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Original Article

Zinc deficiency exacerbates pressure ulcers by increasing oxidative stress and ATP in the skin

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ABSTRACT

Background: Zinc deficiency is believed to be a predisposing factor for the development and intractable healing of pressure ulcers (PUs); however, the mechanisms of this association have not been elucidated.
Objective: Objective was to elucidate the mechanisms of the formation of severe and prolonged PUs under the zinc deficiency condition.
Methods: We assessed PUs formation after cutaneous ischemia-reperfusion (I/R) injury in mice fed with a zinc-adequate (ZA) or a zinc-deficient (ZD) diet from 2 weeks before I/R injury. Wound size, vascular damage, apoptotic cells, adenosine triphosphate (ATP) amount, and the number of Langerhans cells (LCs) in I/R area were analyzed. We evaluated the extent of oxidative stress in I/R area in OKD48 mice through bioluminescence detection.
Results: We found that dietary zinc deficiency caused the formation of severe and prolonged PUs in mice. Zinc deficiency increased the vascular disorder, oxidative stress, and apoptosis induced by cutaneous I/R injury. I/R injury-induced oxidative stress signals were significantly higher in ZD OKD48 mice than in ZA OKD48 mice. Additionally, zinc deficiency reduced the number of LCs and increased the amount of ATP in cutaneous I/R-injured skin. Oral supplementation of zinc improved zinc deficiency-associated PUs.
Conclusion: Zinc deficiency might increase cutaneous I/R injury-induced vascular damages, oxidative stress, and apoptosis, as well as ATP amount in I/R area due to the loss of LCs. These mechanisms might partly account for zinc deficiency-induced formation of severe and prolonged PUs. Oral supplementation of zinc might be a reasonable therapeutic choice for patients with PUs and zinc deficiency.

1. Introduction

Owing to age-related changes, such as loss of fat and muscle mass, bone protrusion, perceptual loss, and impaired immune function and wound-healing ability, elderly people are prone to develop pressure ulcers (PUs), also known as decubitus ulcers. In aging society, the prevention, treatment, and management of PUs has become important more than ever. In the early stage of PUs formation, non-blanchable erythema and/or purpuric lesions appear in the skin subjected to external pressure, and skin ulcers subsequently develop after 2–3 weeks [1]. Ischemia-reperfusion (I/R) injury is characterized by reperfusion of blood to previously ischemic tissue and induces cell damage [2,3]. I/R injury is associated with vascular infarction or vasospasm in various organs, such as the brain, heart, liver, and kidneys [2,3]. There is also growing evidence that cutaneous I/R injury is associated with the development of PUs [4–12]. By using a cutaneous I/R injury mouse model, we and others have previously demonstrated that various events, such as endothelial cells (ECs) injury, thrombus formation, edema formation, and production of proinflammatory cytokines by infiltrating leukocytes and macrophages, occurred at an early stage

Abbreviations: Abs, antibodies; ATP, adenosine triphosphate; ECs, endothelial cells; HO-1, Heme Oxygenase 1; I/R, ischemia-reperfusion; LCs, Langerhans cells; NOX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; Nrf2, NF-E2-related factor 2; PUs, pressure ulcers; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotide transferase dUTP nick end-labeling; ZA, zinc-adequate; ZD, zinc-deficient; Zn, Zinc.

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of PUs formation induced by cutaneous I/R [4–12]. Furthermore, we identified that subcutaneous administration of secreted MFG-E8 protein, mesenchymal stem cells, and botulinum toxin inhibited oxidative stress, endoplasmic reticulum stress, apoptotic cells, inflammatory cytokine production, and inflammatory macrophages, and subsequently prevented the development of PUs [8,5–12].

Zinc (Zn) is an essential trace element in living organisms. Many elderly persons are prone to be Zn deficiency because of inadequate intake, decreased absorption in the intestinal tract, and lifestyle-related diseases [13]. There have been a variety of clinical studies about the relationship between Zn deficiency and PUs. It has been reported that serum Zn levels were lower in patients with PUs than in healthy individuals without PUs and that increasing the serum Zn level can accelerate the healing of PUs, suggesting that Zn deficiency may be associated with the pathogenesis of PUs [14]. However, there has been no basic scientific research examining the role of Zn in the pathogenesis of PUs. In addition, there is no established evidence supporting the effectiveness of Zn supplementation.

It has been shown that CD39, which is exclusively expressed by Langerhans cells (LCs) in the epidermis, plays a protective role against adenosine triphosphate (ATP)-mediated inflammatory signals by hydrolyzing extracellular nucleotides released by keratinocytes in irritant contact dermatitis responses [15]. Kawamura et al. investigated the mechanisms by which Zn deficiency induces dermatitis in dietary Zn-deficient (ZD) mice, and found that Zn deficiency reduced $CD39^+$ LCs and increased the ATP release induced by exposure of the skin to irritant stimulation, resulting in severe and prolonged dermatitis [16,17]. There is recent evidence suggesting that extracellular ATP can serve as a damage-associated molecular patterns (DAMPs) molecule that initiates an inflammatory response through autocrine/paracrine signaling [18]. ATP can be released from cells by various stimulators, including mechanical stress, tissue injury, inflammation, and hypoxia [19,20]. Further, in PUs, it is assumed that ATP is released extracellularly by physical and hypoxic stimulation due to skin pressure. However, no study has shown the changes in the amount of ATP in PU lesions in normal and ZD states. Therefore, the objective of this study was to elucidate the mechanisms of the formation of severe and prolonged PUs induced by Zn deficiency by using dietary Zn-adequate (ZA) and ZD mice. Additionally, we investigated the therapeutic effect of oral supplementation of Zn on the development of PUs in ZD mice.

