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SHORT COMMUNICATION

Hemorrhage Trauma Increases Radiation-Induced Trabecular Bone Loss and Marrow Cell Depletion in Mice

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Exposure to high-dose radiation results in deleterious effects on skeletal tissue. However, the effects of combined trauma such as radiation and hemorrhage on skeletal properties have yet to be elucidated. The purpose of this study was to evaluate the effects of radiation injury combined with hemorrhage on trabecular bone properties and biomarkers of bone metabolism, and to determine whether hemorrhage enhances radiation-associated bone loss. Male CD2F1 mice (10 weeks old) were exposed to one single dose of gamma radiation (^{60}Co): 0 or 7.25 Gy. Two hours after irradiation, animals were bled 0% (n = 8) or 20% (n = 8) of total blood volume via the submandibular vein. Mice were euthanized 30 days after irradiation, and distal femora were analyzed using standard histomorphometry to determine changes in trabecular bone volume (BV/TV), thickness (Tb.Th), spacing (Tb.Sp), number (Tb.N) and marrow adipocyte density. Femurs from mice euthanized 1, 7 and 15 days post injury were flushed and total bone marrow cells were counted. Radiation exposure resulted in deleterious effects on distal femur BV/TV (–63%), Tb.Th (–34%), Tb.N (–45%), Tb.Sp (+125%) and adipocyte density (+286%) compared with the sham-irradiated mice (0 Gy; $P < 0.05$). Hemorrhage after irradiation resulted in greater deleterious effects on the distal femur with BV/TV (–13%), Tb.Th (–44%), Tb.N (–26%), Tb.Sp (+29%) and marrow adipocyte density (+33%) compared with radiation exposure only ($P < 0.05$). Analysis of the biomarkers of bone metabolism in serum from irradiated and hemorrhaged mice revealed significantly lower levels of osteocalcin (–60%) and procollagen type 1 amino-terminal propeptide (–36%; P1NP, biomarkers of bone formation activity), as well as elevations in sclerostin (+56%; SOST, an inhibitor of bone formation) compared with serum from irradiated only mice ($P < 0.05$). Additionally, the onset of bone marrow cell depletion in irradiated and hemorrhaged mice occurred earlier and to a greater extent compared to that in irradiated only mice. This study provides definitive, preliminary evidence that hemorrhage further exacerbates trabecular bone loss associated with nonlethal high-dose gamma radiation. © 2015 by Radiation Research Society

INTRODUCTION

Radiation exposure events have historically demonstrated that irradiated victims are often subjected to other trauma as well, such as hemorrhage, wounds, burns, brain injuries and skeletal fractures. For example, a significant number of these combined traumas were observed after the bombings at Hiroshima and Nagasaki (~60–70% of all victims) (*1, 2*). In addition, ~10% of the 237 victims at the Chernobyl reactor meltdown were exposed to radiation plus another trauma (*3*). In rodent models of combined trauma, burns and wounds have been shown to increase morbidity and mortality after otherwise nonlethal radiation exposures (*4–6*). Secondary consequences to radiation exposure combined with another trauma in survivors of these injuries may include bone atrophy and increased risk of bone fracture, presenting further long-term health complications in first responders and survivors of a nuclear attack, nuclear accident or exposure to a radioactive dispersal device (RDD) (*7, 8*).

Most of the current understanding of radiation effects on bone pertains to skeletal health after radiation therapy for cancer treatment. It is well established that long-term consequences of radiation therapy include increased bone loss and incidence of skeletal fracture (*9–11*). Even at low doses (1 Gy) significant reductions in bone volume fraction are demonstrated within 10 days postirradiation (*12*). The predominant bone damage after whole-body gamma irradiation originates from a rapid loss of trabecular bone tissue, demonstrated as reduced trabecular number and connectivity within cancellous bone compartments (*13*). This acute loss of bone mass after irradiation is due to the

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immediate elevations in osteoclast activation and activity (*14*) coupled with decreased osteoblast activity (*15*), resulting in reduced bone formation activity and volume (*16*), which can last for months after exposure (*17*). Although investigations have uncovered significant evidence of the effects of radiation on skeletal tissue, to our knowledge, there have been no published investigations detailing the effects of ionizing radiation combined with hemorrhage trauma on bone. An understanding of the effects of these two traumas on skeletal tissue is necessary to determine and utilize preventative measures for reducing subsequent bone loss and risk of fracture.

