраску на апоптоз осуществляли посредством ApopTag. Разницу между выборками оценивали используя t–критерий Стьюдента.

Результаты. Увеличение количества митотических клеток в тонком кишечнике экспериментальных животных отмечается на 3–е сутки после воздействия γ– и нейтронного излучения. Гистологический анализ нейтронно–активированного ^{56}Mn выявил высокий уровень апоптоза в исследованном органе. Апоптоз как признак разрыва цепи ДНК, коррелирует с повреждением клеток, наблюдаемой на 14–е сутки после облучения.

Выводы. Таким образом, воздействие ^{56}Mn на тонкий кишечник крыс выявил высокий уровень риска облучения, что подтверждено наличием апоптоза.

Ключевые слова: радиоактивный ^{56}Mn, желудочно–кишечный синдром, кишечные крипты, апоптоз.

Түйіндеме

РАДИАЦИЯ ӘСЕРІНЕН ЕГЕУҚҰЙРЫҚТАРДЫҢ ЖІҢІШКЕ ІШЕГІНДЕ ТУЫНДАҒАН АПОПТОЗ

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Кіріспе. Әдеби мәліметтерге сәйкес, иондағыш сәуле әсеріне душар болғандардың көптеген бүліндіргіш салдарымен қоса асқазан–ішек жолдарына да ерекше мән бөлінеді. Хиросима мен Нагасакидағы атомдық бомбалаудың салдарын зерттеген жапон ғалымдарының еңбектеріндегі нейтронды–белсенді Марганец–56 (^{56}Mn) радионуклидінің басым рөлі заманауи жағдайда да қызығушылық арттырады.

Зерттеу мақсаты. γ– мен нейтронды сәуле әсеріне ұшыраған егеуқұйрықтардың жіңішке ішегіндегі микроскопиялық өзгерістерді зерттеу.

Материалдар мен әдістер. Тәжірибе жүзінде «Вистар» тұқымды 220–330 гр салмағы бар аталық және аналық жынысты 36 егеуқұйрық пайдаланылған. 4 топқа іріктеу жүргізілді: 1) ^{56}Mn, яғни 100 мг MnO_2 ұнтағын «Байкал–1» атом реакторы арқылы 4×10^{14} н/см² нейтрон флюенсінде нейтрондық белсендіру жүзінде алынған элемент; 2) бейрадиоактивті MnO_2; 3) ^{60}Co γ–сәулелер; 4) бақылау тобы. Жануарларға сәулелеуден кейін 3–ші, 14–ші және 60–шы тәуліктерде некропсия жүргізу барысында жіңішке ішегін алып, 10 %–тік формалинде фиксацияладық. Тін фрагменттерін парафинге құйып, қалыңдығы 4 мкм көлденең сериялық кесінділер дайындап, әрі қарай гематоксилин мен эозинмен (H&E) боядық. Апоптозға арнайы бояуды ApopTag арқылы жүзеге асырдық. Топтар арасындағы сынамаларды Стьюденттің t–өлшемі бойынша бағаладық.

Нәтижелер. Тәжірибелік жануарлардың жіңішке ішегіндегі митоздық жасушалар саны γ– мен нейтрондық сәулелеуден кейін 60–шы тәулікте жоғарлағаны анықталған. Нейтронды–белсенді ^{56}Mn–тің гистологиялық талдауына сай зерттелген ағзадағы апоптоздың жоғары деңгейі тіркелген. Апоптоз ДНҚ тізбегі бүлінуінің белгісі ретінде 14–ші тәулікте аңғарылған жасушалар зақымдануымен байланысты болған.

Қорытынды. Сонымен, егеуқұйрықтардың жіңішке ішігіне ^{56}Mn әсері апоптоз белсенуімен расталатын сәулелену қаупінің жоғары деңгейін көрсетті.

Негізгі сөздер: радиобелсенді ^{56}Mn, асқазан–ішек синдромы, ішектік крипталар, апоптоз.

