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Original Research Article

Influence of oxidative stress and effect of topical application of α-tocopherol on wound healing in a diabetic animal model

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ABSTRACT

Background: Understanding mechanisms involved in development of diabetes mellitus-associated ulcers is vital to pioneering alternative care approaches. This study aimed to establish effects of oxidative stress (OS) and α-tocopherol's effect on diabetic wound healing.
Methods: Using two animal experimental designs surgical wounds were created in 4 groups of 9-week-old diabetic and non-diabetic rats. OS was induced through antioxidant enzyme inhibition. In experiment-1 wounds were allowed to heal. In experiment-2 varying concentrations of topical α-tocopherol and/or the ointment-base were administered to diabetic animal wounds. Intermittent comparison of wound morphology, histology and local and systemic OS parameters was done.
Results: Irrespective of diabetic state, OS was associated with delayed wound size reduction and poor granulation-tissue collagen deposition. Delayed and subdued local glutathione peroxidase activity in response to wounding and OS induction was more pronounced in diabetic animals. Diabetic animals also showed higher serum malondialdehyde levels regardless of OS induction. Topical application of α-tocopherol was associated with denser wound granulation tissue collagen deposition but could not affect serum malondialdehyde levels.
Conclusions: OS interferes with wound healing especially collagen deposition and the effect is more pronounced in a diabetic state. Topical α-tocopherol can improve collagen deposition in diabetic wounds but cannot counteract systemic OS, therefore combining systemic and local antioxidant supplementation has potential for use in DFU care.

Keywords: Oxidative-stress, α-tocopherol, Diabetic wound healing, Collagen deposition

INTRODUCTION

Diabetes mellitus (DM) is the most prevalent endocrine disease globally, where about a tenth of global adult population lives with or highly risks developing it. Diabetic foot ulcers (DFUs) are localized injuries to the integument and/or underlying tissue of the foot/lower limb in a patient with DM.[1] DFUs have ramifications on both the patients and the healthcare system since a significant amount of DFUs become non-healing and some of the non-healing wounds will lead to an amputation.[2,3] Impairment of wound healing in DM is also associated with increased morbidity, mortality and economic expenses for both patients and healthcare institutions.

Therefore, debridement and antimicrobial dressings are critical in management DFUs to excise the colonized bacteria from within wound matrix.[4,5] Foam and alginate or fibroblast growth factor-based dressings have been recommended and shown to effectively promote healing of non-healing DFUs.[6,7] However, these advanced

treatments may not be applicable in low-income healthcare settings, and some antiseptic substances in dressing ointments themselves e.g. povidone-iodine can inhibit fibroblast proliferation prolonging the healing time of these wounds.[8]

Recently Oxidative-stress (OS) also has been reported to have a significant impact on DM complications including impairment of wound healing.[9] Patients with type-2 DM appear to have reduced antioxidant potential (glutathione levels) which could possibly be linked with complications in wound healing.[10] Enhancement of OS seems to be associated with the chronicity of the wound experimentally generated in the diabetic mice.[11] Therefore, it can be hypothesized reduction of OS could become a treatment modality for DFUs. α-tocopherol has been reported as an antioxidant applied medically to improve peripheral blood flow for treatment of frostbite ichthyosis vulgaris and pityriasis.[12,13] This study aimed to investigate the effect of OS on wound healing and to determine whether topical application of α-tocopherol was effective in accelerating wound healing.

METHODS

This study used an experimental design composed of 2 animal experiments conducted between June 2020 and March 2021. All experimental protocols were approved by Chiba University's ethical committee for animal experiments (No: Dou2-400, 2-477, 3-352).

Animals

Eight-week-old male Sprague Dawley rats (Jcl:SD, Clea, Japan) and spontaneously diabetic Torii (SDT fatty) rats (SDT Cg- Leprfa/Jtt, Clea, Japan) were housed separately under a 12-hour light/darkness cycle with ad-libitum food and water access. The SDT fatty rat is model of obese type-2 DM whereas the SD rat is the most genetically similar non-diabetic control to the SDT fatty rat. Blood glucose levels were measured at 9-weeks-old in SDT fatty rats using a portable glucometer (Arkray GT-1670, Japan) and diabetic hyperglycaemia was confirmed if the fasting blood sugar was above 8.3 mmol/dl, 150 mg/dl.

Reagents

3-amino-1,2,4-triazole (ATZ) (Tokyo Chemical Industries, Japan) and mercaptosuccinic acid (MSA) (Tokyo Chemical Industry, Japan) were used to induce OS. ATZ and MSA are potent inhibitors of catalase and glutathione peroxidase, respectively. α-tocopherol (Fujifilm, Wako, Japan) was chosen as an antioxidant to reduce OS within the wound. MSA and α-tocopherol were prepared as an ointment by mixing them with an ointment base, which was a mixture of petroleum jelly (Kenei Pharmaceutical, Japan) and liquid paraffin (Kozakai Paharmaceutical, Tokyo, Japan) in a ratio of 1 part petroleum jelly to 2.3 parts liquid paraffin. MSA was added to the ointment-base to create a concentration that could be administered at approximately 150 mg/kg when 0.1 ml of the ointment was applied per wound (0.2 ml/animal). α-tocopherol ointment of either 2% or 5% concentration were prepared for the experiment 2. These concentrations were determined from a commercially available α-tocopherol containing cream (Juvela®). Anaesthesia was provided using a cocktail of xylazine (10 mg/kg b.w.) and ketamine (25-40 mg/kg b.w.) depending on the animal's weight. Inhaled isoflurane was used during wound dressing.

Wounding and wound management

Two symmetrical 15 mm^2 full thickness wounds were created on the dorsum of the rats using sterile forceps and scissors. Thereafter, wounds were debrided daily if necessary and irrigated with isotonic saline and were covered with a transparent dressing (Tegaderm, 3M) which was changed daily.

