Summary of Doctoral Dissertation

Newer Approaches to Wound care in Diabetes Mellitus: The Role of Oxidative Stress in Clinical Outcomes in Diabetic Foot Complications

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I. BACKGROUND

Diabetes mellitus (DM) is the most prevalent endocrine disease globally, where about 10% of the global adult population lives with or is highly at risk of developing diabetes. While the current annual prevalence of 2% it is anticipated that the incidence will rise by 2025 to 500 million people while

125 million patients will likely develop diabetic foot ulcer (DFU) complications (Dhall et al., 2014). A DFU is a localized injury to the integument and/or underlying tissue of the foot in a patient with DM (Mavrogenis et al., 2018). This has severe ramifications on both the patients and the healthcare system. Though the majority (60–80%) of DFU will eventually heal, 10-15% of DFU will remain non-healing, and 5–24% of these non-healing wounds will lead to an amputation at least 6 months after the first evaluation (Alexiadou & Doupis, 2012; Smith et al., 2016). Studies have shown that even with well-controlled treatments, only 24% of DFUs heal after 12 weeks of care and that 31% take 20 weeks of care until healing (Kavros et al., 2014). Generally, about 50% of diabetic foot ulcers remain unhealed after 6 months, with a median healing time of 147 days for toes ulcers, 188 days for midfoot ulcers and 182 days for plantar ulcers (Pickwell, Siersma, Kars, Holstein, & Schaper, 2013).

The impairment of wound healing in DM causes significant morbidity and mortality worldwide. Apart from reduced quality of life (pain, weight loss, etc.) caused by the chronicity of these wounds, non-healing wounds can pose worse patient outcomes such as systemic infections using the wounds as an entry portal, amputations, and even death. Financial burden arising from the care of non-healing wounds can be unbearable for the patient and healthcare systems alike, for instance in 2001 in the USA and UK, $10.9 billion and £3 billion respectively was used in diabetic foot care and treatment.

Mechanical debridement has been associated with a decrease in diabetic ulcer area and bacterial load debridement and has been acknowledged as pivotal for the management of DFUs (together with systemic antibiotics and dressings) once euglycaemia is achieved as it reduces bioburden and opens a time-dependent therapeutic window for topical antimicrobial therapy (Kavitha et al., 2014; Wolcott et al., 2010). Even though debridement is also critical in the management of chronic ulcers and DFUs, the bacterial extracellular polymeric substances (EPS) which are part of the biofilm that promote strong attachment of the biofilm to the wound bed which makes it difficult to completely excise these persister bacterial cells.

Currently utilized methods for managing DM-associated chronic ulcers mainly focus on the antimicrobial aspects of wound management to limit the proliferation of pathogens that gain access to the wound bed. Nonadherent dressings, hydrocolloids, hydrogels, foam-based dressings, alginates, and silver-based dressings while preventing wound contamination also maintain a moist environment necessary for cellular regeneration (Beuker, Miller, Williams, Hilton, & Harding, 2004).

Nevertheless, some of these antiseptic solutions e.g., iodine-containing preparations are associated with inhibited fibroblast proliferation prolonging the healing time of these wounds (Balin & Pratt, 2002). Less emphasis has been placed on striking a balance between antiseptic dressing ointments and wound healing enhancing ointments, hence the need to shift focus onto other possible treatment modalities for DFU. Also, these treatments are not cost-effective meaning that their range of application is limited to healthcare settings in high-income countries. As such, a detailed understanding of pathophysiology mechanisms involved in DFU could help in the identification of wound care agents with minimum disturbance, and maximum promotion of the wound reparative process is needed.

A. Alpha-Tocopherol use in Diabetes Mellitus Complementary Therapy

An ester of retinoic acid and α-tocopherol D-α-tocopheryl retinoate (Tretinoin tocoferil, 0.25%) has been included in an approved ointment for treatment of burns, decubitus ulcers, and lower leg ulcers. After periods of use between 1-14 weeks, the ointment (Olcenon®, Pola Pharma, Japan) was associated with a 76.1% overall wound condition improvement rate (L-300 group, 1990). In other instances α-tocopherol and retinol ointment has been approved for use in alleviating frostbite, progressive keratoderma, ichthyosis vulgaris, pityriasis, palmoplantar keratosis owing to the peripheral circulation promoting effects of α-tocopherol and the suppression of keratin formation effects of vitamin A (Chopra & Flaxman, 1975; Kamimura & Matsuzawa, 1968).