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Introduction. It is known that ^{56}Mn became one of the dominant neutron caused by beta–irradiator during first few hours following A–bomb explosion in Hiroshima [30]. For the dose–effect relationships in atomic bomb survivors to be applied beyond the radiation quality as a generalized measure of risk assessment at a Gy–equivalent basis of reference radiation, neutrons in atomic bomb radiation in Hiroshima and Nagasaki have been conventionally weighted by a constant value [34]. Therefore, atomic bomb effects on health of survivors have been correlated with delayed γ–rays and neutrons [12]. The accidental high–dose radiation exposure induces a series of injury levels in multiple organs [36]. The highly radiosensitive intestine is an important dose–limitative organ in both total body and abdominopelvic radiation [13]. Most of studies regarding the fast neutron effect have focused at intestinal changes [19].

Nuclear factor is pronounced in gastrointestinal tract those that are exposed to the external environment [20], therefore one of outcomes of radiation effects is gastrointestinal (GI) syndrome [13]. The underlying molecular mechanism of radiation–induced intestinal injury is still not well understood. Some researchers suppose that intestinal stem cells, almost always located in crypts subjected directly to ionizing radiation [18]. It is still unclear whether intestinal stem cell apoptosis or endothelial cell apoptosis is the main factor involved in the initiation and development of radiation–induced GI syndrome. Given that intestinal cell apoptosis has major implications in GI syndrome, radiation oncologists and medical researchers have been seeking radioprotective agents for the intestine that would help to limit intestinal cell death and facilitate intestinal crypt reproduction. Several protective substances that minimize radiation–induced intestinal apoptosis have been known for decades [11]. Currently, particular interest is a comparative characteristic of microscopic changes in the immune organs of persons exposed to ^{56}Mn and ^{60}Co [5], allowing in the future to work out the diagnostic criteria for assessing of radiation effect factor on the gastrointestinal tract, depending on the cumulative dose.

The objective of study. Our goal has been to identify and compare the microscopic changes in the small intestine of rats after exposure by single 2.0 Gy dose of γ–radiation and neutron–activated ^{56}Mn powder.

Materials and methods. For this study, it was purchased and raised in a the specific–pathogen–free facility six–month–old both sexes «Wistar» rats (Karaganda State Medical University) in an amount of 36 with mean whole body weight 220–330 g. All rats were acclimatized for 2 weeks before initiation of experiments and kept under normal conditions and fed pellets concentrated diet and vitamin mixtures. They were maintained at constant temperature (22±1°C) on 8 hour light–dark cycle. Then, rats were allocated into 4 groups. The first group of animals (n=9) were subjected to ^{56}Mn which was obtained by neutron activation of 100 mg of manganese dioxide – MnO_2 (Rare Metallic Co., Ltd., Japan) powder using the «Baikal–1» nuclear reactor with neutron flux 4×10^{14} n/cm². Activated powder with total activity of ^{56}Mn 2.75×10^{8} Bq was sprayed pneumatically over rats placed in the special box. The moment of exposition beginning of experimental animals by ^{56}Mn powder is 6 minute after finishing of neutron activation. Duration of exposition of rats to radioactive powder was 3.5–4.0 hours (starting from the moment of spraying of ^{56}Mn powder till surgical extraction of the small intestine) [1].

The second group of rats (n=9) were exposed to not irradiated MnO_2. The spray powder was carried out in a chemical box, which contained boxes of 9 rats. Each portion of MnO_2 was

sprayed in box with lots of biological objects. Then unirradiated powder and incubated biological objects in a container for hour.

The third group of rats (n=9) were irradiated with a total dose of 2 Gy was performed at a dose rate of 2.6 Gy/min using ^{60}Co γ−ray by czech radiotherapy device «Teragam K–2 unit». Before the exposure, topometry and dosimetry of the rats was performed. After irradiation, rats were taken back to the animal facility and routinely cared. All the experiments were followed our institution's guide for the care and use of laboratory animals. During the exposure, animals were placed in a specially engineered cage made of organic glass with individual compartments for each rat.

The fourth group consisted of control rats (n=9) which were placed on shelves in the same facility and shielded from the radiation. All animal procedures were approved by Ethical Committee of Semey State Medical University, Kazakhstan (Protocol №5 dated 16.04.2014) in accordance with Directive of the European Parliament and the Council on the Office in animals protection. Rats were housed in a moderate security barrier.