Induction of OS

OS was exacerbated by administering antioxidant enzyme inhibitors with slight modifications to the procedure reported by Dhall et al.[11] Twenty minutes prior to wounding all animals were treated with a single dose of intraperitoneal AZT (1 g/kg b.w.). Immediately after wounding, MSA containing ointment was topically applied on wounds (0.1 ml/wound, approximately 150 mg/kg). MSA was topically applied for 5 consecutive days since wounding.

Experiment 1: influence of OS on wound healing

Thirty-six 9-week-old non-diabetic (SD) rats and diabetic (SDT fatty) rats were divided into 4 groups (n=9 per group) by stratified randomization based on pre-wounding body weight and blood glucose levels (SDT fatty only). Wounds created in both control group animals were allowed to heal naturally from the onset, while wounds created in both oxidative stress groups were treated with ATZ and MSA up to day 6 then allowed to heal naturally thereafter. Animals were sacrificed intermittently at 2 or 3-day intervals by overdose inhalation of isoflurane. The schematic diagrams for both experiments are on Figure 1.

Experiment 2: effect of α-tocopherol topical application on wound healing of diabetic animals

Twenty-four (24) nine-week-old SDT fatty rats received the treatment for induction of oxidative stress for 5 consecutive days post-wounding. After sacrificing 3 animals to provide baseline variables the remaining 21 animals were equally randomized into either of 4 groups where wounds were topically treated daily with clinical-dose (2%) α- tocopherol, high dose (5%) α-tocopherol, the ointment-base (vehicle control) or left to heal naturally until the end of the experiment.

Outcome measurement

Evaluation of wound healing

Wound size and gross morphology

Wound morphology was recorded via photographs (Nikon Coolpix S9100, Japan) at 2-day intervals. Wound area was calculated by use of an image analysis software (Image J, U. S. National Institutes of Health, USA). Images of wound photographs were also processed using Image J. Wound areas on day 0 (experiment 1) and day 5 (experiment 2) were regarded as initial wound areas, therefore their respective wound area percentages were 100%. Wound area percentages on subsequent days was calculated in relation to the initial day wound area.

Collagen deposition within granulation tissue

Tissue specimens were stained with Masson's trichrome for visualization of collagen within the granulation tissue between the epidermis and the panniculus carnosus muscle layer.[14,15] Micrographs were taken to include whole wound area for each wound using a microscope camera (Nikon Digital sight DS-L3, Tokyo, Japan). Using Image J software binarized micrographs were analysed according to an established technique whereby collagen fibres highlighted red while all non-collagen structures highlighted grey.[16] Collagen deposition for each micrograph was expressed as the percentage of red stained collagen fibres to whole area of granulation tissue in the micrograph. Collagen deposition in layers beneath an area of an open wound were termed active wound collagen, while areas beneath the regenerated epidermis were termed peripheral wound collagen.

Oxidative stress parameters

Glutathione peroxidase activity

Wound tissue was immediately frozen in liquid nitrogen after mincing and stored until the extraction of assay sample. Tissues were homogenized in a cold buffer (<4 ºC) consisting of 50 mm Tris-HCl (pH 7.5), 5mM EDTA and 1mM Dithiothreitol using a beads crusher (µT-12, Taitec, Saitama, Japan). The ratio of tissue and buffer was such that 100 mg of tissue was homogenized in 1 ml of buffer. The homogenized material was centrifuged, and the supernatant was collected and stored at -800 ºC until the assay.

Activity of GPx was measured using a colorimetric assay kit (NWLSSTM product NWK-GPX01, Vancouver, Canada) which detected the NADPH concentration as GPx catalyzes redox reaction of H_2O_2 and glutathione. GPx activity was calculated from the reduction slope of NADPH at an optical density (OD) of 340 nm, at 30 second intervals for 5. The activity of the enzyme normalised to protein levels (200 µg/ml) evaluated using the Micro-BCA™ Protein Assay Kit (ThermoFisher Scientific, Illinois, USA).

Serum malondialdehyde levels

At each point of sacrifice serum levels of malondialdehyde (MDA) were measured using the TBARs (TCA assay kit) (Cayman, USA) serving as an oxidative stress marker. Sera were separated after centrifuging for 15 minutes and frozen and stored at -80 ºC until the assay. Serum was incubated with thiobarbituric acid (TBA) and colour reagent in boiling water for 1 hour to make TBA-MDA adduct from which MDA concentration was calculated from the absorbance at an OD 535 nm.

Data analysis

Parametric tests (t-tests, ANOVA) were used to determine significance of differences in ratio data between and within groups. Statistical tests were conducted using the statistical software package IBM SPSS Statistics version 27 (IBM). A $p<0.05$ was considered statistically significant.

RESULTS

Experiment 1

Wound size and gross morphology

The change of wound size is shown in Figure 2. There was a marked difference in wound size changes between the OS groups and control group of both non-diabetic and diabetic animals.

For control groups, both non-diabetic and diabetic control group animals showed continuous reduction in wound size since the day of wound creation. However, from days 2 to 7 wound contraction was marginally slower in diabetic control animals compared to non-diabetic control animals with statistical significance being found on day 2 (72.9±16.8%, n=9 versus 58.9±7.3%, n=10, p=0.027). By day 11 wounds in remaining 2 animals from both control groups had virtually closed with mean wound area percentage being less than 10% of the original wound size (non-diabetic control=6.7±4.2%, n=2; diabetic control=7.0±4.8%, n=2). On day 11, for the non-diabetic control group one of the 2 dorsal wounds to be assessed in the 2 observed animals had fully closed (Figure 2A).

Conversely, wound size in continued to increase during the oxidative stress induction (MSA application) period. The non-diabetic group OS showed a slower increase in wound size compared to the diabetic group oxidative stress especially on day 2 ($p<0.001$). Thereafter wound area changes were similar between the two oxidative stress groups. On day 15 wound area in the diabetic group was slightly lower (39.9±14.7%, n=4) than that in the non-diabetic group (46.1±26.2%, n=2) on day 15.

day 17 none of the wounds observed had fully closed and 29.5%±3.1 (n=2) of wound area remained open in the diabetic oxidative stress group. Morphologically, wounds from oxidative stress groups showed more debris and produced more exudate compared to the control groups at each time point (Figure 3).