The aim of this study was to determine whether hemorrhage, when combined with nonlethal ionizing radiation, exacerbates the effects of radiation exposure on skeletal tissue. We hypothesized that the combined trauma of hemorrhage after irradiation would be more detrimental to bone and marrow than either injury alone.

MATERIALS AND METHODS

Animals and Experimental Design

Ten-week-old male CD2F1 mice (Harlan® Laboratories Inc., Indianapolis, IN) were acclimated to their surroundings for 14 days prior to initiation of the study. All animals were randomly group housed in a temperature-controlled (68–75°F) room on a 12:12 h light-dark cycle and divided into four experimental groups (n = 8/group): sham irradiation (0 Gy), hemorrhage (20% total blood volume), irradiation (7.25 Gy) and combined irradiation and hemorrhage. After irradiation and/or hemorrhage, mice were assigned to clean cages and provided with standard rodent chow (8604 Teklad Rodent Diet; Harlan Laboratories) and acidified water *ad libitum*. The health status of animals was monitored daily and the research was conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI). All animal procedures were reviewed and approved by the Armed Forces Radiobiology Research Institute (AFRRI) Institutional Animal Care and Use Committee (IACUC). The experiment was conducted in duplicate to ensure reproducibility of the data.

Radiation Injury

Mice were placed in well-ventilated acrylic restrainers and one whole-body dose of 7.25 Gy ^{60}Co gamma rays was delivered at a dose rate of approximately 0.6 Gy/min. The 7.25 Gy dose was determined to be the highest nonlethal dose of radiation when combined with hemorrhage (unpublished preliminary data). Dosimetry was performed using the alanine/electron paramagnetic resonance system. Calibration of the dose rate with alanine was traceable to the National Institute of Standards and Technology and the National Physics Laboratory of the United Kingdom. Sham-irradiated mice were placed in the same acrylic restrainers, taken to the radiation facility and restrained for the time required for irradiation.

Hemorrhage

Within 2 h of irradiation with 0 or 7.25 Gy, mice were anesthetized with isoflurane (~3%) and either sham bled or bled 20% of total blood volume via the submandibular vein as previously described (*18*). Briefly, the jaw of the anesthetized mouse was cleaned with a 70% EtOH wipe, and glycerol was applied to the surface of the jaw to allow for ease of collection and measurement of blood loss. A 5 mm Goldenrod animal lancet (MEDIpoint, Inc., Mineola, NY) for facial vein blood samples was used to puncture the submandibular vein of the mouse and heparinized hematocrit collection tubes (75 mm; Drummond Scientific Co., Broomall, PA) were marked and used to collect the appropriate amount of blood to ensure 20% of total blood volume was extracted during the hemorrhage process. The volume of blood collected was based on the body mass of each individual mouse (*19*).

Serum Biomarker Analysis

Whole blood was collected by terminal cardiac puncture from mice anesthetized by isoflurane on day 30 and serum was separated into separate aliquots in 1.5 mL Eppendorf tubes for each biomarker and stored at −80°C until assayed. Tartrate-resistant acid phosphatase 5b (TRAP 5b), a osteoclast number (Immunodiagnostic Systems, Fountain Hills, AZ), osteocalcin, a biomarker of bone formation activity (ALPCO® Diagnostics, Salem, NH), procollagen type 1 amino-terminal propeptide (P1NP), a biomarker of bone formation activity (Immunodiagnostic Systems) and sclerostin (SOST), a inhibitor of bone formation (ALPCO Diagnostics) were determined according to the manufacturer's instructions. The inter-assay coefficient of variation for each biomarker assay was found to be 1.3–2.4%.

Histomorphometry

Left distal femur tissue was demineralized in Immunocal™ (Decal® Chemical Corp., Tallman, NY) according to the manufacturer's instructions, embedded in paraffin and serial frontal sections were cut 8 μm thick and mounted on slides. Slides were stained with Masson's trichrome (Sigma-Aldrich® LLC, St. Louis, MO) for measurement of cancellous bone volume normalized to tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular spacing (Tb.Sp). Adipocyte density was calculated as number of adipocytes (Ad.N) divided by the marrow area (Ma.Ar) of the region of measurement. The histomorphometric analyses were performed by using the OsteoMeasure Analysis System, Version 1.3 (OsteoMetrics Inc., Atlanta, GA). A defined region of interest was established ~0.5 mm proximal to the distal growth plate and extended a further 0.5 mm, all within the endocortical edges at 40× magnification.

