RESEARCH ARTICLE

Ultraviolet B (UVB) Radiation Induces Skin Alterations, Emperipolesis and Decreases the Erythroid-to-myeloid Ratio in Rats

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Received date: Jul 24, 2024; Revised date: Oct 24, 2024; Accepted date: Oct 28, 2024

Abstract

BACKGROUND: Ultraviolet B (UVB) radiation is commonly known to be related to skin inflammation. The inflammation process is orchestrated by many cell types, including immune cells. Changes in bone marrow cellularity can also be an indicator of inflammation. Megakaryocytes, myeloid immune cells progenitor and erythroid progenitor cells, are at high risk of changes upon UVB irradiation. However, there are still limited study observing the change of bone marrow cell population after UVB irradiation. Therefore, this study was conducted to investigate the alteration of the skins, emperipolesis, and the change in erythroid and myeloid cell population in bone marrow after UVB irradiation.

METHODS: Ten Wistar rats were divided equally into control and UVB-irradiated group. The skin superficial condition before and after UVB irradiation was observed with a skin analyzer camera. On the 9th day, skin tissues were processed for the observation of general skin structure with hematoxylin-eosin (HE) staining, mast cells infiltration with toluidine blue staining, and collagen fibers with Mallory staining. Bone marrow and peripheral blood samples were collected and proceeded for Giemsa-staining to observe the cell population.

RESULTS: Erythema appeared on the skin as marked by orange-red spots. There were hyperkeratosis and pigment accumulations in the skin of UVB-irradiated group. The depletion of collagen-density and hemorrhage were clearly observed in the skin of UVB-irradiated group. There were higher mast cell numbers in the UVB-irradiated skin compared to non-treated skin. The erythroid-to-myeloid ratio in the bone marrow was decreased to around 1.6:11.2 from the normal ratio of 1:4. In addition, emperipolesis was observed in the bone marrow induces by UVB-irradiation.

CONCLUSION: These results indicate that UVB-irradiation alters the skin structure, erythroid-to-myeloid ratio, and induced emperipolesis.

KEYWORDS: emperipolesis, erythroid, myeloid, skin, UVB radiation

Indones Biomed J. 2024; 16(6): 510-6

Introduction

Excessive continuous sun exposure contributes to external body damage, especially to the skin.(1) Sun transmitted ultraviolet (UV) light, and the human body increased melanin production (the pigment that gives skin color) by melanocytes as a protective response. In addition, it also increases melanocyte differentiation and proliferation. (1) The adverse effects of sunlight on the cells are mainly contributed by UV radiation from light with wavelengths 320-400 nm (UVA) and 280-320 nm (UVB).(1,2) Ultraviolet radiation results in the thickening of the stratum corneum, epidermal hyperplasia, depletion of Langerhans cells, and

dermal inflammation.(1) Furthermore, UV also induces DNA damage (1,2), skin-resides immune cell modulation (*i.e.*, mast cells, macrophage), and the production of pro-inflammatory mediators (1-4). Chronic UVB exposure causes oxidative stress that may induce damages to the collagen matrix and thus plays a role in the wrinkle formation.(5)

UV radiation affects cells and tissues that directly amend their energy and might also affect the immune cells. The study in mice also shows the overt effects of UV in increasing global oxidative stress conditions.(6) On the other hand, UVB exposure is also vital for immune suppression, for example, psoriasis, atopic dermatitis, and cutaneous T cell lymphomas. This immune suppression was correlated with the increased production of immune suppression cytokine.(2,7) It was reported that skin dendritic cells (DCs), Langerhans cells, and mast cells migrate to the lymph node after UV radiation.(2,8) UVB radiation increased the release of extracellular traps by a neutrophil.(7) In addition, it inflated the production of pro-inflammatory cytokine, and chemokines expressions from immune and non-immune cells.(2,7) Low UVB exposure was also reported to contribute to the lower diversity of commensal bacteria composition through the modulation of vitamin D conversion to its active form.(9)