Granulation tissue collagen deposition

As shown in Figure 4, active collagen deposition in both control groups showed a gradual increase with time. After day 11 active wound collagen was no longer observable in both control groups due to wound closure. Mean values of both active and peripheral collagen were much higher in the non-diabetic control animals than diabetic control animals at almost all time points of the measurement.

For OS groups, collagen deposition within the wound granulation tissue was delayed and decreased in both of non-diabetic and diabetic animals compared to the control group animals. Increase in active collagen deposition was not observed until day 11 in oxidative stress groups and increase in peripheral collagen deposition in both oxidative stress groups was also marginal. Peripheral collagen deposition in the diabetic oxidative stress group was always lower than that in the non-diabetic oxidative stress group on all time points especially on days 11 and 15.

On comparison between non-diabetic and diabetic animals it was evident that wounds from non-diabetic control group showed higher peripheral collagen deposition compared to both diabetic groups. Also, collagen deposition in both diabetic groups was low regardless of induction of oxidative stress.

OS parameters

Glutathione peroxidase activity

GPx activity for control groups on day 7 and 11 could not be obtained due to missing sample data (Figure 6A). Concerning control groups, GPx activity on day 5 showed similar increases from that on day 2 in both non-diabetic and diabetic animals. When oxidative stress was induced, wound tissue GPx activity on day 2 was slightly lower in both control groups compared to the two oxidative stress groups.

Figure 6A also shows that diabetic oxidative stress animals exhibited a much weaker and slower reaction to oxidative stress induction compared to non-diabetic animals at all measurement points especially form day 5 to day 11. Maximum GPx activity in the diabetic oxidative stress group was reached on day 15 while elevated Gpx activity was recorded by day 5 and reached peak by day 11 in the corresponding non-diabetic animals. Moreover, high GPx activity in non-diabetic animals was maintained high over a longer period (days 7, 11 and 15).

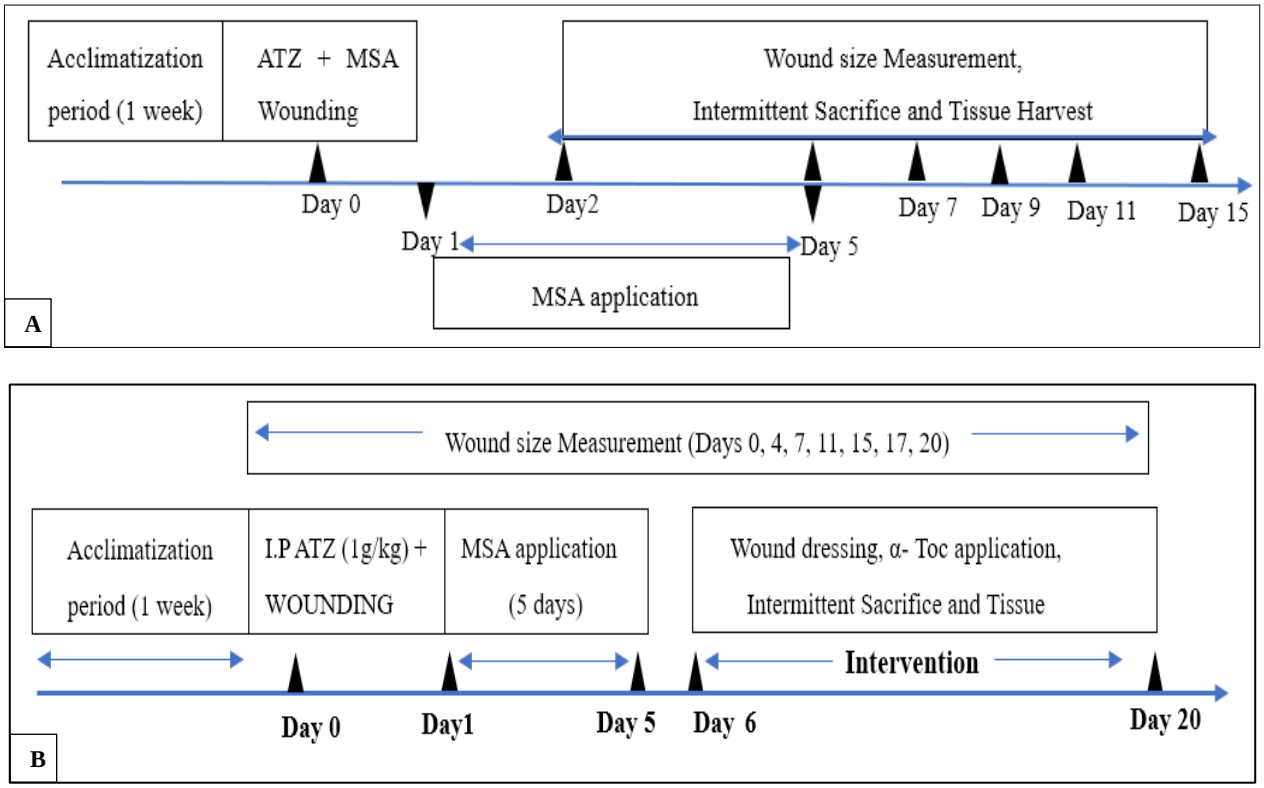

Figure 1 (A and B): Schematic representation for A: experiment 1, B: experiment 2.