2. Methods

2.1. Animals

All experiments were approved by the Gunma University Animal Care and Experimentation Committee (#18-004), and carried out in accordance with the approved guidelines. C57BL/6 mice were purchased from the SLC (Shizuoka, Japan), and OKD48 (Keap1-dependent oxidative stress detector, NO-48) mice were kindly provided by Dr. T. Iwawaki (Department of Life Science, Kanazawa Medical University, Ishikawa, Japan). Seven-to 12-week-old mice were used for all experiments. The mice were maintained in the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions and were handled in accordance with the animal care guidelines of Gunma University.

2.2. Dietary Zn-deficient mice

ZA and ZD diet were purchased from CLEA Japan Inc. The nutritional quality of both diets was the same, except that the Zn content was different (Zn; 6.00 mg/100 g in ZA diet, 0.11-0.38 mg/100 g in ZD diet). Mice were fed with a ZD or ZA diet from 7 weeks of age. Serum Zn level was quantified using a Zink Quantification Kit (abcam) according to the manufacturer's protocol.

2.3. Cutaneous ischemia-reperfusion injury mice model

Cutaneous I/R model that has been previously reported was used [6–12]. Briefly, mice were anesthetized, and hair was shaved. Dorsal skin was gently pulled up and trapped between two round ferrite magnetic plates that had a 12-mm diameter (113 mm^2) and 5 mm thick, with an average weight of 2.69 g and 1180 G magnetic forces (NeoMag Co, Ichikawa, Japan). Epidermis, dermis, subcutaneous fat layer and subcutaneous loose connective tissue layer, but not muscles, were pinched by magnetic plates. Dorsal skin was trapped between magnetic palates for 12 h, and then plates were removed. All mice developed two round ulcers separated by a bridge of normal skin. For the analysis, each wound site was digitally photographed after wounding, and the wound areas were measured on photographs using Image J (version1.48, NIH, Bethesda, MD), as previously reported [8,7–12]. To examine the effect of Zn deficiency on the development of PUs after cutaneous I/R injury, the mice were fed a ZA diet or ZD diet from 2 weeks before the beginning of ischemia to the end of the experiment. To examine the effect of Zn supplementation, the mice were fed with a ZD diet for 2 weeks before the beginning of ischemia, and then, mice were fed a ZA diet to the end of the experiment.

2.4. Histological examination

For immunofluorescence staining, the skin samples were taken from the peripheral area of I/R site, and frozen sections of mouse skin were prepared and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). After blocking with 3% dry milk-PBS supplemented with 5% normal donkey serum or 5% normal goat serum for 1 h at room temperature, skin tissues were stained with rabbit anti-mouse NG2 polyclonal Ab (pAb) (5 μg/ml; Millipore, Billerica, MA) and rat anti-mouse CD31 monoclonal Ab (mAb) (5 μg/ml; MEC13.3; BD Bioscience, San Jose, CA) followed by staining with Alexa Fluor 568- or Alexa Fluor 488- conjugated secondary antibodies (Invitrogen, Carlsbad, CA). Skin tissues were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei, mounted in ProLong Gold antifade reagent (Life Technologies). Immunofluorescence images were collected and visualized with an FV10i-DOC confocal laser scanning microscope (Olympus). To analyze LCs, epidermal sheets from the skin in the peripheral area of I/R site were prepared. The skin were surgically removed and then incubated with a 0.5 M ammonium thiocyanate in PBS for 45 min at 37 °C to separate the epidermis from the underlying dermis and fixed in acetone (−20 °C for 10 min). After blocking with 3% solution of dry milk in PBS, epidermal sheets were stained with Alexa 568- conjugated anti-mouse I-A/I-E mAb (5 μg/ml; BioLegend, San Diego, CA). Sections of mouse skin in paraffin were stained with hematoxylin and eosin (H&E). Skin fibrosis was quantified by measuring the thickness of the dermis, which was defined as the distance from the epidermal-dermal junction to the dermal-subcutaneous junction, at randomly selected microscopic fields.

2.5. RNA isolation and quantitative reverse transcription-polymerase chain reaction

To analyze the mRNA expression levels in the I/R site by using real-time reverse transcription-polymerase chain reaction