Bone Marrow Cell Counts

Bone marrow cells from femurs were collected on day 1, 7 and 15 and washed with 10 mL 1× phosphate buffered saline (PBS). The cells were then centrifuged at 800*g*, resuspended in 10 mL 1× PBS buffer, then counted using a hemocytometer.

Statistical Analyses

All data are presented as mean ± SEM and their statistical relationships were evaluated using the SPSS® statistical software package (v.15, Chicago, IL). All data were analyzed using a two-way ANOVA (hemorrhage and radiation), and when a main effect was found, a Tukey's post hoc test was performed for pairwise comparisons. For all data, statistical significance was accepted at $P \leq 0.05$.

RESULTS

Radiation Injury, either Alone or in Combination with Hemorrhage, Inhibits Normal Body Mass Accrual in Mice

Hemorrhage alone resulted in 6% lower proportional body mass (percentage difference vs. day 0 body mass for each animal) on day 1 after injury (vs. sham irradiation only; $P < 0.05$), but no detectable differences in body

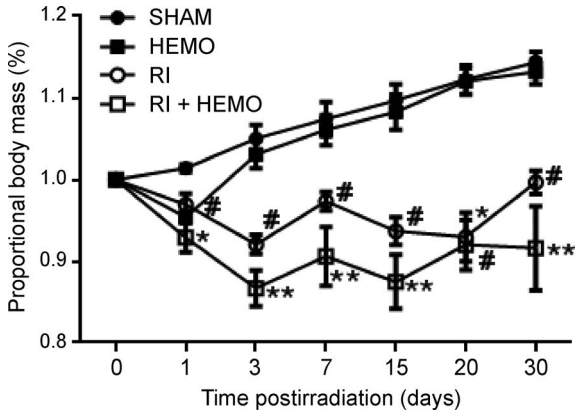

FIG. 1. Radiation injury only (RI), hemorrhage only (HEMO) and combined trauma (RI + HEMO) effects on proportional body mass changes after irradiation (0 or 7.25 Gy). Proportional body mass (percentage difference in body mass after day 0) was calculated for each mouse to determine the effects of HEMO, RI and RI + HEMO on normal body mass gains. All values are mean ± SE. #vs. RI (0 Gy; $P \leq 0.05$); *vs. HEMO (0 Gy; $P \leq 0.05$); **vs. all groups ($P \leq 0.05$).

mass between the hemorrhaged only and sham-irradiated groups were demonstrated after this time point (Fig. 1). As expected, radiation exposure only immediately reduced body mass (by day 1; −4% vs. sham irradiation) and remained significantly lower compared to sham-irradiated mice through day 30 (−9% to −17%; $P < 0.05$). Irradiated and hemorrhaged mice exhibited further deficits in proportional body mass through day 30 compared to sham-irradiated mice (−16% to −25%; $P < 0.05$) and never fully recovered to their preirradiated weight.

Hemorrhage after Radiation Injury Exacerbates Alterations in Trabecular Bone Microarchitecture and Accelerates Reductions in Bone Marrow Cells

The irradiated only mice had significantly altered distal femur microarchitecture, resulting in reduced BV/TV (−63%), Tb.Th (−34%), Tb.N (−45%), and increased Tb.Sp (+125%; $P < 0.05$) and adipocyte density (+286%) compared to sham-irradiated mice (Fig. 2A–D). Although hemorrhage only increased BV/TV, combined irradiation plus hemorrhage exacerbated radiation-induced effects on trabecular bone microarchitecture. BV/TV (−73%), Tb.Sp (+29%) and Tb.N (−61%) were significantly different in the irradiation plus hemorrhage group compared to the irradiated only group ($P < 0.05$).