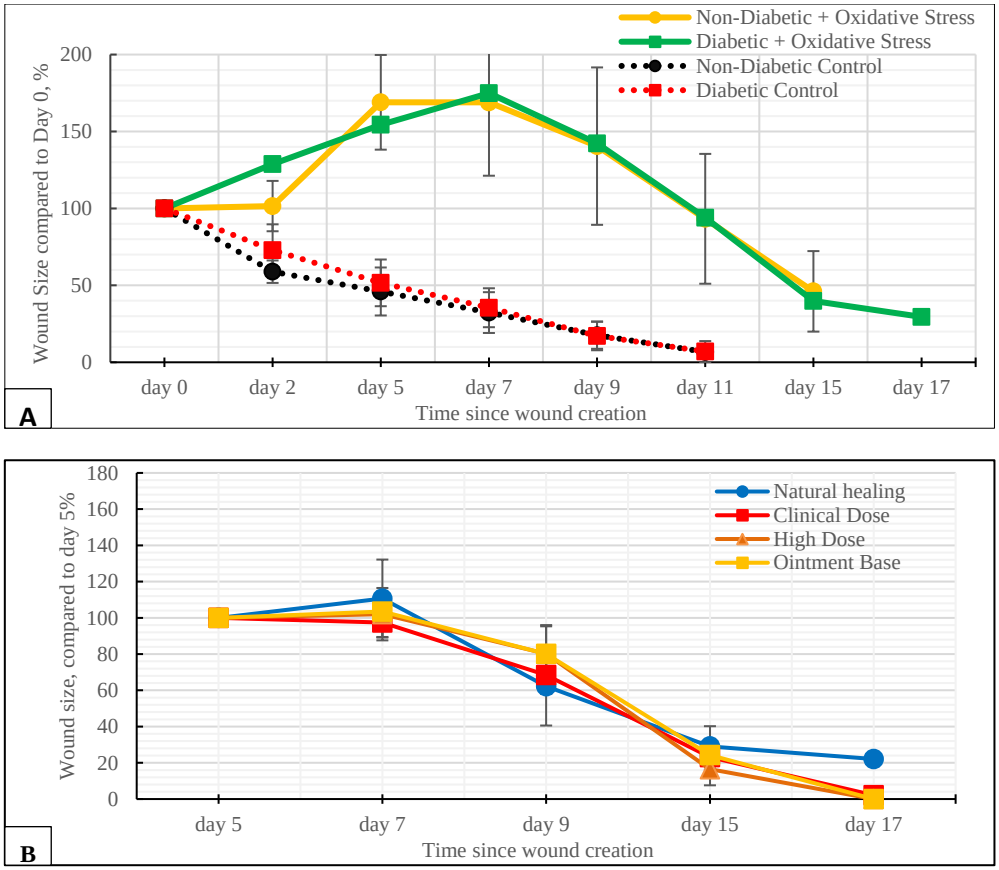

Figure 2: Wound size (%) vs duration since wounding (A: experiment 1, B: experiment 2); A: wound size reduction was significantly slower in animal groups subjected to exacerbated oxidative stress irrespective of the animals' diabetic state; similarly, control groups achieved wound closure much faster than their OS counterparts; B: wound size reduction was similar between both α-tocopherol groups and the ointment-base group; wounds in the natural healing group (absolute control) remained open at the end of the experiment.

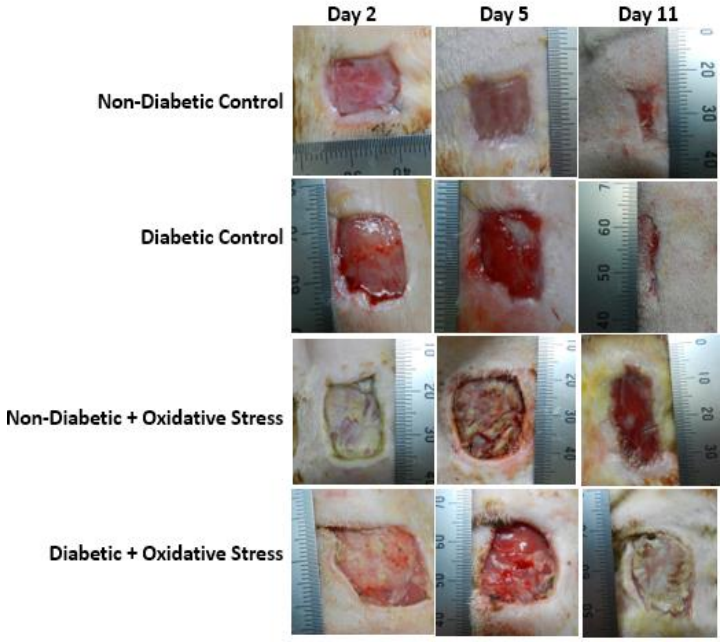

Figure 3: Time series on wound size reduction by group, experiment 1; wounds from animals that were subjected to OS showing more debris and delayed closure than respective control groups; wound healing appeared to be more delayed in the diabetic oxidative group than in the non-diabetic oxidative stress group.