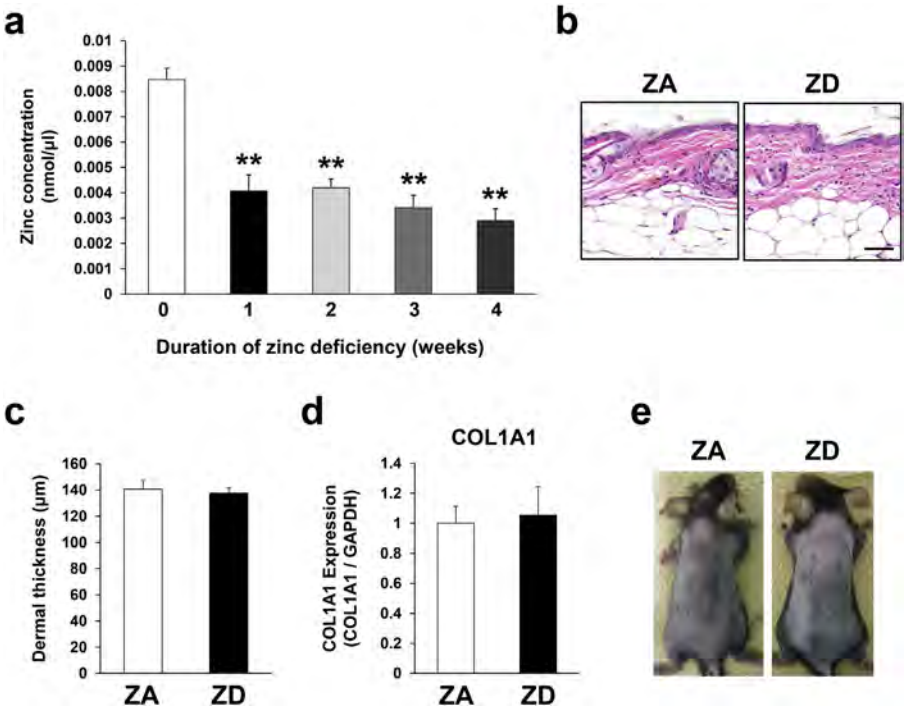

Fig. 1. Physical characteristics of mice fed with ZD diet for 2 weeks. (a) Changes of serum Zn levels in mice fed with ZD diet (n = 5–10 mice per group). (b) Representative images of hematoxylin and eosin staining of the dorsal skin at 2 weeks after starting the ZA or ZD diet. Scale bar = 50 μm. (c) Quantification of dermal thickness of dorsal skin in mice at 2 weeks after starting the ZA or ZD diet. Values were determined in three random microscopic fields in n = 6 mice per group. (d) Expression of mRNA levels of type I collagen in the dorsal skin in mice at 2 weeks after starting the ZA or ZD diet. mRNA level in ZA mice was assigned a value of 1. (e) Representative images of the cutaneous changes of dorsal skin in mice at 2 weeks after starting the ZA or ZD diet. All values represent mean ± SEM. $^{**}P < 0.01$.

(RT-PCR), whole skin samples from the I/R site were used. Total RNA was isolated using RNeasy Mini Kits (Qiagen, Valencia, CA) and was subjected to reverse transcription with a GoScript Reverse Transcriptase (Promega) according to the manufacturer's instructions. Quantitative RT-PCR was performed with the SYBR system (Applied Biosystems, Foster City, CA) by using ABI 7300 real-time PCR instrumentation (Life Technologies) according to the manufacturer's instructions. SYBR probes and primers for type I collagen, heme oxygenase 1 (HO-1), NADPH oxidase (NOX) 2 and 4 and GAPDH were purchased from Sigma and Takara Bio Inc. (Otsu, Japan). As an internal control, levels of GAPDH were quantified in parallel with target genes. Normalization and fold changes were calculated using the comparative Ct method.

2.6. Detection of luminescent signals

Detection of luminescent signals in mice was performed as described previously [11,12,21]. OKD48 mice were sacrificed and the skin was surgically removed and immersed in 0.3 mg/mL VivoGlo™ Luciferin, *in vivo* Grade (Promega, Tokyo, Japan) dissolved with PBS. As soon as possible, the collected skin was placed in the imaging chamber of the *in vivo* imaging system (IVIS; Perkin Elmer, Waltham, MA). Data were collected with high-sensitivity/10-min exposure and analyzed using Living Image software (Xenogen).

2.7. Apoptosis assay

The presence of apoptotic cells in the skin sections were assessed using a terminal deoxynucleotide transferase dUTP nick end-labeling (TUNEL) staining kit (Roche Diagnostics, Indianapolis,

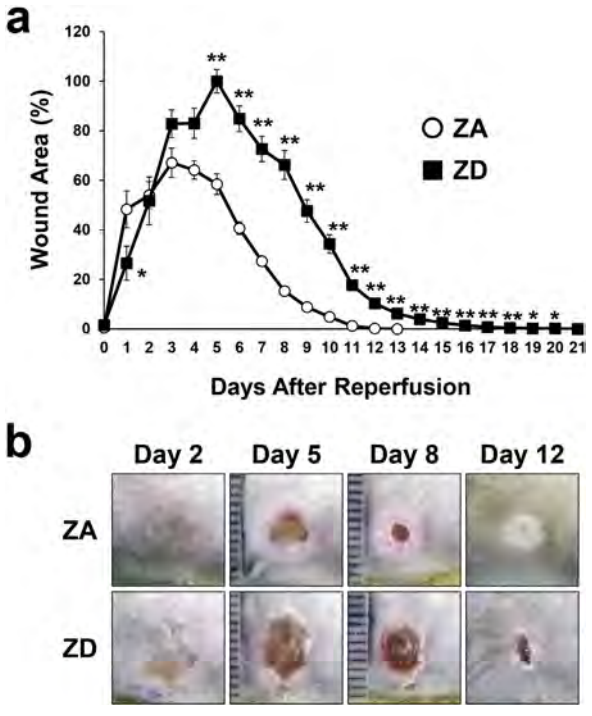

Fig. 2. Dietary Zn deficiency caused severe and prolonged PUs formation in mice. (a) Percent wound area at each time point relative to the wound area in ZD mice at 5 days after reperfusion (n = 18 wounds for each time point and groups). All values represent the mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$. (b) Representative images of the wound after cutaneous I/R in ZD or ZA mice at 2, 5, 8 and 12 days after reperfusion.

IN) as described previously [11,12,22]. Images (eight to nine fields per section) were taken and visualized with an FV10i-DOC confocal laser scanning microscope (Olympus). The number of apoptotic cells was determined by counting TUNEL and DAPI double-positive nuclei in the field (×600), as previously reported [11,12,22].