Although hemorrhage only did not affect marrow adipocyte density, bone marrow cellularity was lower by day 1 after hemorrhage (−26%; $P < 0.05$) in these mice compared to the sham-irradiated mice (Fig. 3A–C). Radiation exposure only increased adipocyte density on day 30 (+286% vs. sham irradiation) and reduced bone marrow cellularity by day 15 (−59% vs. sham irradiation; $P < 0.05$). Irradiation plus hemorrhage exacerbated the effects of radiation exposure, resulting in 339% increase in marrow adipocyte density (vs. hemorrhage only; $P < 0.05$), in addition to reductions in bone marrow cellularity by day 1 (−18% vs. irradiation only) extending through day 15 (−59% vs. hemorrhage only; $P < 0.05$). The significant increase in adipocyte proliferation in bone marrow after irradiation only and irradiation plus hemorrhage was apparent in distal femur samples (Fig. 3C).

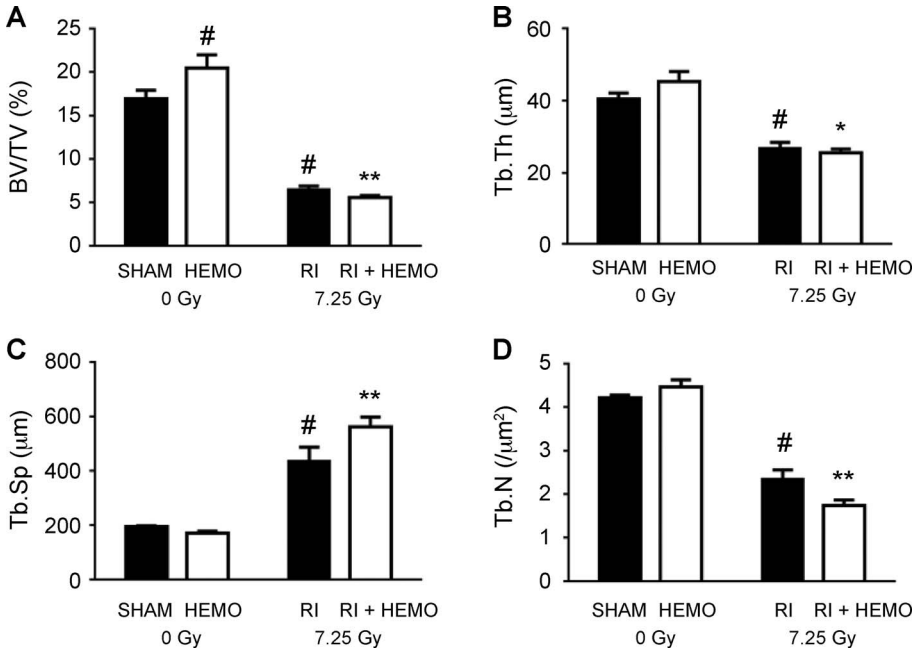

FIG. 2. Radiation injury only (RI), hemorrhage only (HEMO) and combined trauma (RI + HEMO) effects on trabecular bone microarchitecture (panels A–D) day 30 postirradiation (0 or 7.25 Gy). All values are mean ± SE. #vs. sham (0 Gy; $P \leq 0.05$); *vs. HEMO (0 Gy; $P \leq 0.05$); ‡vs. RI (7.25 Gy; $P \leq 0.05$); **vs. all groups ($P \leq 0.05$).