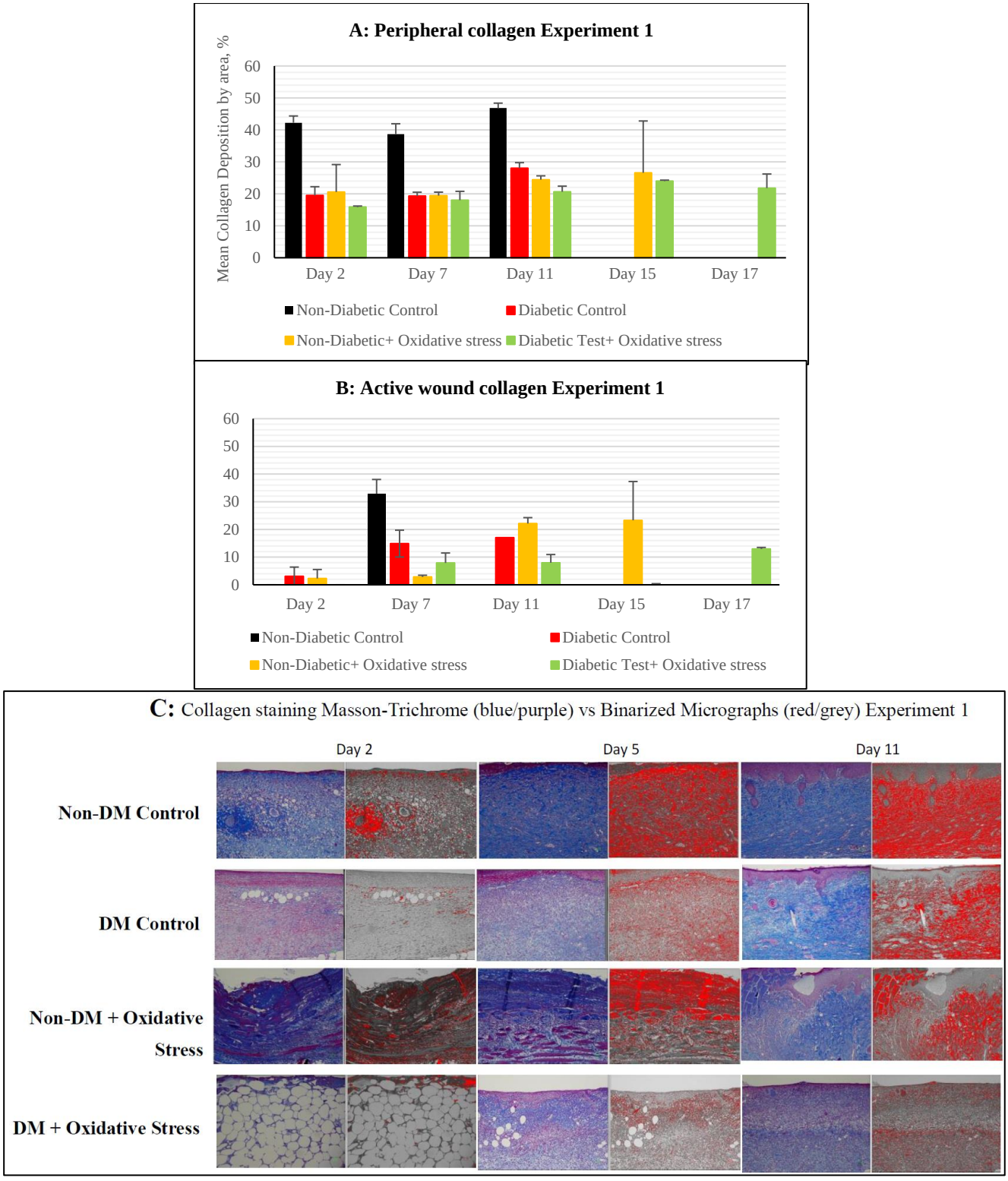

Figure 4: (A and B) Granulation tissue collagen deposition across groups, experiment 1; (C) collagen staining Masson-trichrome (blue/purple) vs binalized micrographs (red/grey) experiment 1; (A) control groups showing more peripheral collagen deposition than OS group regardless of diabetic state; (B) active collagen for non-diabetic control group higher than that for OS groups (day 7); persistent open in wounds in oxidative stress groups showing poor healing; wound closure in control group wounds by as the experiment progressed shown by absence of active wound collagen in these groups after day 11; (C) collagen fibres are stained red; diabetic oxidative groups showing less collagen deposition than either control groups. Magnification ×10.

