Wound Healing Effect of Slightly Acidic Electrolyzed Water on Cutaneous Wounds in Hairless Mice via Immune-redox Modulation

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Directed by Prof. Hyun Kyo Lim

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ABBREVIATIONS

ACC	Available chlorine concentration
AEW	Acidic electrolysed water
AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
BET	Betadine
CAT	Catalase
DCFH-DA	2', 7'-dichlorodihydrofluorescein diacetate
ERK1/2	Extracellular signal-regulating kinase
GPx	Glutathione peroxidase
HCl	Hydrochloric acid
HOCl	Hpochlorous acid
HRP	Horseradish peroxidase
IL	Interleukin
KC	Keratinocyte chemoattractant
MMP	Matrix metalloproteinase

MPO	Myeloperoxidase
NaCl	Sodium chloride
NC	Normal control
NO	Nitric oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
ORP	Oxidation reduction potential
PBS	Phosphate buffered saline
MAPK	Mitogen-activated protein kinase
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
SAEW	Slightly acidic electrolysed water
SAL	Saline
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor-alpha
TW	Tap water
WBC	White blood cell

ABSTRACT

Wound Healing Effect of Slightly Acidic Electrolyzed Water on Cutaneous Wounds in Hairless Mice via Immune-redox Modulation

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Acidic electrolyzed water is an innovative sanitizer having a wide-spectrum of applications in food industry, livestock and agriculture management, and healthcare industry. It is also known to have benefits in the medical field but little is known on its effect and mechanism in wound healing. The study was conducted to identify the effect of slightly acidic electrolyzed water (SAEW) on cutaneous wounds in hairless mice. SAEW (pH: 5.0-6.5, ORP: +800 mV, chlorine

concentration: 25 ppm) was prepared through electrolysis of water and was applied to the wounds of hairless mice three times a day for seven days. Wound size, oxidative stress markers, calcium concentration, immune response related tests and possible signal pathways were explored and compared to control and potential agents such as povidone-iodine (Betadine) and alcohol. We found that SAEW showed the highest percentage of wound reduction from days 2 to 6 ($p < 0.01$). Antioxidant activities such as glutathione peroxidase, catalase and myeloperoxidase activities of SAEW treated group surpassed the total reactive oxygen species in skin. Nuclear factor erythroid-2-related-factor-2 and aryl hydrocarbon receptor, both important in the oxidative stress pathway, were upregulated in SAEW group. Further, SAEW recruited the production of intracellular calcium and promoted its utilization for faster healing. In line, SAEW induced more release of inflammatory cytokines (interleukin (IL)-1β, IL-6, keratinocyte chemoattractant, and tumor necrosis factor-α) vital to wound healing. Other hallmarks of wound healing, matrix metalloproteinases (MMP1 and MMP9) which are responsible for keratinocyte and cell migration were also upregulated. Collectively, our study indicates that SAEW is

effective in wound healing of hairless mice via immune-redox modulation, and heals better/faster than other conventional agents.

Key Words: Slightly Acidic Electrolyzed Water, Wound Healing, Oxidative Stress, Immune Response

I. INTRODUCTION

Skin injuries, such as cutaneous wounds, need to undergo a complex mechanism to repair the damage. Wound healing is comprised of four sequential but overlapping stages; homeostasis, inflammation, proliferation, and remodeling, strongly regulated with the goal of restoring the integrity of the skin.[1] Several studies reveal that different factors can influence wound healing such as immune response and inflammation, redox homeostasis, and also pH.[2-5] In relation to this, there is a rise in the use of topical applications or dressings to control and alter skin pH which can make wounds heal faster. The use of alcohol and povidone-iodine (Betadine) are some of the proven and tested agents in wound healing. Different phyto-extracts are being used and currently being evaluated for their wound healing efficacy.[6] However, some of those treatments can be expensive and can possess side effects.