The rats were sacrificed on the third, fourteenth, sixtieth day after irradiation and the small intestine was immediately surgically extracted for further histological study. The small intestine sections were deparaffinized and dehydrated in graded 10 % formalin solutions. Paraffin sections performed with 4 μm thickness. For routine pathology, sections were hydrated and stained with hematoxylin–eosin (H&E). Identification of apoptosis was confirmed using a TUNEL technique. The TUNEL assay (Terminal deoxynucleotide transferase dUTP Nick End Labeling) was performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit according to the manufacturer's instructions. The incidence of cell death and number of mitotic cells in the small intestine was quantified by counting the number of cells in each crypt in H&E–stained sections at 40 magnification by light microscopic analysis.

All values were expressed as the mean ± standard error (S.E.) of results obtained from experimental animals per data point. Differences between samples by the level of trait measured quantitatively were estimated for statistical significance using the Student's t–test. A $P<0.05$ value was considered to be of statistical significance.

Results. In the present study, we performed experiment with neutron–activated ^{56}Mn powder exposed laboratory rats. Although the level of radioactivity received from ^{56}Mn was rather low, the observed biological effects were consistent in experiment. It was previously reported the internal dose estimates in organs of ^{56}Mn–exposed rats. The highest doses were recorded in the small intestine [2]. According to finding, mitosis in this organ was enhanced for an extended period after exposure to ^{56}Mn. For count of mitotic cells in the intestinal crypt was used longitudinal sections of the crypt. On the figure 1, there was a sharp increase the number of mitotic cells in the intestinal crypts of ^{56}Mn–induced (A) and γ–ray–induced (B) rats on the 3rd day after irradiation when compared with MnO_2 and control rats.

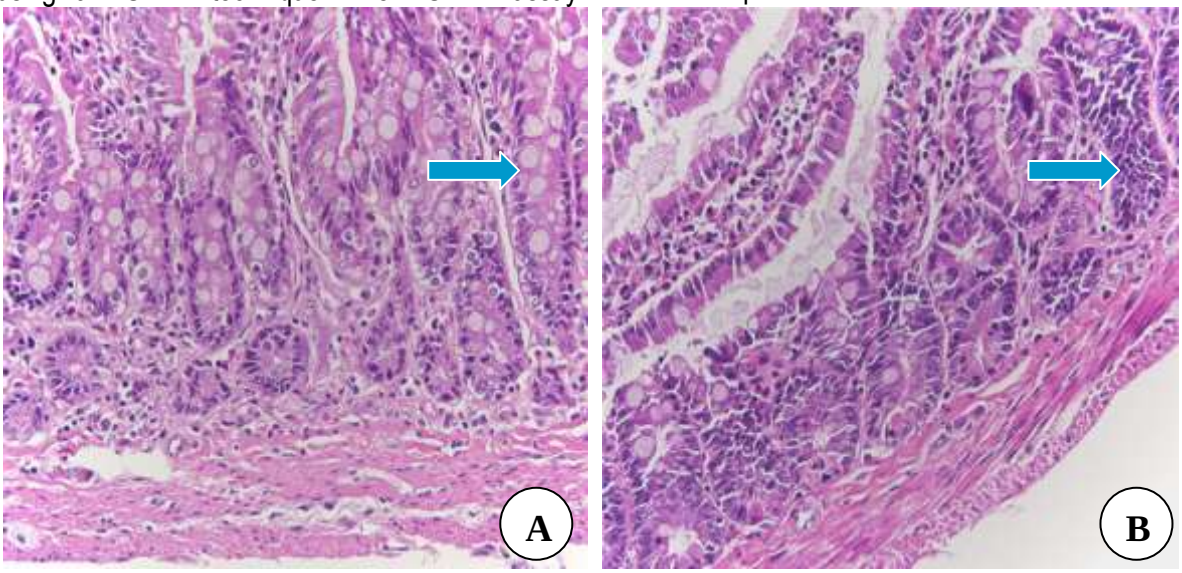

Fig. 1. Photomicrograph of rat small intestine. A number of mitotic cells per intestinal crypt were noted in the ^{56}Mn (A) and ^{60}Co (B) groups on the 3rd day after exposure; H&E staining, original magnification ×10.

Exposure–related histological changes were noted in the small intestine of rats after neutron and γ−radiation. On the 60th day after irradiation the mitotic process could be observed only in rats exposed to ^{56}Mn (Fig. 2 A, B).