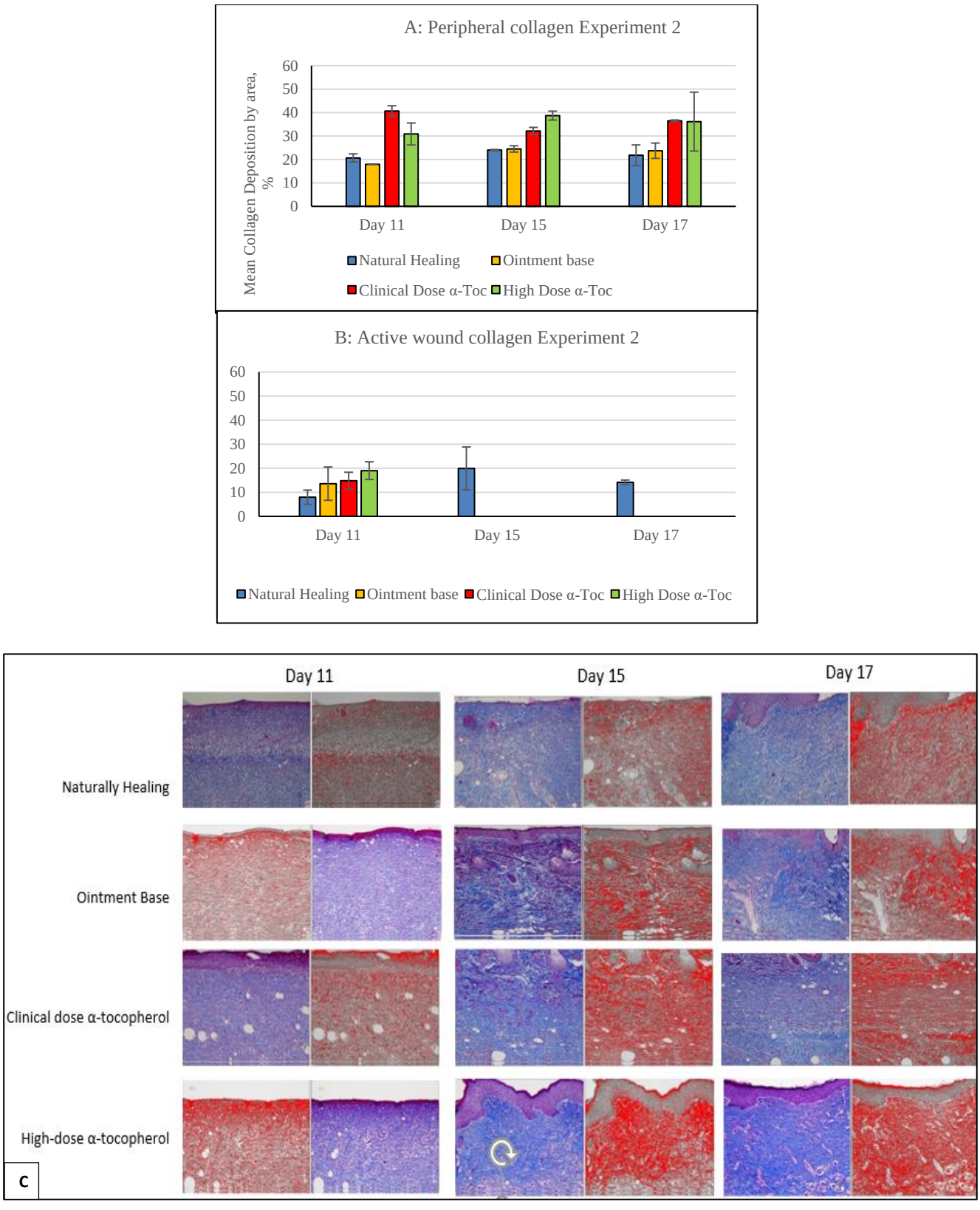

Figure 5: (A and B) Granulation tissue collagen deposition across groups experiment 2; (C) collagen staining Masson-Trichrome (blue/purple) vs binalized micrographs (red/grey); (A) α-tocopherol groups showing more peripheral collagen deposition than both control groups; (B) active collagen for natural healing group was shown on all 3 measurements since it was the only group remaining with active wounds whereas wounds in all 3 groups had reepithelialized; (C) collagen fibres are stained red; α-tocopherol groups showing denser collagen deposition than ointment-base and natural healing micrographs; magnification ×10.

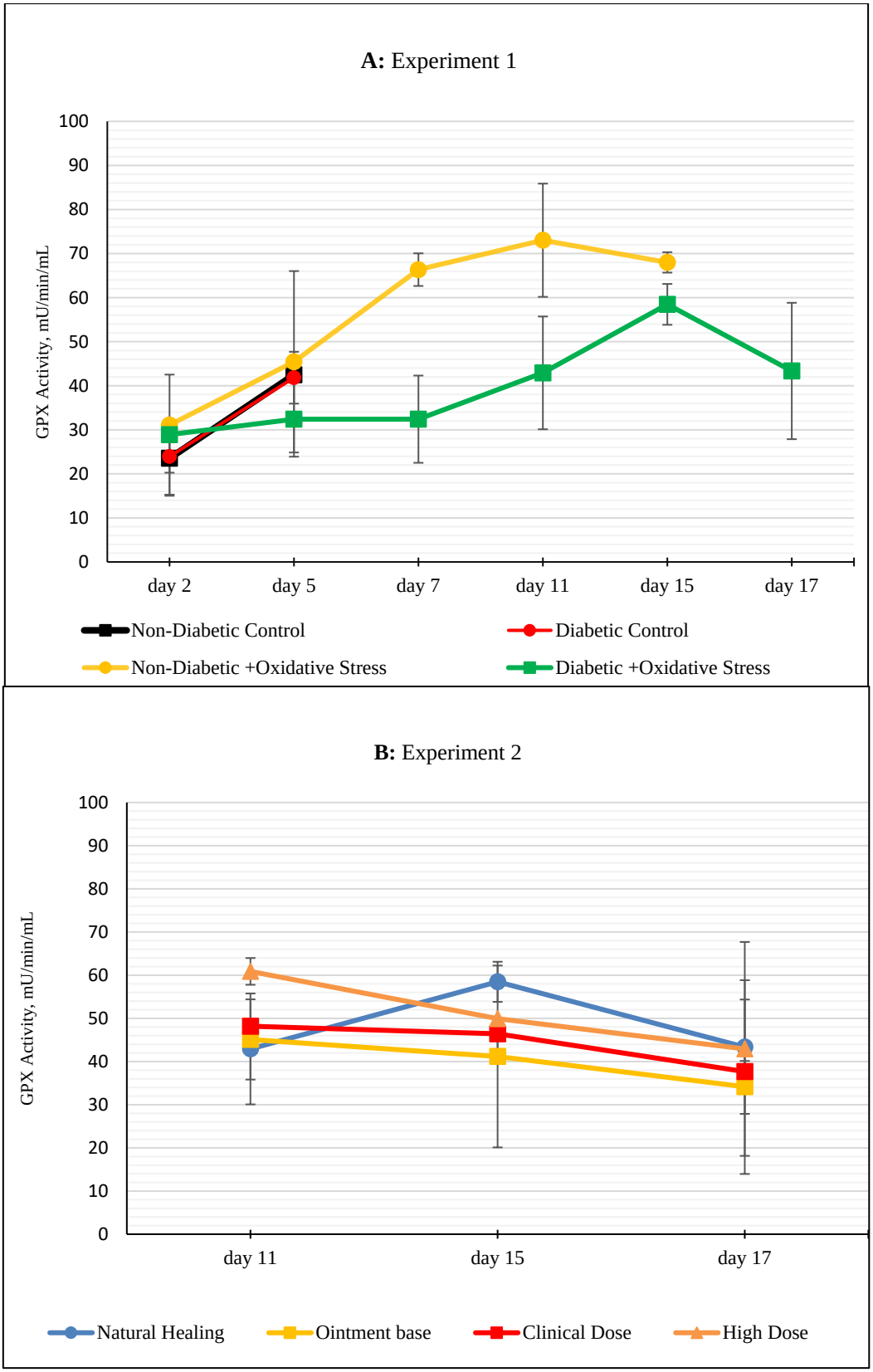

Figure 6: Mean GPx activity vs time; (A) diabetic oxidative stress group showing a delayed and subdued GPx activity response to wounding compared to the non-diabetic oxidative stress group; (B) GPx activity was fairly similar between α-tocopherol groups and the 2 control groups however initial trajectory suggests both α-tocopherol groups achieved higher peak GPx activity than either control group.