Acidic electrolyzed water (AEW) is produced by electrolysis of water. It is produced by a machine where water and low concentration of solution (i.e HCl or NaCl) pass through and generates water containing oxygen gas, and available chlorine concentration such as

Cl_2, HClO, ClO^- as components.[7,8] This generally corresponds to acidic water with a general characteristic of having low pH (2.2-6.5), high oxidation-reduction potential (+800-1100 mV), high available chlorine concentration (10-60 ppm) and high content of dissolved oxygen.[8] Spectroscopic analysis revealed that acidic water has microbial activity and is maximum between pH 4 and 5, wherein hypochlorous acid also becomes maximum in killing *E.coli* and *B.subtilis*.[7] Furthermore, acidic water has been known to inactivate microbes, fungus, viruses, and toxins in vitro, and it is also used for disinfecting food equipments, vegetables, fruits, poultry and meat.[8] It is also studied for its efficacy on hand-washing, hospital bactericidal effect, and even cleaning medical apparatus such as endoscope.[9-11] Single study has also been done wherein electrolyzed water functions as a bactericide in burn injury with Pseudomonas aeruginosa infection in a rat burn-wound model.[12]

With this in mind, the development and current applications of acidic water in the medical field could be further explored. Current in vitro studies hinted that functional acidic water might be correlated to epithelial signalling pathway regarding hBD2 and NF-kB regulation on human oral squamous cell carcinoma.[13,14] This signal path might be

plausible to elucidate the molecular mechanism of acidic water on wound healing. Up to date, three studies have been involved in using electrolyzed water in wound healing. An *in vivo* study mentioned above was performed to test the effect of acidic water in burn-wound with bacterial infection, and results showed that the mortality rate was lowest in the acidic water group.[12]Another study testing different waters concluded that the wound healing effect may be due to the free radicals, and the other *in vivo* study proposed that acidic water enhances epithelialization, collagen deposition and increase of inflammatory cells.[15,16] Despite these studies in relation to wound healing, the exact mechanism of the healing effect is still unknown.

This study was performed to investigate the healing effect of slightly acidic electrolyzed (SAEW) on the cutaneous wound in hairless mice, and if so, how. Different mechanisms involved in wound healing process such as oxidative stress, immune response and other plausible pathways were explored. Further, we compared the efficacy of SAEW with other conventional disinfectants such as Betadine and alcohol.

II. MATERIALS AND METHODS

2.1. Preparation of SAEW

SAEW was generated from an electrolyzing apparatus (HOCLER Cosmic Round Korea Co. Ltd., Seongnam, Korea). SAEW was prepared by electrolysis of tap water mixed with 4.5% HCl solution in an electrolytic cell without diaphragm (Fig. 1).

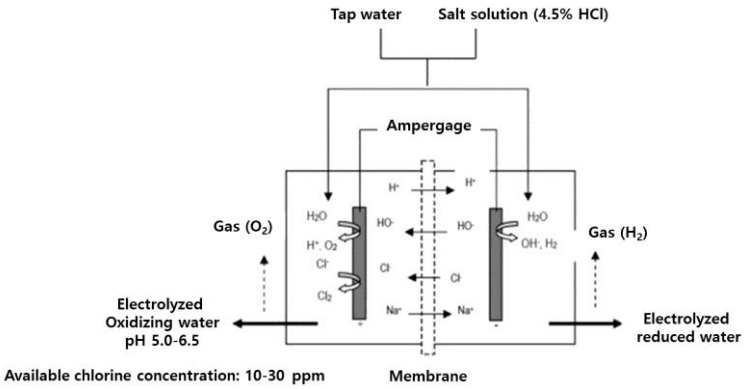

Figure 1. Electrolysis of water to produce SAEW.

Tap water had a pH of 7.0, oxidation reduction potential (ORP) of approximately 400 mV and chlorine concentration of 3.0±0.5 ppm. SAEW produced had properties of pH 5.5±0.5, ORP of approximately +800 mV and available chlorine concentration of 25.0±0.2 ppm (mainly hypochlorous acid) (Table 1). The main component of this

water was 98-100% of HOCl and 0-2% of NaOCl. Saline solution (Life Science Co. Ltd., Dangjin, Korea), Betadine (10% povidone-iodine) (Green Pharmacy Inc, Jincheon, Korea), and 70% alcohol (SK Chemical, Ulsan, Korea) were prepared as control groups.