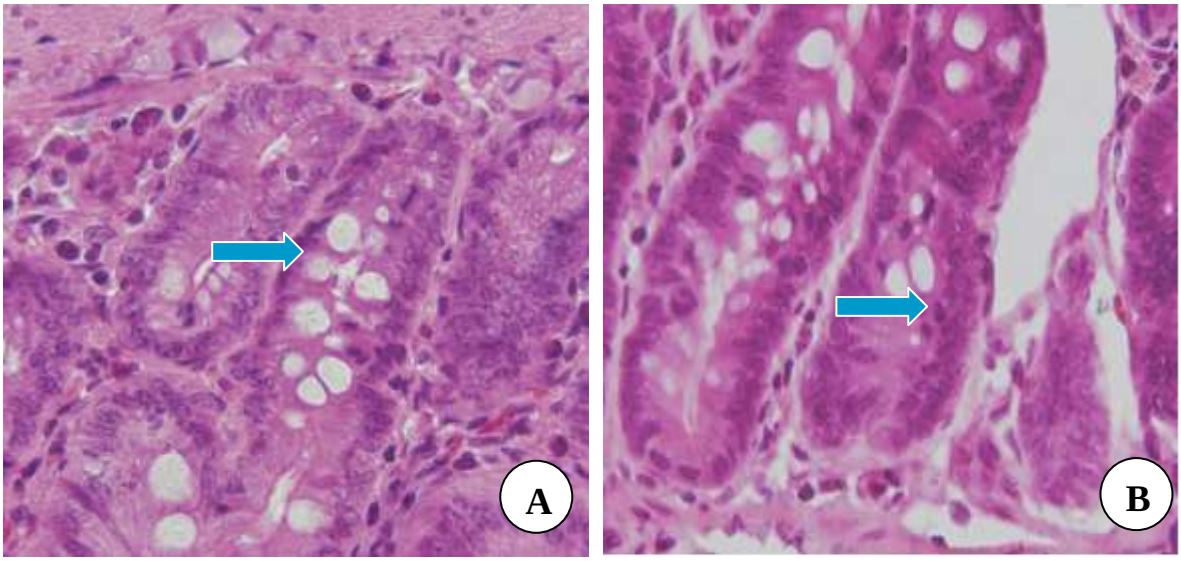

Fig. 2. Light microscopy of ^{56}Mn–induced rat small intestine on the 60th day after exposure; H&E staining, original magnification ×40.

The small intestine is among the most quickly self–renewing tissues in adult mammals [38]. The number of mitotic cells per crypt in the small intestine are summarized on Table 1. The number increased in both the ^{56}Mn and ^{60}Co groups on the 3rd day after exposure. While it returned to the control level by 14th day in the ^{60}Co group, it was still high on the 60th day in the ^{56}Mn group.

Table 1.

Number of mitotic cells per crypt in rat small intestine.

№	Group	3rd day	14th day	60th day
1	^{56}Mn	1.81 ± 0.26*	1.14 ± 0.14	2.83 ± 0.24*,#
2	MnO2	1.07 ± 0.20	0.98 ± 0.13	1.71 ± 0.24
3	^{60}Co	2.19 ± 0.25*	0.89 ± 0.11	1.38 ± 0.18
4	Control	0.95 ± 0.18	1.06 ± 0.22	1.32 ± 0.20
Mean ± S.E. * p<0.05 vs. MnO2 and Control, # p<0.05 vs. ^{60}Co				

Mitotic index, on the other hand, gradually increased and peaked on the 3rd day after exposure, which coincides with our data showing increases in mitotic cell numbers on the 3rd day in both the ^{56}Mn and ^{60}Co groups. Interestingly, an increase in mitosis was still observed on the 60th day after exposure to ^{56}Mn, while it returned to the control level in the ^{60}Co group, suggesting that the effects of internal radiation of ^{56}Mn were more persistent.

Figure 3 shows that apoptosis was observed in the small intestinal crypts in the rats exposed to neutron–irradiation. On the 14th day after irradiation in rats from the first group, a large number of apoptotic cells was observed in the intestinal crypts, as determined by TUNEL staining (Fig. 3 A, B). Apoptotic cells different small dimensions comparable with lymphocytes dimensions with high nuclear–cytoplasmic ratio, rounded contours and condensed chromatin and cytoplasm in experimental animals of the first group on the 3rd and 60th day after irradiation, whereas the third group of data changes were identified by three day after irradiation. The distinctive morphological features of apoptosis were used to recognize apoptotic cells. Small clusters of dead cell fragments were assessed as originating from one cell and any doubtful cells were disregarded. Apoptosis was measured on the basis of nuclear image morphology and were able to correlate TUNEL positive staining with measurable nuclear fragmentation.