It was suggested that sunburn and nonmelanoma skin cancer etiologies are immunologically linked.(10) UV radiation can trigger a cellular senescence and inflammatory state in the skin. Chronic low-grade inflammation stimulates a counteracting immunosuppression involving an expansion of immunosuppressive cells, *e.g.*, regulatory T cells, myeloid-derived suppressor cells, and regulatory dendritic cells. This increased immunosuppressive activity not only suppresses the function of effector immune cells, but also induces bystander degeneration of neighboring cells.(4)

Bone marrow stem cells developed into multi-potent progenitor cells of lymphoid and myeloid cells that can further differentiate into lymphocytes and granulocytes that are irreplaceable in the immune system.(11) Immune cells are mobile and able to be regulated by mediators released by distance cells. Oxidative stress and changes in cytokine production caused by UVB irradiation could increase the potency of changes of myeloid immune cells progenitor and also erythroid progenitor. Other hematopoietic stem cells-derived cell component which is less studied upon UVB irradiation is megakaryocytes. The development of megakaryocytes depends on thrombopoietin (12), and its characteristic might changes in several pathologic conditions (13).

Changes in the bone marrow cellularity can be an indicator of systemic toxicity and inflammation. Most of bone marrow changes observed in inflammation process are in response to hematologic modification or lesions elsewhere in the body. Therefore, consideration of all tissue changes in the body is necessary to distinguish pathologic effects versus physiologic responses in the bone marrow, including the identification of erythroid, myeloid, megakaryocyte cells, that can be achieved from conventional staining.(14) However, studies observing the change of bone marrow cellularity after UVB irradiation is still limitedly available. Therefore, this study was performed to investigate the effect of UVB irradiation on skin conditions, change in megakaryocytes, erythroid and myeloid cell population in the bone marrow.

Methods

Animal Preparation

The animal experiment method performed in this study has been reviewed and approved by the animal research ethics committee in the Faculty of Veterinary Medicine, Universitas Gadjah Mada (No. 0017/EC-FKH/Eks/2020). Ten male Wistar rats (2 months old), 200-300 grams, were obtained from Animal Care Unit, Universitas Gadjah Mada, Yogyakarta, Indonesia. The rats were acclimated for 7 days, and the dorsal area was cleanly shaved. The rats were divided into a control group without UVB radiation (n=5) and an UVB-irradiated group (n=5).

Animal Exposure to UVB

The UVB irradiation was performed in a closed cage that provided enough space for 5 rats with the UVB intensity equal to 225 $\mu W/cm^2$. The UVB irradiation was performed 30 min/day with a UVB light (Reptile UVB200, Exo Terra, Rolf C. Hagen, Montreal, QC, Canada) for 9 consecutive days. Before and after single UVB exposure, the rats were fed and supplied with sufficient water to prevent dehydration.

Skin Observation

The skin superficial condition was observed before and after (the last 9 day) UVB irradiation with a skin analyzer camera (Skin Analyzer EH900U, Panasonic, Osaka, Japan). On the 9^{th} day, erythema occurrences marked by orange-red spots on the skin of shaved area were observed macroscopically. The existence of orange-red spots should be confirmed by at least 3 different observers before in can be concluded as orange-red spot in this report.