Table 1. Water characteristics of TW and SAEW

Water	pH	ORP (mV)	ACC (mg/mL)
TW	6.90±0.35	410±166	0.9±0.1
SAEW	5.0 – 6.5	800	10-30

Tap water (TW), Slightly acidic electrolyzed water (SAEW), Oxidation reduction potential (ORP), Available chlorine concentration (ACC)

2.2. *In vivo* study

2.2.1. Animal groupings

Ten-week-old female hairless mice (n=50) weighing 18±2 g were purchased (Orient Bio Inc., Seongnam, South Korea) and kept at 22±2℃ and 40-60% humidity under a cycle of 12:12 h light and dark.

The mice were put in plastic cages (W 172 mm × D 240 mm × H 129 mm) with five mice in one cage, were acclimatized for one week, and were assigned randomly to five groups: no wound induction: normal control (NC) group (n=10), wound-induced groups; saline-treated group (n=10), Betadine-treated group (n=10), alcohol-treated group (n=10) and SAEW-treated group (n=10).

The animal use and protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Yonsei University Wonju Campus (YWC-150827-2).

2.2.2. Wound induction

Mice were anesthetized using Isoflurane (Piramal Critical Lab, Inc., PA, USA) and wounds were induced using 5 mm biopsy punch (Integra-Miltex, PA, USA), creating a total of six wounds equally distributed on the dorsal part of the mice.

2.2.3. Wound treatment

Treatment was done by spraying 2 mL of the treatment groups on the wounds 3 times a day for 7 days.

2.2.4. Wound size measurement

The length and width of two wounds at the middle were measured daily using a vernier scale (Mitutoyo Co., Japan). Wound area reduction was calculated using the following formula: Wound contraction (%) = 100 × [(zero day wound size – specific day wound size)/zero day wound size].[17]

2.2.5. Wound gross examination

Gross morphology was observed by taking a photo of the wounds using a digital camera at a constant focusing distance every day for 7 days to check and compare the wound state of each mouse.

2.2.6. White blood cells (WBC) and their differential counts

On the 7th day, blood was collected from the retro-orbital plexus in tubes coated with anticoagulant and was mixed with an automatic mixer for 5 min. Thereafter, WBC and its differential members such as lymphocytes, monocytes, and neutrophil were measured using an automatic blood analyser (HEMAVET HV950 FS, Drew Scientific Inc., Dallas, Texas, USA).

2.2.7. Preparation of serum

Serum was prepared after the blood collection and was put into BD Microtainer tube (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.), was centrifuged at 14000 rpm for 5 min at 4℃ to get the serum and was kept in at −80°C until further use.

2.2.8. Preparation of skin lysate

Skin tissues (1×1 cm) were cut from the wound area of the mice. The skin tissue was placed in ice-cold RIPA buffer (Pierce Biotechnology Inc., IL, U.S.A.) with protease inhibitor cocktail (Sigma Chemical Co., St Louis, U.S.A.) and was homogenized at 25 rpm for 15 min. Thereafter, the crude skin lysate was centrifuged at 14000 rpm for 10 min at 4℃ and the supernatant was collected. Skin lysate was checked for protein concentration by Pierce BCA Assay Kit (Thermo Scientific, Rockford, IL, U.S.A.).

2.2.9. Total reactive oxygen species (ROS) detection

Total ROS was measured using 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA) (Abcam, Cambridge, MA, U.S.A.) by following manufacturer's manual. In brief, 50 μL of samples were put in the 96-well plate. One hundred microliters of 10 μM DCFH-DA was added and the plate was incubated for 30 min in the dark. Fluorescence at 488

nm excitation/525 nm emission was analyzed using DTX-880 multimode microplate reader (Beckman Counter Inc., Fullerton, CA, U.S.A.).