2.8. Measurement of ATP concentration

The amount of ATP in the mouse skin was measured using "Tissue" ATP assay kit (TOYO INK) according to the manufacturer`s protocol and the previously described protocols [20].

2.9. Statistical analysis

P values were calculated using the Student's *t*-test (two-sided) or by analysis of one-way ANOVA followed by Bonferroni's post test as appropriate. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.

3. Results

3.1. Physical characteristics of mice fed with ZD diet for 2 weeks

To confirm the deficiency of Zn in mice fed with ZD diet, we examined the serum level of Zn. We found that the serum Zn levels were significantly decreased and almost the same from 1 to 4 weeks after the initiation of the ZD diet (Fig. 1a), suggesting that serum Zn levels might be sufficiently reduced at 2 weeks after starting the ZD diet. It has been reported that Zn deficiency affected the development of connective tissues, including collagen in the skin and bone [23–25]. Therefore, we next examined the amount of collagen in the skin. There was no significant difference in dermal thickness and the mRNA expression of type I collagen in the dorsal skin between ZD and ZA mice at 2 weeks from the initiation of the ZD or ZA diet (Fig. 1b–d). At 2 weeks after starting the ZD diet, ZD mice did not exhibit visible cutaneous symptoms, such as inflammatory

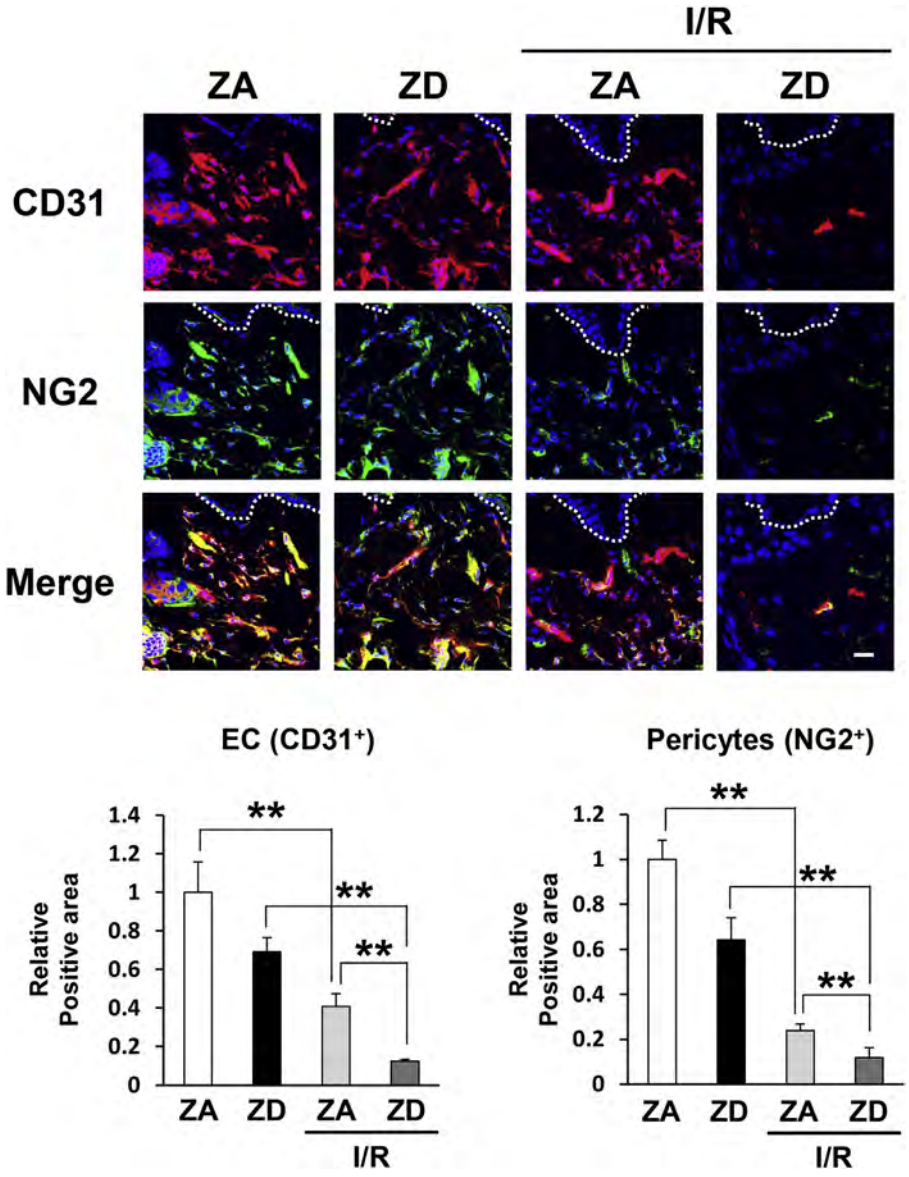

Fig. 3. Zn deficiency increased the vascular disorder induced by cutaneous I/R injury. The amount of $CD31^+$ EC and $NG2^+$ pericytes in cutaneous I/R area at 4 days after reperfusion. Quantification of the $CD31^+$ and $NG2^+$ areas in 9–16 random microscopic fields from the periphery of I/R area in n = 3 mice per groups was performed using Image J software. Positive area in ZA mice without I/R was assigned a value of 1. Values represent mean ± SEM. $^{**}P < 0.01$. Scale bar =20 μm.

dermatitis, alopecia, and hyperkeratosis (Fig. 1e). These results suggest that although the serum Zn level might be sufficiently lowered in mice at 2 weeks after ZD feeding, there might be no visible skin changes. Therefore, for cutaneous I/R injury experiments, we started cutaneous I/R injury at 2 weeks after starting the ZD diet.