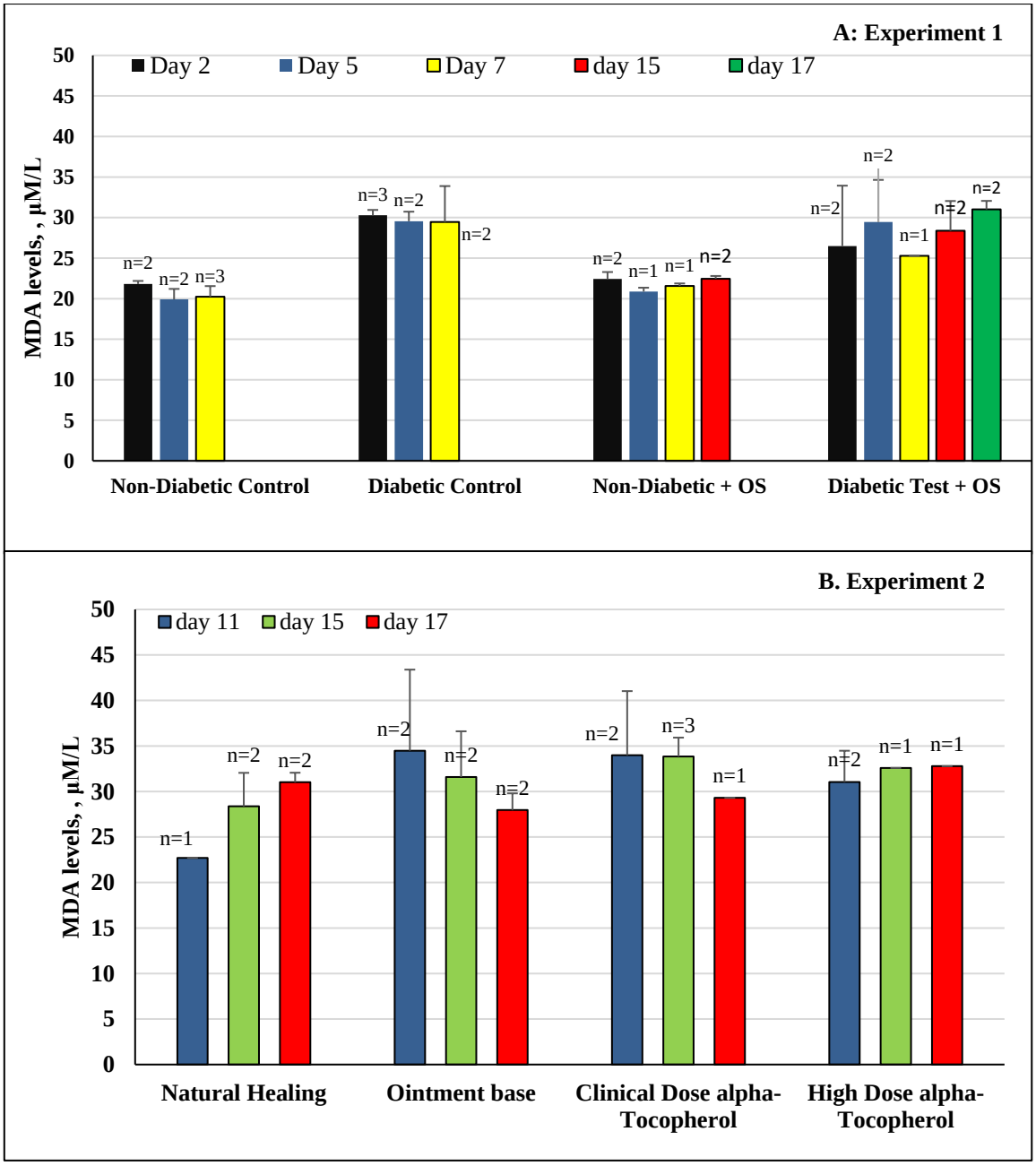

Figure 7: Mean MDA levels; (A) systemic oxidative stress was higher in diabetic animals than in non-diabetic animals regardless of exacerbation of OS; (B) local administration of α-tocopherol did not influence any change in systemic states of OS as all 4 groups of diabetic animals had similar serum MDA levels.

Serum malondialdehyde (MDA) concentrations

According to Figure 7A, diabetic animals appeared to consistently show higher levels of serum MDA than non-diabetic animals irrespective of oxidative stress induction. In addition, serum MDA levels were relatively unchanged during the progress of wound healing in both of control and oxidative stress animals. When expressed as an average of each time point until day 7, serum MDA levels were noticeably higher in diabetic animals than in non-diabetic animals [day 2: diabetic=28.7±4.2 µmol/l versus non-diabetic=22.1±0.6 µmol/l (n=9, p=0.019); day 5: diabetic=29.5±3.1 µmol/l versus non-diabetic=20.4±1.0 µmol/l (n=8, p=0.001); day 7: diabetic OS=28.1±4.0 µmol/l versus non-diabetic=20.8±0.8 µmol/l (n=8, p=0.078)]. However, statistical significance of these differences could not be calculated due to small sample sizes used per time point.

Experiment 2

A total of 33 animals were used in this experiment, whereby 11 animals from the diabetic OS group in

experiment 1 were included as the natural healing control in experiment 2.

Wound size and gross morphology

Wound size changes after the last day of OS induction (day 5) are shown in Figure 2B. Until day 15 (10 days since commencing the topical application), α-tocopherol groups were showing similar wound retraction to natural healing and ointment-base groups.

The only exception was that on day 17 only wounds from the natural healing group were still open (22.1%±0.62, n=2) whereas wounds form the clinical and high dose α-tocopherol group and ointment-base group had virtually or completely closed (clinical dose 2.1%, n=2, ointment-base 0% n=3: high dose 0%, n=2).

Granulation tissue collagen deposition

Granulation tissue collagen deposition of each group from day 11 to day 17 in experiment 2 is summarized in Figure 5. On day 11 active wound collagen deposition was noticeably higher in the α-tocopherol and ointment-base groups than that in the natural healing group with collagen being highest in the high dose α-tocopherol group although the values of clinical dose group, and ointment-base group were almost similar. On days 15 and 17 active wound collagen deposition was only observed in the natural healing group since this was the only group remaining with active wounds.