2.2.10. Antioxidant endogenous enzyme activities

The activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and myeloperoxidase (MPO) in skin lysate were measured using Biovision kit (Milpitas, CA, U.S.A.) and following each of the manufacturer's instruction. In brief, the normalized protein concentrations together with each reaction mix and solutions were used to measure the activities of different antioxidant enzymes and was analyzed at the following observance: SOD (450 nm), GPx (340 nm), CAT (510 nm), and MPO (412 nm).

2.2.11. Nitric oxide assay

NO production was measured using Griess reagent (Promega Corp., Madison, WI, U.S.A.) and the assay was done according to manufacturer's instruction. Briefly, 50 μL of the reagent was added to the same volume serum in a 96-well plate and incubated at room temperature for 15 min. The absorbance was analyzed at 540 nm using

a DTX-880 multimode microplate reader (Beckman Counter Inc., Fullerton, CA, U.S.A.).

2.2.12. Inflammatory cytokines analysis

Serum inflammatory cytokines were analyzed using Bio-Plex Cytokine Assay. Inflammatory cytokines such as interleukin (IL)-1β, IL-6, keratinocyte chemoattractant (KC), and tumor necrosis factor (TNF)-α in serum were analyzed using a Bead Array Suspension Multiplex Kit (Bio-Rad, San Diego, CA, U.S.A.) according to the manufacturer's instructions. In brief, conjugated beads were diluted 1 : 25 per well using assay buffer, transferred into each plate well and washed on the wash platform 2 times. Samples, standards and controls respectively were mixed with anti-cytokine conjugated beads, and incubated for 1 hr. After washing, specific biotinylated detection anti-cytokines were added and incubated for 30 min. Streptavidin-PE solution diluted with assay buffer A was added to each well, incubated for 15 min, and washed 3 times with wash buffer A. One hundred microliters assay buffer A was added into each well containing the beads and was read in multi-plex bead suspension array system (Bio-Plex 200, BIO-RAD® , U.S.A.).

2.2.13. Western blotting

The prepared skin lysate with the normalized protein concentration was equally loaded and separated by electrophoresis on sodium dodecyl sulfate- polyacrylamide gels, was transferred to nitrocellulose membranes, and was blocked in 5% skim milk. Then, the membranes were incubated with primary antibodies overnight at 4℃, and were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Chemiluminescent detection was done using the Chemiluminescence Western blot Detection System (BioSpectrum® 600 Imaging System, Upland, CA, U.S.A.). Primary antibodies used for housekeeping: B-actin; for oxidative stress related mechanism: nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Cell Signaling, Danvers, MA, U.S.A.) and aryl hydrocarbon receptor (AHR) (Abcam); and for remodeling stage mediation: Matrix metalloproteinases (MMP)1 and MMP9 (Abcam). Immunoblotting was done three times in each target protein to confirm results and check statistical significance.

2.2.14. Total calcium assay

The total calcium concentration was measured using Cayman (Ann Arbor, USA) and following manufacturer's instructions. In brief, serum and skin lysate were prepared as mentioned above. Calcium standard in two-fold dilution was prepared and added to the microplate wells. Samples were then added into the wells followed by the working detector reagent. The plate was incubated for 5 minutes and was read at 590 nm (Biotech Instrument Inc., VT, USA).

2.2.15. Statistical analysis

Data values were expressed as the mean ± S.D. The mean values among the groups were analyzed and compared using one-way analysis of variance (ANOVA) followed by subsequent multiple comparison test (Tukey) with GraphPad Prism version 5.0 software packages (GraphPad, La Jolla, CA, USA). Differences were considered statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

III. RESULTS

3.1. Wound size reduction

To check the wound healing efficacy of SAEW, the wound size reduction was calculated and gross examination was observed for 7 days. The result shows that Betadine-treated group showed an increased wound area as compared to saline group. While saline and alcohol-treated groups had a decreased wound area, SAEW-treated group showed the highest percentage of wound reduction from days 2 to 7 (Fig. 2). The wound size reduction was significantly reduced on the third day ($p < 0.001$). By the end of day 7, the wound size of SAEW-treated group was reduced to 22.4%, as compared to 26.1% of alcohol, 31.4% of Betadine and 28.8% of saline-treated groups.