3.2. Dietary Zn deficiency caused severe and prolonged PUs formation in mice

To assess the effects of Zn deficiency on the development and healing of PUs after cutaneous I/R injury *in vivo*, we compared the wound area in cutaneous I/R injury model mice fed with a ZD diet or a ZA diet. The wound areas were significantly larger in ZD mice than in ZA mice from 5 to 20 days after reperfusion (Fig. 2a, b). Furthermore, the wound closure time in ZD mice was longer than that in ZA mice (17.2 ± 0.5 *vs.* 12.3 ± 0.1 days, $P < 0.01$) (Fig. 2a, b). These results suggest that Zn deficiency might cause severe and prolonged PUs formation.

3.3. Zn deficiency increased the vascular disorder induced by cutaneous I/R injury

We have previously identified that the number of blood vessels was reduced after cutaneous I/R injury in a mouse model, and that hypoxia and oxidative stress were induced at the I/R site [11,12]. Therefore, we investigated the effect of Zn deficiency on the number of blood vessels in the cutaneous I/R area. We confirmed that the numbers of $CD31^+$ ECs and $NG2^+$ pericytes in the dermis were significantly reduced by cutaneous I/R injury at 4 days after reperfusion (Fig. 3). In addition, the I/R injury-induced reduction of ECs and pericytes was significantly suppressed by Zn deficiency (Fig. 3). These results suggest that Zn deficiency might have an incremental effect for the vascular injuries caused by cutaneous I/R.

3.4. Dietary Zn deficiency increased the oxidative stress and apoptosis induced by cutaneous I/R injury in vivo

We further investigated the effect of Zn deficiency on oxidative stress induced by I/R injury in OKD48 mice. OKD48 mice have a