Leukocytes and Bone Marrow Smears

Peripheral blood and bone marrow samples were collected and proceeded for Giemsa-staining to observe the cell population. One-hundred microliter of the blood samples were collected to heparinized tube from anesthetized rats via retro-orbital venous plexus. For leukocyte count, bloods were diluted in Türk`s solution (Merck, Darmstadt, Germany) and counted with a haemocytometer. Bone marrows were extracted by aspiration of crista proximal femoral from euthanized (intramuscular administration of ketamine 75 mg/kg and xylazine 5 mg/kg) rats. The collected bone marrow was then centrifuged at 1000 rpm at 25°C for 10 min. The cell pellet was smeared on glass slides and allow to dry for 2–4 hours at room temperature prior to staining with May-Grünwald–Giemsa dyes (Cat. No. MG500; Sigma-Aldrich, Darmstadt, Germany). The evaluation of Giemsa-stained bone marrow tissue included a myeloid/erythroid ratio and megakaryocytes population. The leukocytes of peripheral blood were calculated and compared to the myeloid and erythroid population in the bone marrow smears. Myeloid, erythroid, megakaryocytes, and leukocytes population were calculated from the microscopic image of smears samples.

Tissue Staining

On the 9th day, the skin tissue samples taken from the shaved area were then processed for histopathological staining. This process involves specific steps or techniques, and in this study, hematoxylin-eosin (HE) was used to evaluate the general skin structure, Mallory staining to observe the skin tissue collagen fibers and extracellular matrix, and toluidine blue staining to evaluate the mast cell population. The skin tissue sections were stained with HE following method described before.(15,16) After fixation, the tissues were stained with hematoxylin (Cat. No. MHS32; Sigma-Aldrich) for 15 min followed by 0.5% eosin (Cat. No. 109844; Sigma-Aldrich) staining for 10 min. Mallory staining was performed according to previous work.(17) In brief, 1% of acid fuchsin (Cat No. F8129; Sigma-Aldrich) was used to stain the tissue slide for 10 min, followed by the staining with the combination of aniline blue (Cat. No. 415049; Sigma-Aldrich) and orange G (Cat. No. 861286; Sigma-Aldrich) for 10 min. Tissue sections were incubated in 0.1% Toluidine blue O (Cat. No. 1.04172; Sigma-Aldrich) for 1 min, washed in distilled water and observed under the microscope.(18)

Results

UVB-irradiation Alters Skin Structure

The appearance of orange-red spots (inflammation sign) on the skin surface were clearly observed in UVB-irradiated group (Figure 1B), but not in group without UVB irradiation (Figure 1A). HE staining of UVB-irradiated skin tissues showed an irregular skin layer structure, hyperkeratosis, and the accumulation of melanocytes on the epidermal layer (Figure 2B). Skin pigmentation was marked by the accumulation of melanocytes on the epidermal layer. These melanocytes were responsible for the red spot that appeared on the skin's surface (Figure 1B). All the events were absence in normal skins (Figure 2A).

UVB-irradiation Increased Hyperkeratosis and Erythrocyte Infiltration

The thickness of the epidermis layer was increased (hyperkeratosis), erythrocyte infiltration, and the damage of skin regular structure also observed after staining with Mallory dyes of UVB-irradiated skin tissues section (Figure 2D). Normal skin tissue showed dense and thick collagen, as shown by the thick and uniform blue color. Collagen density was depleted in the skin with UVB radiation as marked by the reduction in blue color intensity (Figure 2D). Hemorrhage characterized by the infiltration of red blood cells into the epidermis layer was observed in the UVB-irradiated group (Figure 2D) but not in the group without

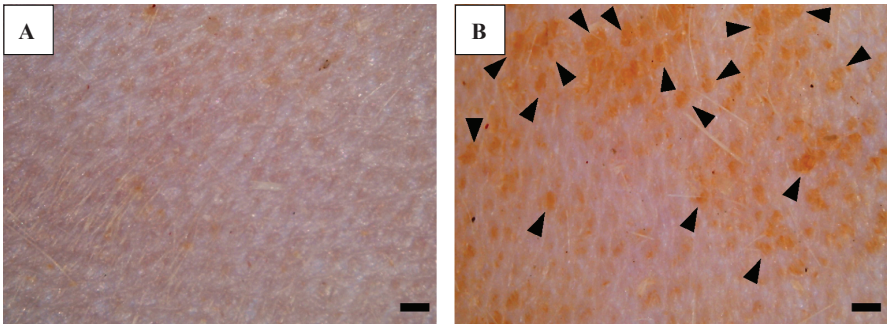

Figure 1. The effect of UVB irradiation on skin erythema formation. A: Skin erythema absence in normal skin. B: Skin erythema presence as marked by orange/red spot (pointed by black arrow head) in UVB irradiated rats. Black bar: 300 µm.