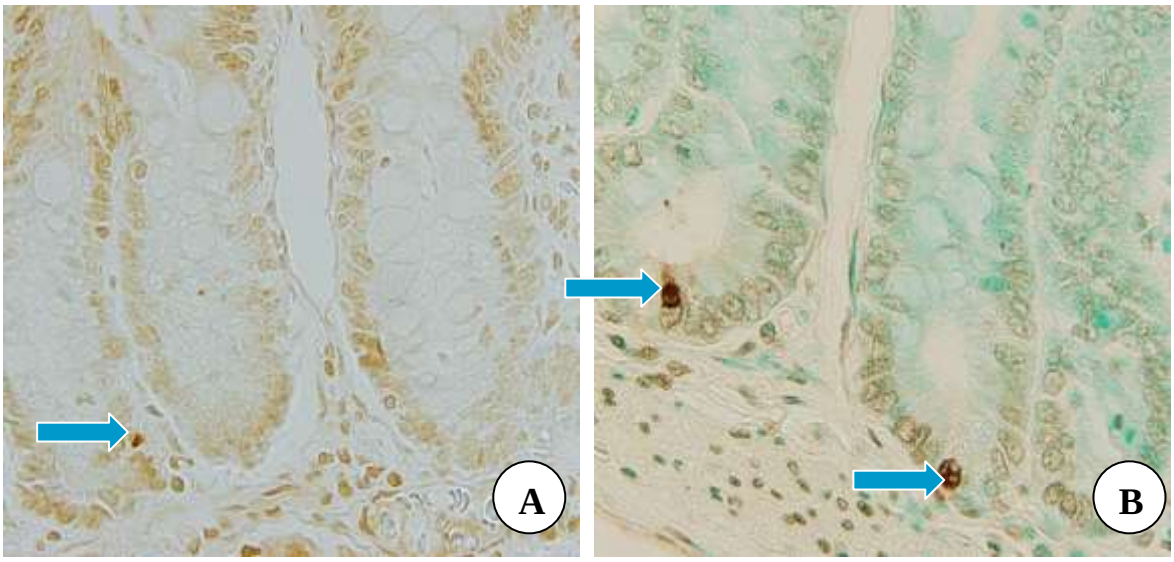

Fig. 3. Histologic sections of the small intestine of rats on the 14th day after ^{56}Mn exposure, stained by TUNEL method to make visible the cells containing DNA fragments, original magnification ×40.

Apoptotic cells look as the rounded or oval accumulations of intensively eosinophil cytoplasm with dense by the fragments of nuclear chromoplasm.

Table 2 shows the number of mitosis in small intestinal crypt were increased in 2.0 Gy ^{56}Mn exposed rats on the 3rd and 14th day after internal irradiation and in 2.0 Gy ^{60}Co exposed rat on the 3rd day after external irradiation. Number of apoptosis in small intestinal crypt were increased only in 2.0 Gy ^{56}Mn internal exposed rats on the 14th day after irradiation.

Table 2.

Mitosis and apoptosis in the small intestinal crypt at different days after irradiation.

№	Group	Mitosis			Apoptosis		
		3rd day	14th day	60th day	3rd day	14th day	60th day
1	^{56}Mn	increase	———	increase	———	increase	———
2	MnO_2	———	———	———	———	———	———
3	^{60}Co	increase	———	———	———	———	———
4	Control	———	———	———	———	———	———

Discussion

Experimentally confirmed that a certain percentage of Mn enters to organism through absorption in the gastrointestinal tract. If Mn not absorbed in the stomach, it is rapidly absorbed in the small intestine [28]. Microscopic examination which proved that acute radiation intestinal damage triggers apoptosis of intestinal crypt [39], being observed within a period of some hours in rodents [27]. Evidence obtained using genetic modification technology has convincingly shown that intestinal stem cells are columnar cells at the crypt base intermingling with Paneth cells [35]. The molecular determinants of intestinal radiosensitivity and GI syndrome are not well understood. Some believe that damage to stem cells plays a critical role in this process [32]. Ionizing radiation leads to the exhaustion of the stem cells pool, increases the load on the differentiated cells, resulting in enhanced processes of apoptosis [22].