Other *in-vitro* studies have shown that alpha-tocopherol promotes HaCaT keratinocyte (an immortalized human keratinocyte cell line) migration during wound repair by regulating protein-C kinase activity. Alpha-tocopherol-related improved artificial wound healing is related to the antioxidant's effect on keratinocyte polarization whereby administration of α-tocopherol in HaCaT cell lines (in *in-vitro* studies) is related to localization of polarity proteins, such as atypical protein-kinase C and Par 3, in the plasmalemma of cells at migrating edge of the microplate (Horikoshi et al., 2018). Alpha-tocopherol has also been demonstrated to affect cellular signaling in MRSA-infected wounds influencing their wound healing, particularly in senescent animals. Regarding human subjects, oral supplementation of α-tocopherol (between 100-200 mg per day) has been associated with elevated blood cytokine levels and improved immunity against infections in elderly patients (Hobson, 2016). However, there is not enough conclusive evidence to prove this effect.

Another double-blind placebo-controlled RCT investigated the effects of supplementing the diet with omega-3 fatty acid plus alpha-tocopherol on biomarkers of inflammation and oxidative stress in chronic hemodialysis patients. For the α-tocopherol group patients received daily α-tocopherol (400 IU) supplements for 12 weeks. Evaluation after intervention revealed that similar to omega-3 fatty acid supplements, α-tocopherol was associated with a significant increase in blood

nitric oxide (NO) levels and decreases in blood malondialdehyde levels (Asemi, Soleimani, Shakeri, Mazroii, & Esmaillzadeh, 2016). Evaluation of biochemical outcomes revealed that a combination of omega-3 fatty acids and α-tocopherol co-supplementation was associated with significant rises in total antioxidant capacity (TAC), and decreases in plasma malondialdehyde concentration (Jamilian et al., 2017).

In one double-blind crossover trial, healthy individuals had significantly higher baseline blood α-tocopherol compared to individuals with metabolic syndrome (MetS). Seventy-two hours after a single oral dose of hexadeuterium-labeled α-tocopherol (15mg) the absorption of α-tocopherol and its bioavailability remained lower in the MetS group compared to the healthy participants (Mah et al., 2015). The findings resonate with the theory that oxidative stress increases α-tocopherol turnover resulting in oxidative stress and inflammation being associated with depleted α-tocopherol levels in metabolic syndrome (Van Guilder, Hoetzer, Greiner, Stauffer, & Desouza, 2006). With knowledge of these metabolic pathways, it can be suggested that direct application of α-tocopherol on the wound bed would have appositive effects on wound healing of DFU.

II. Study Purpose

It is suggested that reducing oxidative stress within chronic wounds might become a new non-invasive and effective strategy in the management of chronic diabetic wounds. Therefore, this study aimed to determine the influence of oxidative stress on diabetic wounds and to determine whether topical application of an antioxidant (α-tocopherol) can accelerate the healing of experimentally created chronic wounds in a diabetic animal model by alleviating oxidative damage.

III. METHODS

This research was composed of 2 studies. Study 1 was composed of two animal experiments investing the effect of oxidative stress on wound healing in a diabetic animal model, as well as the effect of topical application of α-tocopherol on the healing of a diabetic wound model. Study 2 was a clinical observational study that focused on corroborating findings from animal experiments relating to oxidative stress and diabetic ulcer healing in diabetic patients.

IV. STUDY 1 (ANIMAL EXPERIMENTS)

A. Animals

All experimental protocols were approved by Chiba University's Ethical Committee for Animal Experiments (No: Dou2-400, 2-477, 3-352). 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 (MF,

Oriental Yeast Corp). and water access.

B. Reagents

3-amino-1,2,4-triazole (ATZ) (Tokyo Chemical Industries, Japan) and mercaptosuccinic acid (MSA) (Tokyo Chemical Industry, Japan) were used to induce oxidative stress. ATZ and MSA are potent inhibitors of catalase and glutathione peroxidase, respectively. Alpha-tocopherol (Fujifilm, Wako, Japan) was chosen as a material to reduce oxidative stress within the wound. ATZ was dissolved in sterilized saline for peritoneal injection. 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 Pharmaceutical, Tokyo, Japan). The ratio of liquid paraffin to petroleum jelly was 1 to 2.3. MSA was added to an 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 (0.2mL/ animal). Alpha-tocopherol ointment of either 2% (clinical dose) or 5% (high dose) concentration was prepared for Experiment 2. These concentrations were determined from a commercially available α-tocopherol-containing cream.