Peripheral granulation tissue collagen deposition was higher in both α-tocopherol groups compared to the ointment-base and natural healing groups at all 3 time-points. When comparing between the two α-tocopherol groups, peripheral collagen deposition on all 3 time points was relatively similar despite being higher in clinical dose group on day 11.

OS parameters

Glutathione peroxidase activity

Gpx activity data for days 11, 15 and 17 is shown in Figure 6B. Increase in GPx activity was delayed in the natural healing group compared to both α-tocopherol groups reaching its highest measurement (of the evaluated 3 time points) on day 15.

On the contrary the highest GPx activity in the high-dose α-tocopherol, low-dose α-tocopherol and ointment-base groups appeared to have been achieved on day 11 or earlier as it continuously declined through days 15 and 17. Though earlier measurements before day 11 were not taken, a comparison of the GPx activity trajectories for each group suggests that only the α-tocopherol groups had higher initial trajectories GPx activity (for earlier time points not measured) than either the natural healing and ointment-base groups.

Serum malondialdehyde concentration

Serum MDA concentration on days 11 to 17 are presented in Figure 7B. At all 3 time points overall mean values of serum MDA concentration were similarly distributed between all 4 groups. Among all 4 groups over three measurements (days 11, 15 and 17), only natural healing group appeared to show subsequently increasing MDA levels while ointment-base (vehicle control) showed trend of subsequently decreasing MDA levels over the same period.

DISCUSSION

Effect of oxidative stress on wound healing

Data from wound size reduction, wound morphology and collagen deposition within granulation tissue showed that oxidative stress induced by administration of antioxidant inhibitors was linked to deterioration of some aspects of wound condition in both diabetic and non-diabetic animals. Wound area in OS groups increased during oxidative stress induction phase and turned to decrease after the termination of OS induction phase. Wounds in diabetic OS animals showed more debris and exudate and took more time to heal and had poor collagen deposition.

OS occurs when the production of reactive oxygen species (ROS) overwhelms cell antioxidant systems' ability to detoxify these intermediate products of cellular metabolism.[17] Detrimental effect of OS on wound healing, especially on chronic wound has been shown in previous research.[18-20] One study reported that OS might exert an inhibitory effect on fibroblast activity within the wound-bed leading to poor collagen deposition within the granulation tissue.[21] The increase in wound size observed in our study can therefore be attributed to inhibited activity of ROS-neutralizing enzymes which allowed uncontrolled ROS-dependent tissue damage. Our experiment results also supported the notion that OS impaired fibroblast activities and collagen deposition, as OS groups showed reduced granulation tissue deposition.

While morphologically wound healing of non-diabetic and diabetic animals appeared to be similarly affected by OS, granulation tissue collagen deposition in diabetic animals was poor irrespective of ATZ/MSA application (Figure 2A and Figure 3). Therefore it can be interpreted meant that OS in the diabetic state was already significant enough to interfere with dermal collagen deposition in these animals.

Results in our study showed that the diabetic state is associated with higher serum MDA levels meaning that OS in diabetes occured at both local (wound tissue) and systemic platforms. Association of elevated levels of serum MDA with poor outcomes in patients with DFU has also been previously reported.[22] MDA-dependent ROS production counteracted GPx activity levels in diabetic patients thereby lowering the overall antioxidant

potential potentiating the effect of ROS and subsequent delayed wound healing.[23] Experiment 1 results established a delayed and subdued GPx activity in diabetic animals in response wounding and oxidative stress induction (Figure 5B) which corroborated this reasoning.

Effect of α-tocopherol topical application on wound healing of diabetic animals

Despite similarity in wound size reduction between groups, α-tocopherol groups showed higher granulation tissue collagen deposition compared to the natural healing group. In previous studies α-tocopherol had been suggested to effectively increase the skin's antioxidant potential by reducing dermal malondialdehyde levels.[24] It was also reported that α-tocopherol increased expression and activity of GPx in cardiomyocytes.[25] In this present study, prompt and elevated activity of GPx in α-tocopherol groups in response to wounding suggests that OS within the wound tissue in diabetic animals was reduced by this antioxidant capacity of α-tocopherol (Figure 6B). However, reliability of this assertion is questionable due to missing data for control groups.

Lack of significant differences in wound size reduction between α-tocopherol groups and the ointment-base group can be explained by the composition of materials used in both preparations. Petroleum jelly and liquid paraffin used for the ointment-base itself may have preserved moisture within the wound-bed thereby accelerating extracellular matrix regeneration, reepithelialisation and angiogenesis.[26]

The absence of significant differences in serum MDA levels between α-tocopherol groups and the control groups indicated that while topical application of antioxidants may improve local antioxidant potential, this was inadequate to counteract diabetes-associated systemic oxidative stress (Figure 7B). Blood glucose control should be combined to interventions that reduce systemic oxidative stress e.g. oral antioxidants administration, antioxidant rich dietary adjustments etc., to improve systemic antioxidant potential and ultimately promote diabetes-associated wound healing. The potential application of these findings resonates with recent studies which used α-tocopherol metabolites embedded into bacterial nanocellulose dressings for application on diabetes-related wounds.[27,28]

Limitations of the study

The limited number of animals used confounded the ability to make statistical comparisons and draw reliable conclusions from the data. Also, skin tissue GPx activity on intact skin before induction of oxidative stress was not done preventing comparison of baseline GPx activity. Lastly, differences in wound healing mechanisms between human and murine wound models may reduce the applicability of findings in our study to a clinical environment.

CONCLUSION

Results from this study showed that exacerbation of OS interferes with wound healing especially granulation tissue collagen deposition. It was also evident that the diabetic hyperglycemic conditions are associated with higher levels of systemic and local tissue oxidative stress compared to a healthy state. Topical application of an antioxidant ointment (α-tocopherol) could improve granulation tissue collagen deposition however it was ineffective in facilitating wound size reduction and in mitigating systemic oxidative stress. These results suggest a possibility to utilize antioxidant supplementation in DFU care. However there remains a need to determine the effect of combining systemic and topical antioxidant supplementation on wound healing in human diabetic patients.

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Conflict of interest: None declared
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