Previous studies implicated vascular endothelial cell apoptosis in the initiation and development of GI syndrome [10]. The immediate response to damaged DNA is the stimulation of DNA repair machinery and the activation of cell cycle checkpoints, followed by down–stream cellular responses, such as apoptosis. It was observed that 2 Gy irradiation induced apoptosis and cell cycle arrest [9]. Literature data suggest that intestinal crypt stem cell apoptosis dominant over villus vascular endothelial cell apoptosis in

the initiation of radiation–induced GI syndrome [26]. Few studies have focused on a biopolymer whose manipulation significantly regulates GI syndrome via securing stem cell zones and the integrity of intestinal epithelium [23]. Over the past decade, numerous studies have confirmed that multifunctional adaptor proteins have indispensable roles as scaffolds and adaptors in apoptosis–associated signal transduction [24].

Cell death after radiation occurs by mitotic catastrophe and by apoptosis [14]. It should be noted that apoptotic cells are eliminated by the adjacent epithelial cells, endothelial, fibroblasts, macrophages [21, 40]. Apoptosis ensures the removal of dying cells by phagocytosis without inflammation [16]. The most fully the apoptosis role was investigated at tumor growth. Intensification of apoptosis has implications for tumor regression. If the cell is not able to produce apoptosis due the mutation it can start reproducing uncontrollably, resulting to tumors [25]. Radiation–induced apoptosis of intestinal crypts is largely responsible for intestinal tissue damage [29]. In the gastrointestinal system, irradiation induces apoptosis of the small intestinal crypts, contributing to denudation of the intestinal mucosa and reduces the surface for nutrient absorption [31]. The acute morphological changes of intestine by irradiation were consisted of structural changes in the villus–crypt architecture and epithelial transformations associated with radiation–induced apoptosis [13]. Apoptosis is a major pathogenic peculiarity of radiation–induced small intestinal mucosal injury, and apoptosis degree reflects the mucositis degree [7]. Most authors believe that cell death resulting from toxicity of Mn is not a classical apoptosis, and its combination with cessation of ATP synthesis due to mitochondrial damage [33]. Dysfunction or death of intestinal epithelial cells caused by massive apoptosis after radiation influence is considered as dangerous component in the pathogenesis of GI syndrome [15]. The initiation and progression of radiation–induced intestine injury can be caused by disorder of metabolic processes [3, 4, 6, 37] and molecular mechanisms, which form an compounded response [17].

The large increase of apoptotic cells on the 60th day mark in our first experiments revealed a higher turnover of crypt cells for the internal exposure model of crypt cells, as compared to the normal level of apoptosis found in the external exposure model. As the half–life of ^{56}Mn is three hours, understanding the initial damage to stem cells by internally deposited radioactive materials is crucial.

Although whole–body radiation doses from ^{56}Mn were relatively low, higher internal doses were noted in the small intestine, in addition to significant pathological changes that were more severe and prolonged than the effects of ^{60}Co γ–irradiation. These data may indicate the potential for a high risk of internal exposure to ^{56}Mn, which would have existed in airborne dust after A–bomb explosions in Hiroshima and Nagasaki.

Conclusion. Thus, results shown that number of mitotic cells increased in the small intestine on the 3rd day after ^{56}Mn and ^{60}Co γ–irradiation, but the change persisted only in ^{56}Mn–exposed animals. The histological findings show a significantly higher rate of apoptosis in small intestine for the rats irradiated ^{56}Mn when compared to the other group. Apoptosis is an indication of DNA strand breakage and most likely correlates to the continued cell damage observed beyond 14th day.

Interest conflict

All authors declare no conflict of interest.

Authors contributions:

Uzbekov D. – the practical implementation of all phases of the experiment;

Shichijo K. – the practical implementation of histological staining, acquisition of data;

Fujimoto N. – statistical analysis;

Shabdarbaeva D. – histological analysis and interpretation of data;

Sayakenov N. – the practical implementation of rats necropsy;

Chaizhunusova N. – revision of the manuscript;

Hoshi M. – development of methodology;

Kairkhanova Y., Saimova A.. – collection of literature review;

Rakhypbekov T. – administrative, technical and material support.

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