C. Wounding and Wound Management

Wounding was performed under an. anesthesia by intraperitoneal administration of ketamine and xylazine. After depilation with hair removal cream, two symmetrical 15×15mm full-thickness excisional wounds were created on the dorsum of the rats using sterile forceps and scissors. Following the wounding procedure, wounds were debrided daily if necessary and irrigated with isotonic saline, and covered with a transparent dressing (Tegaderm, 3M) which was changed daily.

D. Induction of Oxidative Stress

Twenty minutes before wounding all animals were treated with a single dose of intraperitoneal AZT (1g/kg b.w). Immediately after wounding, MSA-containing ointment was topically applied on wounds (0.1mL/wound, approximately 150mg/kg). MSA was topically applied for 5 consecutive days since wounding. The procedure was performed on both non-diabetic and diabetic animals in the oxidative stress groups in Experiment 1 and all animals in Experiment 2. However, in Experiment 1 both diabetic and non-diabetic control groups received no administration of the antioxidant inhibitors, ATZ, and MSA, before and after wounding. This was to allow for comparative analysis between experimental and control groups concerning the relationship between oxidative stress and delayed wound healing. In Experiment 2 all animal groups received the same antioxidant enzyme inhibitor (ATZ+ MSA) treatment as the objectives of the experiment were different from those in the initial experiment. The difference in protocols between the 2 experiments is shown in the tables below:

Table 1：Animal Group assignment Experiment 1

Group	Animal	n	Antioxidant inhibitor	Characteristics
1	SD	9	-	Natural wound healing in a healthy animal
2	SDT fatty	9	-	Natural wound healing in diabetic animal
3	SD	9	+	Natural healing of chronic wounds in healthy animals
4	SDT fatty	9	+	Natural healing of chronic wounds in diabetic animal

In Experiment 1 it was hypothesized that exacerbation of oxidative stress would cause delayed wound healing that is more pronounced in diabetic rats compared to non-diabetic rats.

Table 2: Animal group assignment Experiment 2

Group	Animal	n	Antioxidant inhibitor	Characteristics
1	SDT fatty	7	+	Wounds treated with 2% α-Toc ointment
2	SDT fatty	7	+	Wounds treated with 5% α-Toc ointment
3	SDT fatty	7	+	Wounds treated with Vehicle control (ointment base)
4	SDT fatty	11	+	Wounds left to heal naturally

In Experiment 2 it was hypothesized that administration of α-tocopherol to wounds in diabetic rats would be associated with accelerated wound healing parameters compared to wounds of rats in either the ointment-base or the naturally-healing group.