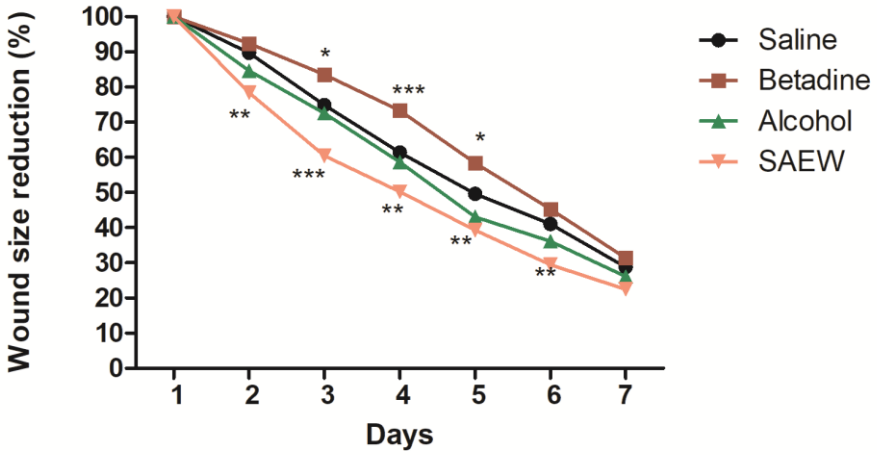

Figure 2. Wound area reduction of different treatment groups from day 1 to 7.

All values are presented as mean ± SD, n=10. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with saline group.

3.2. Gross morphology

Figure 3 shows the digital photograph of a wound representative of the representative mouse from each group from day 0 to day 7. This result was consistent with the wound size reductions as shown in the morphology of the wounds among treatment groups.

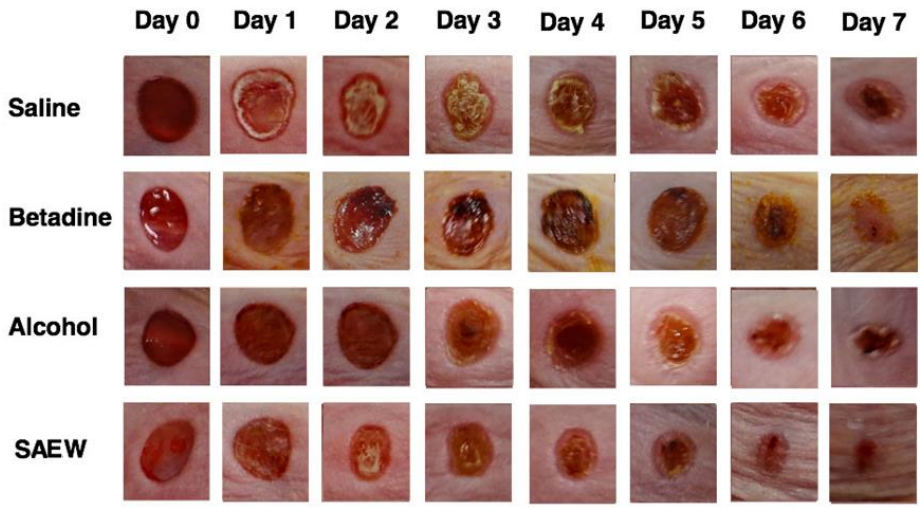

Figure 3. Wound morphology of the representative mouse from different treatment groups from day 1 to 7.