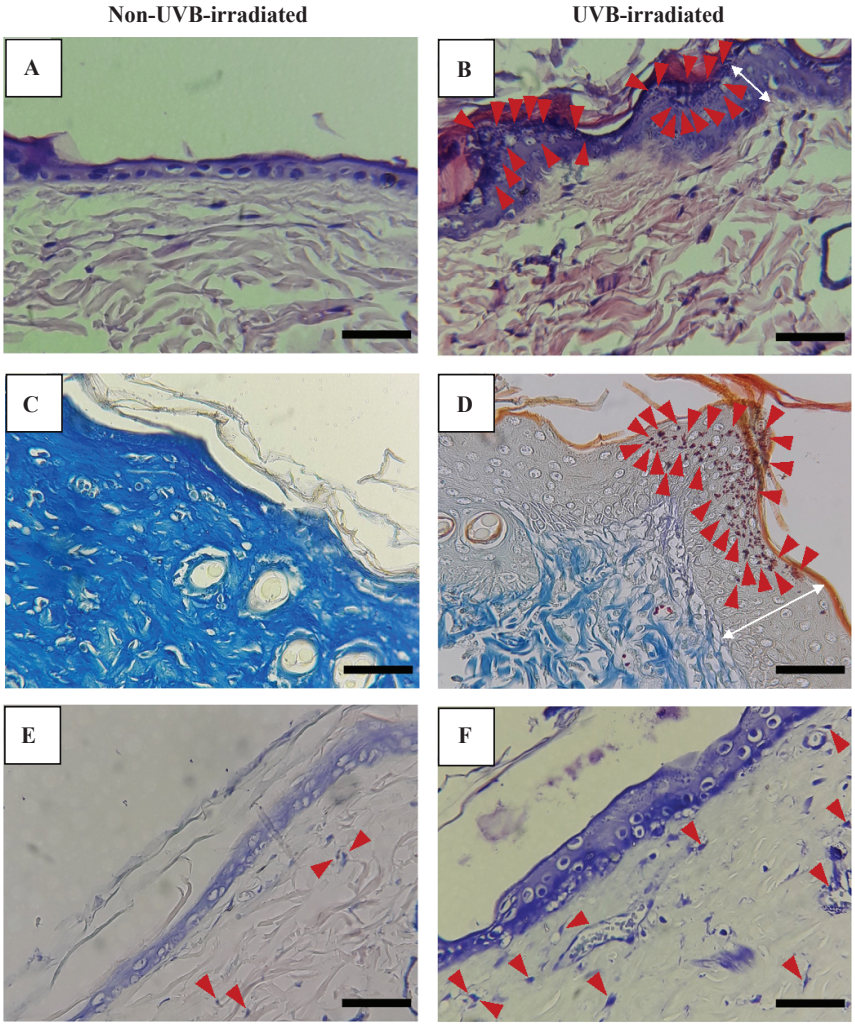

Figure 2. The effect of UVB irradiation on skin tissue condition as observed by various staining methods. A: Normal skin after HE staining showed a well organize skin structure with a thin epidermis layer. B: The skin of UVB irradiated rats after HE staining was less organized with hyperkeratosis (white double-head arrow) and pigment accumulation (pointed by red arrow head). The skin tissue after Mallory staining (C-D). C: Normal skin after Mallory staining showed high collagen (intense blue colour) and no hyperkeratosis or erythrocyte infiltration. D: The skin of UVB irradiated rats after Mallory staining showed low collagen (less intense and discrete blue colour), hyperkeratosis (white double-head arrow) and erythrocyte infiltration (burgundy spot pointed by red arrow head). E: Normal skin after toluidine blue staining showed a few mast cell (pointed by red arrow head) numbers. F: The skin of UVB-irradiated rats after toluidine blue staining showed more mast cell (pointed by red arrow head) numbers than in normal skin. Black bar: 300 µm.

any UVB irradiation (Figure 2C). Toluidine blue staining of UVB-irradiated tissues shows a higher mast cell population (Figure 2F) than in non-UVB-irradiated tissues (Figure 2E).