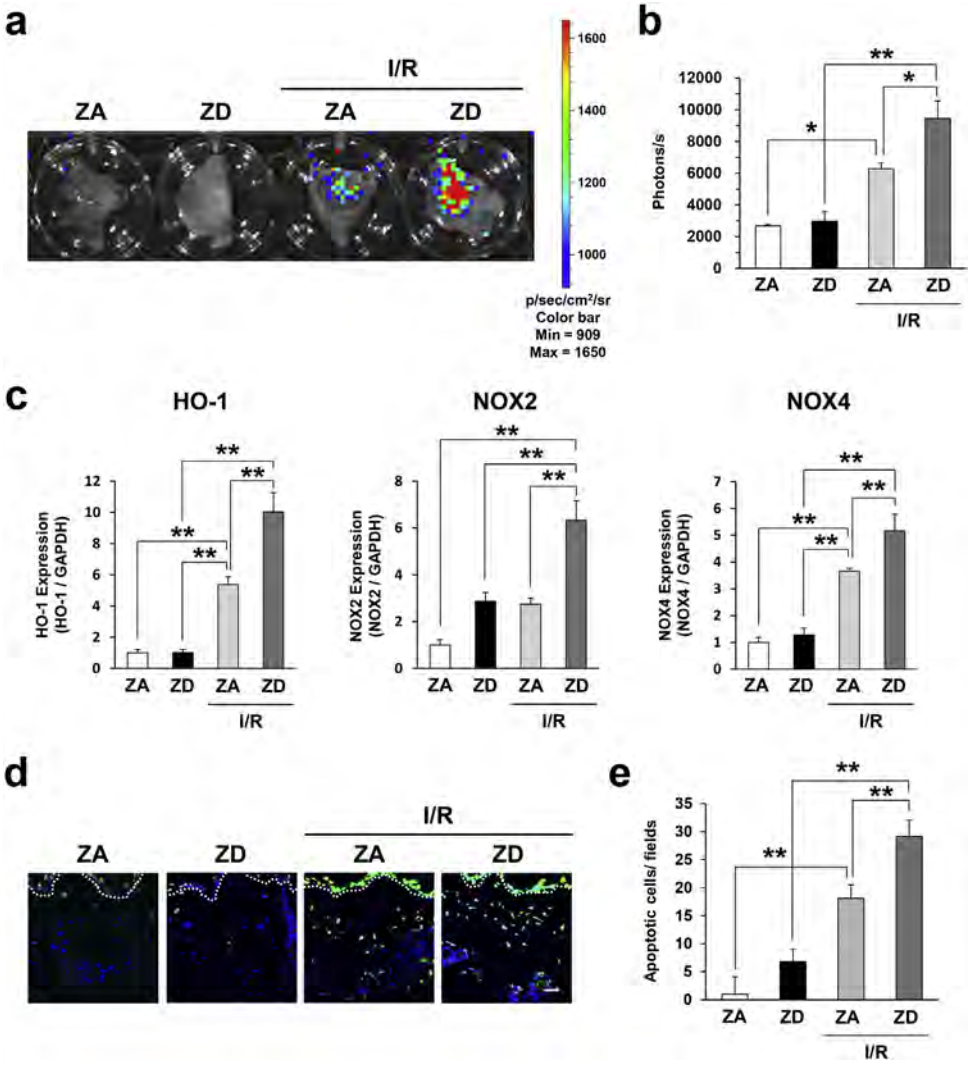

Fig. 4. Dietary Zn deficiency increased the oxidative stress and apoptosis induced by cutaneous I/R injury *in vivo*. (a) Representative image of luminescence signals in cutaneous I/R area in OKD 48 mice fed with ZA or ZD diet just after reperfusion. The color scale bar shows the photon counts (photon (p)/sec/cm^2/sr). (b) Quantification of luminescence signals in cutaneous I/R area in OKD 48 mice fed with ZA or ZD diet (n = 3–6 in each group). (c) mRNA levels of oxidative stress-associated factors, HO-1, Nox2 and Nox4 in the I/R area of ZA or ZD mice at 4 days after reperfusion. mRNA levels in ZA mice without I/R were assigned values of 1 (n = 4–6 in each group). (d) The number of apoptotic cells in the I/R site of ZA or ZD mice at 4 days after reperfusion was determined by counting both TUNEL- and DAPI-positive cells. Values were determined in 8–9 random microscopic fields from the center of I/R area in n = 3 mice per groups. The number of apoptotic cells in ZA mice without I/R was assigned as a value of 1. Scale bar =20 μm. All values represent mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$.

transgene encoding a modified nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) protein, which is an essential transcription factor for the expression of anti-oxidative stress genes [21,26]. By using this strain of mice, oxidative stress can be detected *in vivo* through luminescence signals [11,12,21]. Soon after reperfusion (day 0), a luminescence signal was detected in the I/R area in ZA mice. Furthermore, the I/R injury-induced luminescence signal in ZD mice was significantly higher than that in ZA mice (Fig. 4a, b). In addition, by using real-time polymerase chain reaction, we examined the mRNA levels of the oxidative stress-associated factors, HO-1, NOX2 and NOX4 after I/R. HO-1 is an essential enzyme with an antioxidant effect [27]. The biological function of NOX enzymes is the generation of ROS [28]. It has been reported that the expression of HO-1 and NOX is enhanced by I/R injury in the cerebrum and liver [29–31]. We found that cutaneous I/R injury significantly increased the mRNA levels of HO-1 and NOX4 in ZA and ZD mice (Fig. 4c). The mRNA expression of NOX2 tended to be enhanced by cutaneous I/R injury, although the difference was not statistically significant. Additionally, the I/R injury-induced mRNA expression of HO-1, NOX2, and NOX4 was significantly enhanced by Zn deficiency (Fig. 4c). We previously demonstrated that oxidative stress-induced apoptosis was observed in the cutaneous I/R area [8–12]. Therefore, we next examined the effect of Zn deficiency on the number of apoptotic cells in the I/R area in mice. At 4 days after reperfusion, we confirmed that the number of TUNEL$^+$ apoptotic cells was significantly enhanced by cutaneous I/R injury. In addition, the number of apoptotic cells in the I/R-injured skin in ZD mice was significantly higher than that in ZA mice (Fig. 4d, e). These results suggest that Zn deficiency might increase the oxidative stress and apoptosis induced by cutaneous I/R injury.

3.5. Zn deficiency reduced the number of LCs and increased the amount of ATP in cutaneous I/R-injured skin

It has been reported that CD39$^+$ LCs in the epidermis were reduced and irritant exposure-induced ATP from keratinocytes was increased in ZD mice, resulting in ATP-mediated severe skin inflammation [16,17]. Therefore, we next examined the effect of Zn deficiency on the number of LCs and the amount of ATP in I/R-injured skin in ZA and ZD mice. Two weeks after ZD or ZA diet, there was no difference in the number of LCs between ZD and ZA mice (Fig. 5a, b). In addition, the number of LCs in the skin of ZA mice was not changed by cutaneous I/R injury (2 days after reperfusion) (Fig. 5a,b). However, the number of LCs in the peripheral area of cutaneous I/R injury in ZD mice was significantly lower than that in ZA mice (Fig. 5a,b). Furthermore, the amount of ATP in the peripheral area of cutaneous I/R injury in ZD mice at 3 days after reperfusion was significantly higher than that in ZA mice (Fig. 5c). These results suggest that Zn deficiency might induce the loss of LCs, resulting in increased ATP amount in the I/R area.

3.6. Oral supplementation of Zn improved Zn deficiency-associated PUs

Finally, we assessed the therapeutic effect of oral supplementation of Zn on the development of PUs after cutaneous I/R injury in ZD mice *in vivo*. We confirmed that the wound size and wound closure time in ZD mice were significantly larger and longer than those in ZA mice, respectively (Fig. 6a, b). We also found that oral supplementation of Zn after I/R injury significantly reduced the size of PUs exacerbated by Zn deficiency and shortened the time of wound closure (10.2 ± 0.2 *vs.* 15.9 ± 0.2 days, $P < 0.01$) (Fig. 6a, b).