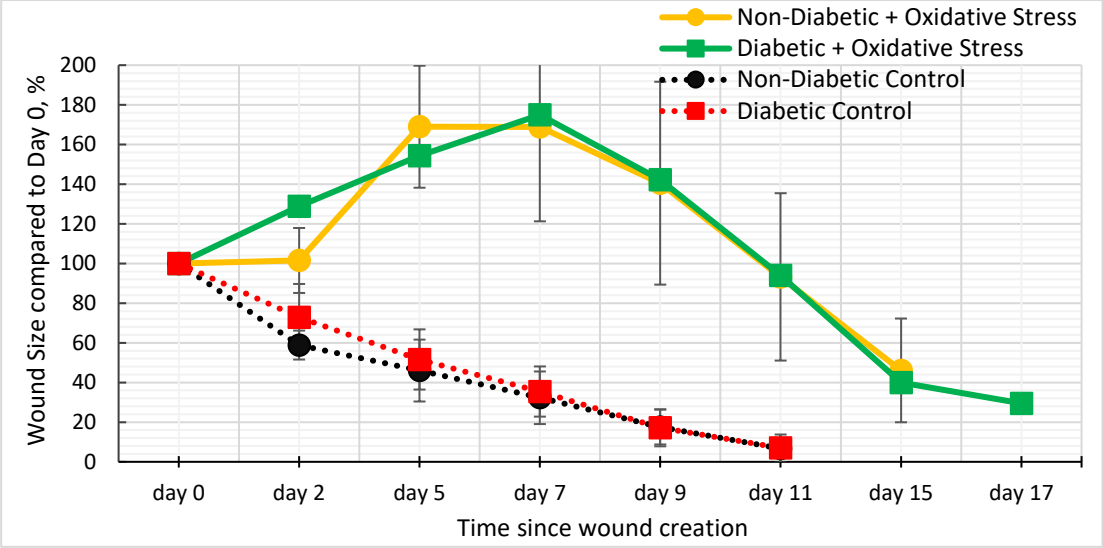
E. Outcome Measurement

Periodically observation of the animals' wound condition (wound size reduction and gross morphology, histological analysis of wound tissue (granulation tissue collagen deposition), biofilm quantification within the wound bed, bacterial burden, and oxidative stress parameters (Glutathione peroxidase activity and serum malondialdehyde concentrations).

F. RESULTS (EXPERIMENT 1)

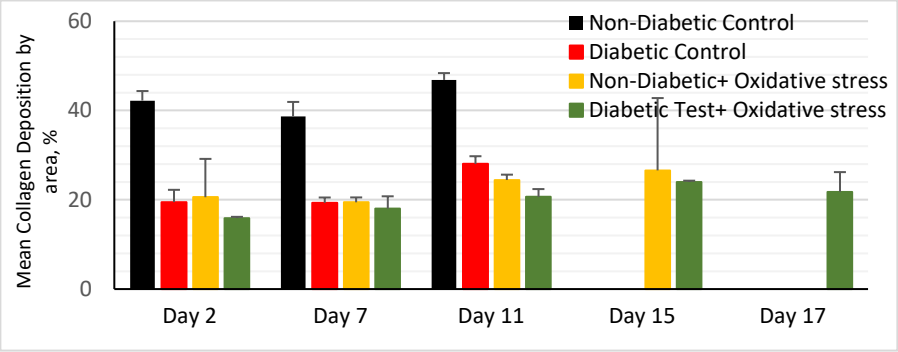
1. Wound Size and Gross Morphology

There was a marked difference in wound size between the oxidative stress groups and control groups of both non-diabetic and diabetic animals during the experiment. Conversely, under oxidative stress induction wound size continued to increase during the MSA application period. The mean 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. On day 17 none of the wounds observed had fully closed, and 29.5% ± 3.1 (n=2) of the wound area remained open in the diabetic oxidative stress group.



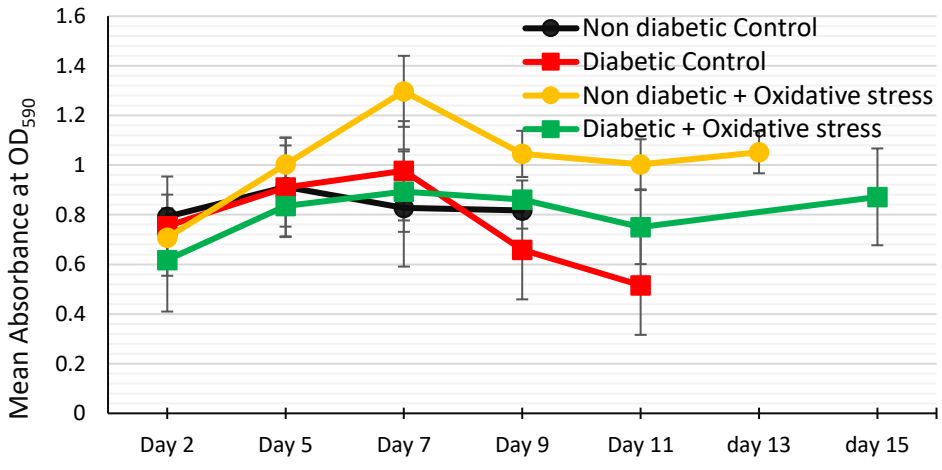
2. Granulation Tissue Collagen Deposition

When oxidative stress was induced, collagen deposition within the wound granulation tissue was poor in both non-diabetic and diabetic animals compared to the control group animals.



3. Biofilm Quantification

Exacerbation of oxidative stress was associated with persistently high biofilm quantification in wounds from both oxidative stress groups irrespective of the presence of a diabetic state.