UVB-irradiation Induced Emperipolesis

Bone marrow swaps of UVB-irradiated rats showed emperipolesis involving one megakaryocyte penetrated by more than five other cells (Figure 3B). No emperipolesis was observed in bone marrow swaps images of normal rats (Figure 3A). In normal rats, four megakaryocytes were observed in 1,000 cells (0.004). Whereas, in UVB-irradiated rats one megakaryocyte was found in 100 cells (0.01). However, the increase of megakaryocytes numbers upon UVB irradiation was not statistically significant (p=0.1143) (Figure 3C).

UVB-irradiation Decreases the Erythroid-to-myeloid Ratio

Erythroid-to-myeloid ratio was significantly decrease (p=0.0079) from 0.25±0.04 in non-UVB-irradiated rats to 0.15±0.03 in the UVB-irradiated rats (Figure 4A). The rise of leukocyte precursors in the bone marrow as indicated by the decrease in erythroid-to-myeloid ratio in UVB-irradiated rat was in line with the changes of leukocyte number in peripheral blood. The leukocyte number was significantly increased (p=0.0321) from 7,060±1,200 cells/mm^3 in normal rats to 17,840±4,800 cells/mm^3 in UVB-irradiated rats (Figure 4B).

Discussion

This study shows the local and systemic inflammation and immune responses after UVB irradiation. UVB exposure causes skin damage in experimental albino animals with characteristics of dry skin, wrinkles, irregular spots (erythema), skin thickening, and decreased elasticity or elastosis.(19) Pigmented skin irradiated to UVB due to increased melanin synthesis. Repeated UVB exposure for 2 weeks has significantly increased eumelanin and