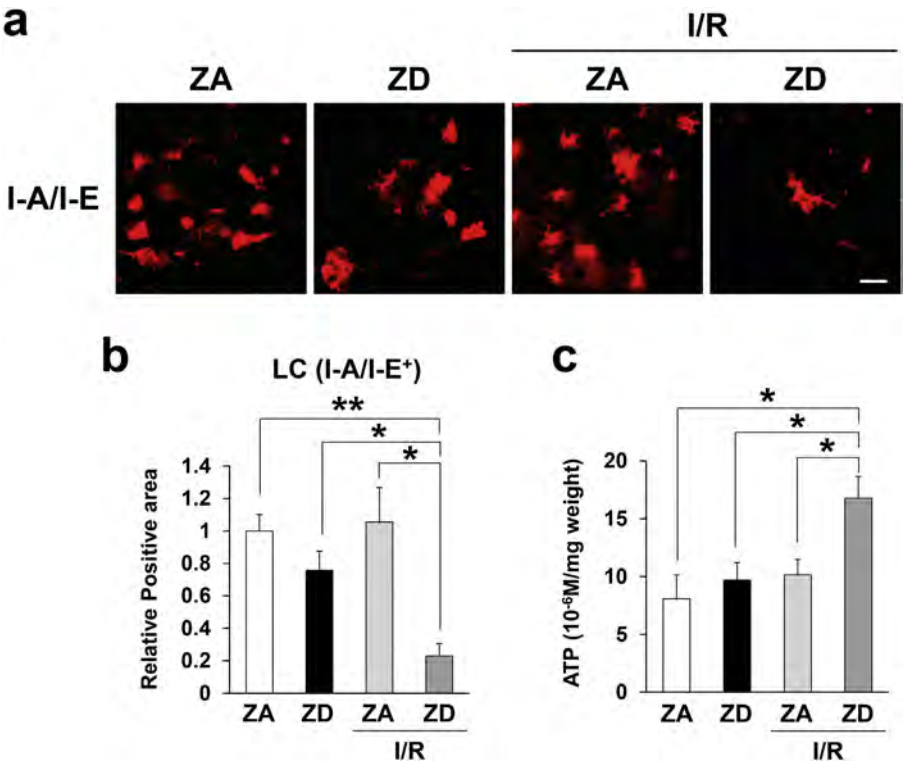

Fig. 5. Zn deficiency reduced the number of LCs and increased the amount of ATP in cutaneous I/R-injured skin. (a) Representative image of immunofluorescence staining of I-A/I-E$^+$ LCs in peripheral area of I/R in ZA or ZD mice at 2 days after reperfusion. Scale bar = 10 μm. (b) Quantification of the I-A/I-E$^+$ areas in 5–10 random microscopic fields from the periphery of I/R area in n = 3 mice per groups was performed using Image J software. Positive area in ZA mice without I/R was assigned a value of 1. (c) The amount of ATP in peripheral area of cutaneous I/R in ZA or ZD mice at 3 days after reperfusion (n = 4–6 mice per group). All values represent mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$.

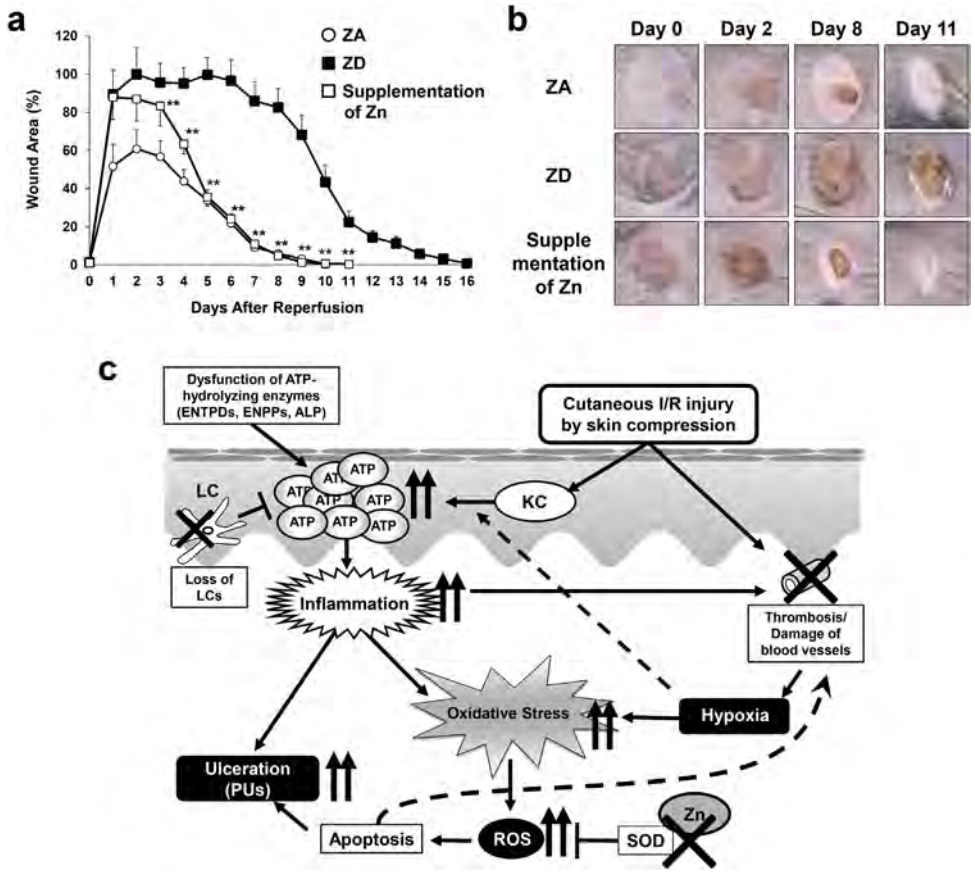

Fig. 6. Oral supplementation of Zn improved Zn deficiency-associated PUs. (a) Percent wound area at each time points in ZA, ZD and Zn-supplemented mice relative to the wound area in ZD mice at 2 days after reperfusion (n = 10–12 wounds for each time point and groups). All values represent the mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$. (b) Representative images of the wound after cutaneous I/R in ZA, ZD and Zn-supplemented mice at 0, 2, 8 and 11 days after reperfusion. (c) Model for the mechanism by which Zn deficiency exacerbates PUs. Cutaneous I/R injury due to repeated or sustained skin compressions induces thrombosis and blood vessel damage, which result in hypoxia. Hypoxia-induced oxidative stress generates ROS in ECs, fibroblasts, keratinocytes, and infiltrating cells. Then, apoptosis of these cells is induced. Zinc deficiency causes SOD dysfunction and enhances ROS production. Apoptosis of ECs initiates a vicious cycle that results in further hypoxia and oxidative stress. On the other hand, skin pressure promotes the release of ATP from keratinocytes and causes inflammation. Zinc deficiency decreases the number of $CD39^{+}$ LCs and ATP-hydrolyzing molecules, such as ENTPDs, ENPPs and ALP, leading to increased amount of extracellular ATP and further skin inflammation.