4. Oxidative Stress Parameters

1. Glutathione Peroxidase Activity

Wound tissue from diabetic animals had a weaker and slower increase in GPx activity in reaction to oxidative stress. Maximum GPx activity in the diabetic oxidative stress group was reached on day 15 while raised Gpx activity levels were achieved by day 5 and reached a peak by day 11 in corresponding non-diabetic animals. Moreover, high GPx activity in non-diabetic animals was maintained high over a longer period (days 7, 11, and 15).

2. Serum Malondialdehyde (MDA) Concentrations

MDA concentration of each measurement time point was similar within each group and diabetic animals appeared to consistently show higher levels of serum MDA than non-diabetic animals irrespective of oxidative stress induction.

G. RESULTS (EXPERIMENT 2)

1. Wound Size and Gross Morphology

The major finding was that topical application of α-tocopherol did not appear to significantly improve wound size reduction compared to either control group.

2. Granulation Tissue Collagen Deposition

Granulation tissue collagen deposition was however higher in wound tissues of animals that received either low or high doses of α-tocopherol

3. Oxidative Stress Parameters

Glutathione peroxidase activity curve trajectories between days 11 to 15 suggest that peak enzyme activity in the naturally healing group might have been delayed compared to both α-tocopherol groups. On the contrary, the highest GPx activity in α- tocopherol and ointment base groups was observed on day 11 and then continuously declined on days 15 and 17 although GPx activities on day 7 for these groups were not evaluated. The highest GPx activity in the clinical dose α-tocopherol group was like the ointment base group and lower than that of the natural healing group.

H. Discussion (Study 1)

1. Effect of Oxidative Stress on Wound Healing

Delayed wound size reduction observed in Experiment 1 for animals subjected to oxidative stress suggests the contribution of oxidative stress in this phenomenon although data on local GPx activity was incomplete due to missing data from control group samples for the period between days 7-11.

Wound area in oxidative stress groups increased during the oxidative stress induction phase and decrease after the termination of oxidative stress induction. Wounds in oxidative stress animals were accompanied by more debris and exudate and took more time to heal. Collagen deposition was also lower in the non-diabetic oxidative stress group compared to the non-diabetic control. Moreover, despite collagen deposition being lower in healed wounds, it was also reduced in the diabetic control group suggesting that the diabetic state itself was associated with levels of oxidative stress high enough to interfere with dermal collagen deposition within these animals. This phenomenon concurs with Experiment 1 results in which systemic levels of oxidative stress (serum MDA concentration) were higher in diabetic rats than non-diabetic rats irrespective of exacerbation of oxidative stress.

While morphologically, wound healing appeared to be similarly affected by oxidative stress in both non-diabetic and diabetic animals, collagen deposition in diabetic oxidative stress animals was less remarkable than in non-diabetic animals since collagen deposition was already decreased in diabetic control animals. Therefore, it can be suggested that oxidative stress was already present in diabetic animals before ATZ and MSA administration. Higher serum MDA levels observed in diabetic control and both oxidative stress groups support this assumption.

Additionally, the trend of persisting increase in absorbance at OD_{560} in both oxidative stress groups after antioxidant inhibitor administration indicates that increased oxidative stress expedited biofilm formation within wounds of affected subjects. Colonization of a wound by biofilm-producing bacteria has been established as one of the critical events that lead to delayed wound healing as it impairs epithelialization and granulation tissue formation (Metcalf & Bowler, 2013).

Initially, during preliminary experiments in this study, it was hypothesized that critical colonization and biofilm formation is the cause of delayed wound healing. Though results from Experiment 1 could not be statistically proven, it can still be suggested that excessive oxidative stress is the initial point that creates conditions conducive for subsequent bacterial colonization and biofilm formation leading to sustained inflammation which delays wound healing through several pathways.

2. Topical α-Tocopherol's Effect on Wound Healing in Diabetic Animals

In Experiment 2 diabetic animals in α-tocopherol groups showed earlier wound closure with increased granulation tissue collagen deposition and reduced biofilm formation compared to the