3.3. Redox balance mechanism

3.3.1. Oxidative stress

ROS and antioxidant enzymes play pivotal roles in wound healing, specifically in defence from pathogens. To identify whether these molecules would affect would healing of SAEW, the levels of ROS and antioxidant enzymes were quantitated. We found that there was a significantly lower ROS level in SAEW as compared to the alcohol-treated group ($p < 0.05$) and saline-treated group ($p < 0.05$, Fig. 4A). A similar trend was observed in ROS level of skin lysate comparing SAEW with alcohol-treated ($p < 0.05$) and saline-treated group ($p < 0.05$, Fig. 4B).

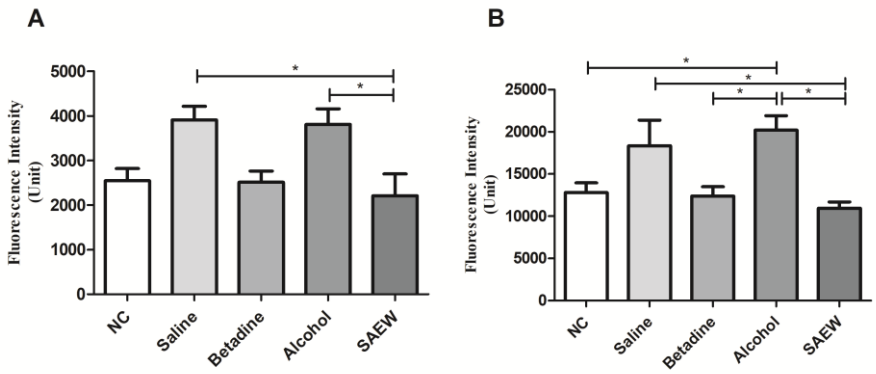

Figure 4. ROS levels in serum (A) and skin lysate (B) among treatment groups.

All values are presented as mean ± SD., n=10. * $p < 0.05$ and *** $p < 0.001$ indicate significant differences when tested with ANOVA. Tukey's test was used for post-hoc tests.

3.3.2. Antioxidant activities

To know the antioxidant activity upon treatment, SOD, GPx, CAT and MPO assays were performed. SOD showed no significant changes among all the treatment groups (Fig. 5A). However, GPx increased among all groups but were significantly higher in Betadine and SAEW-treated groups as compared to NC group ($p < 0.05$, Fig. 5B). It is also observed that there was no significant change observed between the positive control groups and experimental groups as compared to the saline-treated group. On the other hand, there was a significant reduction of CAT in Betadine-treated ($p < 0.05$) and SAEW-treated ($p < 0.001$) versus NC group and SAEW-treated group also had significantly lower CAT activity as compared to saline-treated group ($p < 0.001$, Fig. 5C). MPO also showed significant reduction of expression upon treatment of saline, alcohol and SAEW ($p < 0.001$) but other groups observed no significant difference when compared with saline-treated group (Fig. 5D).