These results suggest that oral supplementation of Zn might have a therapeutic effect on Zn deficiency-associated PUs.

4. Discussion

With respect to the relationship of Zn and wound healing, Zn plays an essential role in hemostasis, inflammation, proliferation, and remodeling during the wound-healing process [32]. In addition, it has been reported that wound healing is delayed by prolonged inflammation and decreased function of fibroblasts in the state of Zn deficiency [33]. Fukada et al. reported that the Zn transporter Zip13 regulated the bone morphogenic protein/transforming growth factor-β signaling and the formation of connective tissue in the skin [25]. Although several reports have demonstrated that Zn controls wound healing, no study has scientifically examined the role of Zn in the development of PUs. We have shown for the first time that PUs were exacerbated by Zn deficiency in a cutaneous I/R injury mice model, and that Zn deficiency increased the cutaneous I/R injury-induced vascular damage, oxidative stress, apoptosis, and the amount of extracellular ATP in the I/R area (owing to the loss of LCs).

As SOD, which is an enzyme that degrades the ROS generated in cells by oxidative stress, requires Zn [34], it is believed that Zn deficiency increases the amount of ROS and promotes oxidative stress disorder. Therefore, it is possible that ROS production and oxidative stress due to skin compression might be enhanced by the dysfunction of SOD in ZD mice. Consistent with this hypothesis, we identified that Zn deficiency enhanced the I/R injury-induced oxidative stress signal in OKD48 mice.

NOX is a family of enzymes involved in ROS generation, acting *via* the transfer of a single electron from NADPH to oxygen [28]. NOX2 appears to be the most widely distributed among the NOX isoforms, as it is found in neutrophils, macrophages, ECs, fibroblasts, and hematopoietic cells. NOX4 is strongly expressed in vascular cells and fibroblasts [35,36]. It has been reported that both NOX2 and NOX4 contribute to ROS generation in fibroblasts in patients with systemic sclerosis [37]. In our results, the cutaneous I/R injury-induced mRNA expression of NOX2 and NOX4 was significantly enhanced by Zn deficiency, suggesting that cutaneous I/R-induced oxidative stress in fibroblasts, ECs, keratinocytes, and infiltrating inflammatory cells in the I/R area might be also enhanced by Zn deficiency.

There are several evidences that Zn deficiency delays extracellular ATP clearance [16,38]. Extracellular ATP is hydrolyzed by 3 groups of molecules: ecto-nucleoside triphosphate diphosphohydrolases (ENTPDs), ectonucleotide pyrophosphatase/phosphodiesterases (ENPPs), and alkaline phosphatase (ALP) [39,40]. LCs strongly express ENTPD-1 (CD39) and weakly express ENTPD-2 and ENPP-1, ENPP-2, and ENPP-3 [41]. It has been reported that the numbers of LCs were decreased in ZD mice as well as in the skin of ZD patients, resulting in increased amount of irritant-induced ATP in the skin [16]. Similar to this previous study, our study also identified that Zn deficiency reduced the number of LCs and increased the amount of

ATP in cutaneous I/R-injured skin. In addition, as ENPPs and ALP are Zn-dependent molecules, Zn deficiency severely impairs the activities of ENPPs and ALP, which leads to increased ATP levels [38]. This indicates one underlying mechanism by which ZD mice show increased ATP in I/R-injured skin.

On the basis of our results, we propose a model of the mechanisms by which Zn deficiency exacerbates PUs (Fig. 6c). Cutaneous I/R injury due to repeated or sustained skin compressions induces thrombosis and blood vessel damage, which result in hypoxia. Hypoxia-induced oxidative stress generates ROS in ECs, fibroblasts, keratinocytes, and infiltrating cells. Then, apoptosis of these cells is induced. Zinc deficiency causes SOD dysfunction and enhances ROS production and ROS-induced apoptosis of these cells. Apoptosis of ECs initiates a vicious cycle that results in further hypoxia and oxidative stress. On the other hand, skin pressure promotes the release of ATP from keratinocytes and causes inflammation. Zinc deficiency decreases the number of $CD39^+$ LCs and ATP-hydrolyzing molecules, leading to increased amount of extracellular ATP and further skin inflammation. These mechanisms might partly account for the formation of severe and prolonged PUs by Zn deficiency.

We also demonstrated that oral supplementation of Zn improved Zn deficiency-associated PUs in our cutaneous I/R injury mice model. A randomized, double-blind, placebo-controlled trial has reported that Zn supplementation increases the enzymatic activity of SOD in blood in patients with type 2 diabetes mellitus, suggesting that supplementation of Zn might also be useful in patients with PUs [42]. Our results strongly suggest the clinical significance of monitoring the serum Zn levels in patients with PUs and elderly patients at a high risk of PUs, and Zn supplementation in patients with PUs and Zn deficiency.

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