natural healing group. Alpha-tocopherol is known to scavenge for lipid peroxyl radicals to stop the continuation of the ROS chain reaction which occurs in lipid peroxidation (Yoshikawa & Naito, 2002). While not proven from the results in this study's Experiment 2, a previous *in vitro* study also showed that α-tocopherol effectively increases the skin's antioxidant potential by reducing dermal malondialdehyde levels (Thomas, Vieira, Hass, & Lopes, 2014). Li et al also reported that vitamin E i.e., α-tocopherol increased the expression and activity of GPx-1 in cardiomyocytes via posttranscriptional stabilization of the mRNA for GPx (Li, Cowan, Mickle, Weisel, & Burton, 1996). There were no significant differences in wound healing parameters (rate of wound size reduction, biofilm quantification) between α-tocopherol groups and the ointment base group indicating that α-tocopherol-containing ointment was superior to the ointment base (vehicle control) in aiding these wound healing aspects. Petroleum jelly and liquid paraffin used in preparing the ointment base themselves may have provided some protective effect on the wound, specifically preserving moisture within the wound bed which is a vital component for accelerating extracellular matrix regeneration, reepithelialization, and angiogenesis (Bryan, 2004). This moisturizing properties of the ointment base might have contributed to the effect of α-tocopherol ointment to a certain extent.

On the other hand, similarity in serum MDA levels between α-tocopherol groups and the control groups shows that improvement of the redox environment by topical application of antioxidants is not enough to promote wound healing in diabetic animals, and that reduction of systemic oxidative stress is equally necessary.

The reason α-tocopherol was chosen as an antioxidant in this study is due to the availability of an already approved ointment (Juvela ®) that contains α-tocopherol in its formulation. However, this does not mean that α-tocopherol is the only antioxidant to be recommended for future DM-associated wounds. Rather the results from Experiment 2 (regarding collagen enrichment effects of α-tocopherol) support the idea of using antioxidants both locally and systemically to compensate for diminished antioxidant potential.

In summary, it can be concluded that topical application of α-tocopherol on wounds in a diabetic animal model was not effective in wound size reduction, nor was it effective in reducing systemic oxidative stress. However, it had had positive effects on collagen deposition within wound tissue. Moreover, there is a possibility to utilize antioxidants locally in the care of DFUs if they are combined with blood glucose control and interventions to reduce systemic oxidative stress such as an oral administration of antioxidants.

V. LIMITATIONS

A. Study 1

Animal experiments in Study 1 were conducted with very small sample sizes, for instance as few as 2 animals per group per observation were sacrificed. This limited freedom to perform all required tests in both experiments for instance biofilm quantification was conducted only once (day 7) in the second experiment compared to at least 5 measurements per group conducted in the first experiment. This inability to make appropriate comparisons between groups over time potentially confounds the ability to make statistical comparisons and derive meaningful conclusions from the data given. A small number of animals had to be used to conform to the reduction principle of animal experimentation (Lewis, 2019). Also, skin tissue specimen GPx activity on intact skin before induction of oxidative stress was not done.

In the methodology of Experiment 1, detailed bacterial composition analysis throughout the wound healing phase was not conducted. As such the findings on mere colony types isolated from the wounds are not comprehensive enough to be compared with some previous literature on the same subject which details bacterial biodiversity. Those studies in question e.g., Kim et al (Kim et al, 2020) used diabetic mouse models in which chronic wound models lasting over 60 days were achieved. In contrast wounds in Study 1 lasted a mere ±20 days only producing a delayed wound healing model instead of a chronic wound mode wherefore detailed bacterial analysis such as biodiversity dynamics can be measured.

Despite noticeable improvement in granulation tissue collagen deposition, application of α-tocopherol was not associated with significantly faster wound size reduction compared to the 2 control groups used. The available data from Experiment 2 cannot be used to conclude causality between topical antioxidant use and improved wound healing but is only sufficient to highlight relationships between topical α-tocopherol application and improved dermal collagen deposition but is still insufficient to ascertain a causal relationship between the application of α-tocopherol and improvement in wound condition. Further studies with larger sample sizes are recommended to provide empirical evidence of this hypothesized causal relationship.

VI. STUDY-2 (CLINICAL OBSERVATIONAL STUDY)

The study was approved by Chiba University's research ethics board (Approval number R2-48, NR3-76) as well as the research ethics committee of the research facility (Approval number TGE01764-017).

A. Methods (Data Collection)

Both diabetic and non-diabetic participants provided urine samples from which oxidative stress marker levels were evaluated, and compared with the data from medical records or wound severity scores if patients had wounds. Measured variables demographic data, blood chemistry data, and complete blood count data to evaluate the patients' state including comorbidities, also retrieved from the patients' hospital records. Wound condition was evaluated from photographs which were used to determine wound severity. Oxidative stress was assessed by assaying the concentration of the participants' lipid peroxidation marker (urinary malondialdehyde)

Data analysis mainly focused on using parametric tests to compare differences in oxidative stress between subgroups based on blood sugar control, wound severity, comorbidities, weight distribution, etc to establish the strength of correlations between these variables to the extent of oxidative stress.