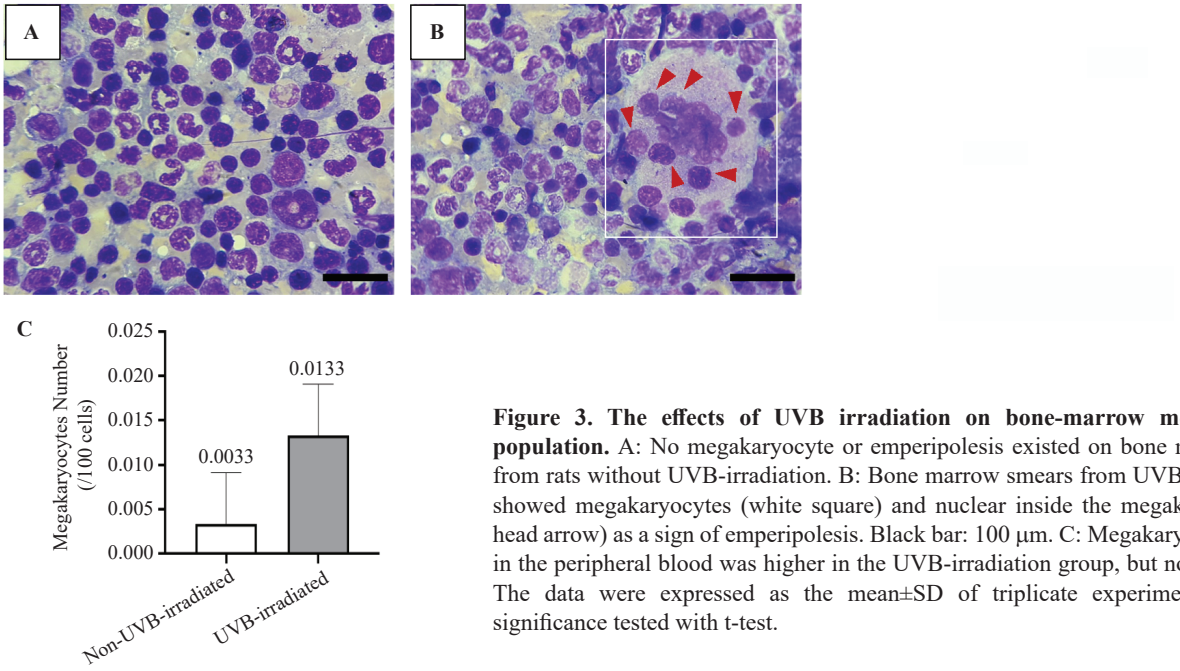

Figure 3. The effects of UVB irradiation on bone-marrow megakaryocytes population. A: No megakaryocyte or emperipolesis existed on bone marrow smears from rats without UVB-irradiation. B: Bone marrow smears from UVB-irradiated rats showed megakaryocytes (white square) and nuclear inside the megakaryocytes (red head arrow) as a sign of emperipolesis. Black bar: 100 µm. C: Megakaryocyte numbers in the peripheral blood was higher in the UVB-irradiation group, but not significantly. The data were expressed as the mean±SD of triplicate experiments. Statistical significance tested with t-test.

pheomelanin levels in mice.(20) Melanocytes accumulation on the skin surface is common signs of skin inflammation caused by UVB irradiation. Melanocytes produce skin pigments such as melanin that are responsible for skin and hair color.(1) The production of melanin is increased in UV treatment, including UVB.(1) Exposure to UVB irradiation increases the number of melanosomes, organelles within melanocytes responsible for the production and distribution of melanin.(21) Red blood infiltration might also contribute to the skin surface redness. Erythrocyte infiltration to the skin tissue demonstrated the increase of vascular endothelial permeability and inflammation processes. Bleeding also appears on the skin of mice because the skin of mice is thin, so it is easily irritated by UVB, which is called contact dermatitis petechial hemorrhage.(22)

UVB irradiation induces keratinocyte proliferation and its accumulation in the epidermis, which results in an increased in epidermis thickness.(23) In addition, its depleted collagen content and deposition. This event can be caused by the increased production and activities of collagen degrading enzymes or protein that contribute to collagen deposition by cells that located in the skin *i.e.*, neutrophil, macrophage, mast cells, and fibroblast.(3,24) These cells produces matrix metalloproteinases that are responsible for collagen and extracellular matrix degradation. An increase in the expression of matrix-metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-9, and MMP-13 were reported after UVB radiation.(3,25) The possibility of involvement of MMP in collagen and extracellular matrix degradation was supported by the increase of mast cells population in skin tissue of UVB-radiated rats observed in this study and also previous studies.(3,26) Chronic UVB exposure causes oxidative stress that may induce damages to the collagen matrix and thus plays a role in the wrinkle formation. UVB radiation induce the downregulation of type 1 procollagen production in the skin. UV radiation may cause irreversible