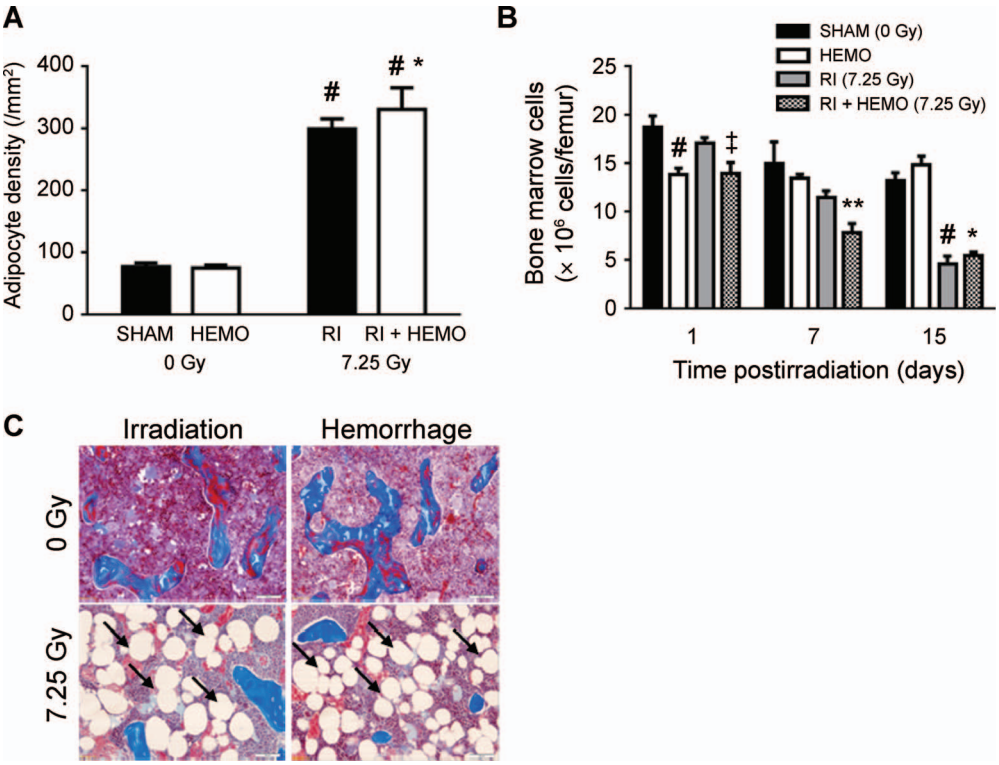

FIG. 3. Panel A: Radiation injury only (RI), hemorrhage only (HEMO) and combined trauma (RI + HEMO) effects on bone marrow adipocyte proliferation day 30 postirradiation (0 or 7.25 Gy). Panel B: The effects of RI, HEMO or RI + HEMO on bone marrow cellularity were determined day 1, 7 and 15 postirradiation (0 or 7.25 Gy). Panel C: Representative images after Masson's trichrome staining within the distal metaphysis of the femur from all treatment groups on day 30. Original magnification 200×. Blue indicates bone or collagen, red (in marrow) indicates cytoplasm and erythrocytes and white indicates adipocytes. Black arrows indicate adipocytes; note the extensive adipocyte density in the irradiated only (7.25 Gy) and the 7.25 Gy irradiated plus hemorrhage samples. All values are mean ± SE. #vs. sham (0 Gy; $P \leq 0.05$); *vs. HEMO (0 Gy; $P \leq 0.05$); ‡vs. RI (7.25 Gy; $P \leq 0.05$); **vs. all groups ($P \leq 0.05$).

Biomarkers of Bone Formation and Resorption are Deleterious Affected by Hemorrhage after Radiation Injury

Irradiated only mice showed reduced serum levels of osteocalcin (−54%) and P1NP (−18%), and elevated TRAP 5b and SOST (+62 and +90%, respectively) compared to sham-irradiated mice (Table 1; $P < 0.05$). The irradiated and hemorrhaged mice showed further reductions in osteocalcin and P1NP (−36% and −60%, respectively) and significantly elevated SOST (+56%) compared to irradiated only mice ($P < 0.05$). Surprisingly, there was a 91% increase in circulating serum SOST levels in the hemorrhage only mice compared to sham-irradiated mice (0 Gy; $P < 0.05$).

DISCUSSION

The main purpose of our study was to test the hypothesis that combined trauma of hemorrhage and ionizing radiation exposure would be more detrimental to skeletal tissue and marrow cellularity than either injury alone. In

TABLE 1
Serum Bone Metabolism Biomarkers Day 30 after Radiation Injury, Hemorrhage and Combined Trauma

	0 Gy		7.25 Gy	
Serum biomarker	Sham irradiation	Hemorrhage only	Irradiation only	Irradiation plus hemorrhage
Osteocalcin (ng/ml)	79.66 ± 11.91	65.93 ± 14.43	36.42 ± 6.94[a]	15.23 ± 3.07[c]
P1NP (ng/ml)	54.11 ± 2.02	48.99 ± 4.44	44.26 ± 1.52[a]	28.21 ± 0.74[c]
TRAP 5b (U/L)	7.29 ± 0.26	7.53 ± 0.18	13.86 ± 0.93[a]	13.56 ± 0.62[b]
Sclerostin (ng/ml)	0.34 ± 0.05	0.65 ± 0.05[a]	0.55 ± 0.07[a]	0.86 ± 0.10[c]