B. Ethical Considerations

The study was approved by Chiba University's research ethics board (Approval number R2-48, NR3-76) as well as the research ethics committee of the research facility (Approval number TGE01764-017). Data was stored electronically stored for easy retrieval. For data storage and analysis purposes, the participant names were substituted with number codes to preserve the participants' anonymity

C. RESULTS (STUDY 2)

Compared to the control, markers for oxidative stress were significantly higher in diabetic patients. Patients with DFU showed significantly higher urinary oxidative stress marker concentrations compared to patients without wounds and there was a strong correlation between wound severity and urinary oxidative stress marker levels. Deteriorating glycemic control was associated with higher urinary oxidative stress marker levels.

Results from averaging data from patient subgroups were also reflected in the case studies from a group of selected patients.

1. Participant Demographics

For diabetic participants, forty-four participants that agreed to participate in the study were included in the data analysis. Among the participants who were excluded from the analysis, 1 had a history of smoking, another participant had no conclusive diagnosis of type-2 DM, 6 participants failed to provide a viable urine sample, and 1 participant withdrew consent to participate.

Ten patients (22.7%) were assessed over 1 hospital visit, two visits in 21 patients (47.7%), and three times in 13 patients (29.5%), respectively. As a result, 92 urine samples were collected. For non-diabetic participants, 33 of 35 solicited attendees of an elderly welfare course at a senior citizens welfare centre agreed to participate. Among diabetic and non-diabetic participants, the most mutually inclusive conditions were hypertension, dyslipidaemia, and gastroesophageal reflux disease (GERD) in decreasing order.

Mean ankle-brachial index (ABI) was evenly distributed between participants with well-controlled, slightly uncontrolled, and poorly controlled blood sugar, while it was significantly lower in participants with severely uncontrolled blood sugar. The distribution of underlying illnesses among participants appeared to be unrelated to their status of glucose control.

2. Wound Condition and Oxidative Stress Marker

The severity of wounds was classified according to the Wagner classification system. Among all 13 participants with diabetic foot ulcers during the whole study, the majority of wounds were classified as either stage 0 (n=4) or stage 1(n=4) according to the Wagner classification system. Mean malondialdehyde values of the urine sample collected from diabetic patients with DFUs were significantly higher than values of the urine sample collected from diabetic participants without any wounds.

In a comparison of wound severity, superficial wounds (stages 0 and 1) were associated with noticeably lower mean urinary MDA compared to urine samples from patients with more severe foot ulcers. Also, despite limited sample sizes elevated white blood cell count was present among

patients with poorly controlled to severely uncontrolled blood sugar levels.

3. Other Factors Influencing Wound Condition/ Severity

Ordinal regression analysis for the patients that had DFUs showed that none of the listed comorbidities had a significant influence on DFU severity.

Ordinal regression with DFU severity score as the outcome variable and ratio data variables (age, HbA1C, GFR, albumin, blood urea nitrogen, BMI, leukocyte count) as predictor variables still showed none of the aforementioned predictor variables having a significant effect on the patients' DFU severity. Ankle-Brachial Index, leukocyte count, and obesity were also found to not contribute to the DFU severity.

D. Comorbidities

Since it had already been established that the presence of wounds significantly influenced oxidative stress marker levels in patients' urine samples, only samples taken when patients had no wounds were included in this study to determine if underlying conditions could affect oxidative stress marker values independent of wound condition. All underlying medical conditions did not influence the outcome of urinary MDA measurement.

D. Discussion (Study 2)

1. Demographic Characteristics

The slight difference in weight categories between the nondiabetic and diabetic participants is most likely due to mechanisms involved in diabetes mellitus as a metabolic syndrome and/or insulin-associated weight gain in type-2 DM (Brown, Guess, Dornhorst, Taheri, & Frost, 2017).