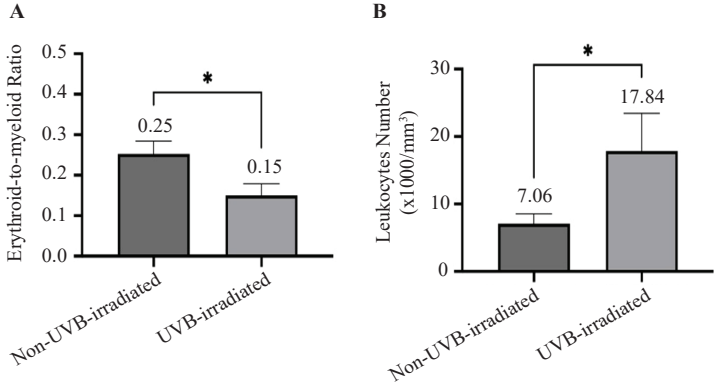

Figure 4. The effects of UVB irradiation on erythroid-to-myeloid ratio and leukocyte number. A: The erythroid-to-myeloid ratio was lower in the UVB-irradiation group. B: Leukocyte`s numbers in peripheral blood increased in the UVB-irradiation group. The data were expressed as the mean±SD of triplicate experiments. Statistical significance tested with t-test; *significant if $p<0.05$.

damage to the cellular and molecular mechanism regulating collagen synthesis and degradation.(5)

The rise of leukocyte in UVB-irradiated rat also affect the ratio of erythroid-to-myeloid. The normal erythroid-to-myeloid ratio observed in this study and also reported previously are 1:4.(14) This study observed the decrease of this ratio caused by the increase of leukocyte numbers after UVB irradiation. The increase of leukocyte numbers is suggested to be directly correlated with skin erythema and inflammation after UV irradiation.(23) Comparison of the cellular changes observed in the bone marrow should always be compared with the complete blood count. Bone marrow changes that are observed in inflammation process are the physiological responses of the bone marrow to hematological changes or lesions elsewhere in the body.(14) UVB-induced inflammation is characterized by redness, swelling, pain, necrosis and loss of tissue function, which result from local immune, vascular and inflammatory cell responses to injury. Important microcirculatory events that occur during the inflammatory process include vascular permeability changes, leukocyte recruitment and accumulation, and inflammatory mediator release. In response to tissue injury, the body initiates a chemical signaling cascade that stimulates responses aimed at healing affected tissues. These signals activate leukocyte chemotaxis from the general circulation to sites of damage. These activated leukocytes produce cytokines that induce inflammatory responses.(10,27) Circulating bone marrow mesenchymal progenitors are peripheral blood cells with a fibroblast-like shape that are able to migrate to regions of tissue injury. These cells exhibit mixed morphological characteristics and express both hematopoietic stem cell antigens and markers of mesenchymal stem cells (MSCs). Mature MSCs play an important role in the immune system since they possess both anti-inflammatory and pro-inflammatory properties.(28)

This study observed emperipolesis in UVB-irradiated rats. Emperipolesis is an uncommon biological process where cells penetrate other living cells. This process might occur naturally in the healthy marrow of humans and other mammals.(29) The occurrence of this process could be correlated with inflammation conditions induced by UVB irradiation. Emperipolesis is increasing in gray platelet syndrome (GPS) and in disease/conditions with an increased demand for platelet, *i.e.*, immune thrombocytopenia, blood loss, and myeloproliferative diseases. Emperipolesis also increases with inflammatory stimuli (29) and x-ray radiation (30), which is in line with the result of this study. Emperipolesis might indicate the ability of UVB irradiation to drive the megakaryocytes to contribute to the immune responses. It is suggested that emperipolesis serve as immune cell, such as neutrophil, transport to re-enter the bone marrow from the blood.(29) A longer-term study with a larger sample size would provide more robust evidence for the observed effects and could be suggested as future work.

Conclusion

UVB-irradiation with the dose and exposure duration used in this study causes inflammation in the skin and alteration in peripheral blood leukocyte compositions. In addition, the UVB irradiation also changes in bone marrow erythroid-myeloid cell population and induces emperipolesis of megakaryocytes in bone marrow, indicating that response in bone marrow cellularity is necessary to distinguish pathologic effects.

Acknowledgments

The authors thanks to Department of Clinical Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada for their assistance to perform this study.

Authors Contribution

MNAS involved in research design, data analysis, writing and review the manuscript, resources. VF involved in research design, methodology, data analysis, and writing initial manuscript. DAD involved in perform the animal experiment and data analysis. SIOS involved in supervising the animal experiment and results interpretation.

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