Notes. Procollagen type 1 N-terminal propeptide = P1NP; tartrate-resistant acid phosphatase 5b = TRAP 5b. All values are mean ± SE.
[a] Versus irradiation only (0 Gy; $P \leq 0.05$).
[b] Versus hemorrhage only (0 Gy; $P \leq 0.05$).
[c] Versus all groups ($P \leq 0.05$).

this study, we have demonstrated (in agreement with our hypothesis) that an acute, nonlethal dose of ionizing radiation, when combined with nonlethal hemorrhage, exacerbates cancellous bone loss and accelerates bone marrow cell depletion associated with radiation only. Thirty days after exposure, irradiation plus hemorrhage resulted in significantly reduced cancellous bone volume and increased marrow adipocyte density. Furthermore, nonlethal irradiation combined with hemorrhage resulted in lower serum biomarkers of bone formation activity (osteocalcin and P1NP) and increased levels of biomarkers indicative of osteoclast number and bone resorption activity as indicated by increased TRAP 5b and SOST concentrations in serum.

This study demonstrates initial confirmatory evidence that significant blood loss (without resuscitation) immediately after ionizing radiation exposure results in more deleterious effects on bone than radiation only. These deleterious effects on skeletal tissue appear to arise from a combination of elevated osteoclast activity and reduced osteoblast-induced bone formation (Table 1). In addition, the phenotypic changes in bone after irradiation plus hemorrhage injury coincide with early and continued reductions in bone marrow cell proliferation and increased bone marrow adipocyte density (Fig. 3). To our knowledge, no previous investigations on radiation exposure combined with bleeding effects on bone exist. However, studies of combined trauma involving hemorrhage and fracture effects on bone have been published. For example, several studies examining acute hemorrhage combined with femur or tibia fracture demonstrated exacerbated increases in osteoclast number (through 5 days post injury) (*20*), reduced plasma osteocalcin, increased osteocyte necrosis, and reduced fracture healing (*21*), all resulting in more deleterious effects on skeletal tissue than fracture alone. Although these studies use a different model of combined hemorrhage trauma, they are in agreement with our data and demonstrate the detrimental effects of combined injury on bone.

In our experiment, we demonstrated that hemorrhage did not negatively affect skeletal tissue. Although hemorrhage increased serum sclerostin (Table 1) and reduced femur bone marrow cell count (day 1 only, Fig. 3), femur cancellous bone volume was significantly greater compared to sham-irradiated mice (Fig. 2A). Previous studies have demonstrated that nonlethal hemorrhage results in a beneficial response to bone tissue, evoking a systemic osteogenic response. Acute blood loss in rodents results in immediate and sustained increases in biomarkers of bone formation (i.e., osteocalcin, osteoprotogerin) and lower bone resorption activity (i.e., RANKL) (*22, 23*). Furthermore, acute hemorrhage has demonstrated the ability to increase mineral apposition rate and osteoblast number and reduce osteoclast number (*22*). Blood loss stimulation of hematopoietic stem cell mobilization from bone marrow and the resultant reduction in osteoclast activation within this compartment, as previously demonstrated in rodents (*24–26*), may explain the increased BV/TV and after hemorrhage in our current experiment.

In summary, our data suggest that hemorrhage trauma after radiation exposure exacerbates radiation-induced bone atrophy, causing deleterious effects on trabecular bone microarchitecture and altering bone metabolism to favor resorption and accelerating bone marrow cell depletion. These effects could be mediated by enhanced sclerostin production by osteocytes as well as increased differentiation of mesenchymal stem cells to adipocytes rather than osteoblasts. The underlying cellular and molecular mechanisms responsible for these effects induced by hemorrhage trauma, which by itself did not negatively affect bone quantity or metabolism, when combined with ionizing radiation exposure requires additional study. In addition, the data provided in this experiment depict the complexity of understanding the etiology of skeletal diseases arising from these types of combined traumas and the need to develop effective countermeasures.

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