Diabetic patients were stratified into subgroups according to their level of glycaemic control because HbA1c is an accurate indicator of the patients' diabetic condition, and it was important to determine if the prognosis of the patients' condition could influence the detection of oxidative stress markers. The similarity of ABI values, diabetic medication options, and underlying conditions across diabetic patient subgroups confirms the homogeneity of characteristics within the diabetic group. Increased hyperglycaemia-dependent chronic inflammatory processes can trigger leucocytosis, and the extent of these inflammatory processes will determine whether the leucocytosis is clinical or subclinical.

There were no age differences between diabetic and non-diabetic participants showing that relatively similar participant groups were chosen in this study. The slight differences in weight categories between the control and diabetic participants is most likely due to mechanisms involved in diabetes mellitus as a metabolic syndrome and/or insulin-associated weight gain in type-2 DM

(Brown et al., 2017).

2. Wound Condition and Oxidative Stress Marker

The presence of DM was significantly associated increased urinary MDA quantities compared to the absence of the condition. This finding confirms earlier results from Study 1 where the findings suggested that oxidative stress was not localized to wounds but rather a systemic phenomenon in a diabetic condition. A similar study that measured serum MDA among type-2 DM Moreover, there was a strong correlation between the severity of those ulcers (as per the Wagner classification system) and the corresponding MDA levels. These results suggest how central oxidative stress is in direct and indirect pathways that determine clinical outcomes for DFU patients.

There is also a generalization that people with long-term type-2 DM patients suffer and possess reduced antioxidant enzyme activity (Deng et al., 2021). Since these extracellular matrix proteins have a slow turnover, increased oxidative stress could extensively alter structural integrity in these collagen-dense areas e.g., the dermis thereby creating a possible basis for initiation and/or delayed healing of diabetic foot ulcers as proven in the results of the animal experiments Study 1.

Observations on the relationship between wound severity and urinary MDA levels also resonate with data from animal experiments in Study 1. Also, the strong relationship between HbA1c and corresponding wound severity (Wagner scores) in Study 2 emphasizes the intrinsic connection between glycemic control and oxidative stress in diabetes mellitus. Circulating oxidative stress marker levels including MDA are also regarded as predictors for the progression of peripheral arterial disease (Krishna, Moxon, & Golledge, 2015). Thus, it is plausible that oxidative stress-induced MDA production could decrease peripheral perfusion which indirectly affects wound healing. Therefore, such literature offers a background for explaining the various aspects from which oxidative stress influenced wound condition in Study 2. Similar to Study 2, MDA concentration was remarkably higher in diabetic patients than non-diabetic participants.

VII. Significance to Nursing Practice

Apart from establishing from the 2 experiments conducted in this study that oxidative stress was associated with poor wound healing these results alone are of low evidence quality to be translated into nursing care practice for diabetic patients. While mechanisms between animal and human subjects were not examined, relationships identified between systemic oxidative and DFU severity in Study 2 offer a substantial basis for making conclusions that oxidative stress is central to the worsening of diabetic wounds in both the animal model and human subjects. As such, application of this study's results to clinical nursing entails addressing high levels of oxidative to either prevent

the onset of DFU complications or to facilitate wound healing in these patients.

Results from this study managed to reiterate the intrinsic nature of the relationship between glycaemic control, oxidative stress, and ultimately DFU severity. As such while it can be concluded that compensation of diminished antioxidant potential by either local or systemic antioxidants can improve wound healing or prevent worsening of DFUs, more research on human subjects needs to be conducted to prove this efficacy. Similar sentiments are shared with conclusions from a literature review on the current evidence of the role of antioxidants in wound healing (Comino-Sanz et al., 2021). This offers an opportunity for further studies to improve wound healing or even prevent cases of non-healing of wounds in diabetic patients. However, there has not been sufficient literature on human subjects supporting antioxidant supplementation in improving DFU outcomes therefore clinical trials would need to be performed before translating this concept into clinical practice.

VIII. SUMMARY AND CONCLUSIONS

This study was designed to determine how oxidative stress influences wound healing in diabetes, as well as to investigate whether topical application of antioxidants (α-tocopherol) would improve wound healing. From all research questions investigated over two studies, it was established from both animal experiments that exacerbation of oxidative stress was associated with delayed wound size reduction and poor collagen deposition. Also, it was determined that since oxidative stress occurs at a systemic level therefore topical application of topical antioxidants alone is insufficient to significantly improve wound healing. Henceforth further research on targeting systemic oxidative stress through systemic and local antioxidant administration is required.

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