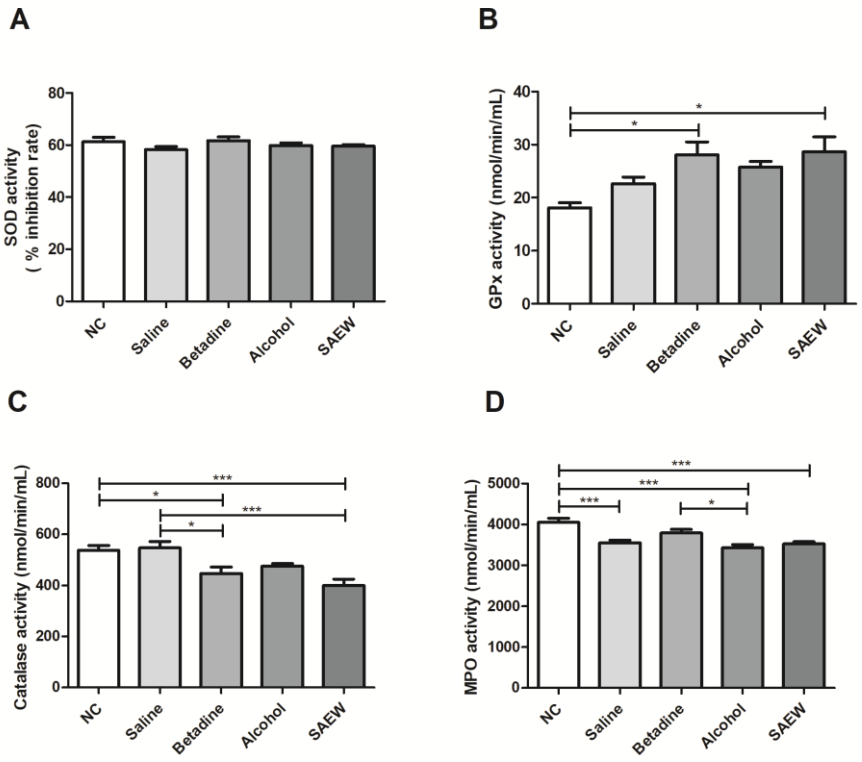

Figure 5. Skin lysate's SOD (A), GPx (B), CAT (D) and MPO (E) levels among treatment groups.

All values are presented as mean ± SD., n=10. *p<0.05 and *** p < 0.001 indicate significant differences when tested with ANOVA. Tukey's test was used for post-hoc tests.

3.3.3. Antioxidant signalling pathway

To further elucidate the oxidative stress pathway, Nrf2, a main regulator of antioxidant enzyme production, and AHR, a protein with adaptive response in oxidative stress, were also checked. Nrf2 level in wounds treated with SAEW was upregulated as compared to NC group ($p < 0.05$) but not statistically different compared to saline-treated group. Similarly, AHR was also more expressed in SAEW treated group as compared to NC group ($p < 0.01$), and an increasing trend was also observed though not statistically significant between SAEW and saline-treated groups (Fig. 6).

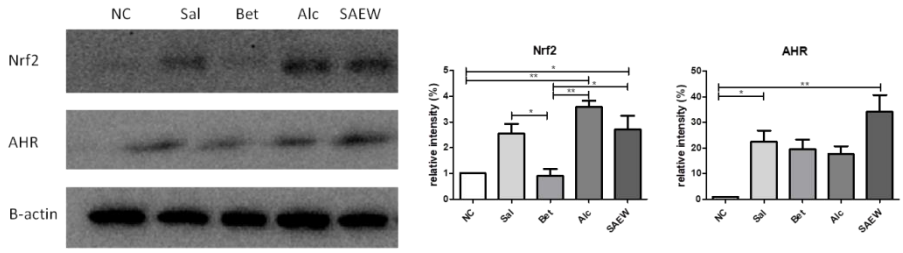

Figure 6. Expression of Nrf2 and AHR on skin lysate of different treatment groups.

* $p < 0.05$ and ** $p < 0.01$ indicate significant differences when tested with ANOVA. Tukey's test was used for post-hoc tests. NC: normal control, Sal: saline, Bet: Betadine, Alc: alcohol, SAEW: slightly acidic electrolyzed water.

3.4. Immune response modulation

3.4.1. Total white blood cells and their differential counts

To identify how SAEW mediates the immune response which can contribute in its rapid healing process, WBC and its differential counts were analyzed. Table 1 shows that there was a significant reduction of the total WBC in all treatment groups (saline, alcohol and SAEW-treated groups ($p < 0.05$) and Betadine-treated group ($p < 0.01$). Also, there was no significant difference in neutrophil counts among all groups. Lymphocytes of saline, alcohol and SAEW-treated groups were similarly reduced ($p < 0.01$) and Betadine-treated group was greatly reduced ($p < 0.001$). In addition, upon treatment of saline, alcohol and SAEW, monocytes were also significantly reduced ($p < 0.001$) and Betadine-treated group had also decreased monocyte counts ($p < 0.05$). In addition, there was no observed statistical difference between saline-treated group and positive control groups and experimental group.

Table 2. Total white blood cells and their differential counts

WBC count (K/μL)	NC	Saline	Betadine	Alcohol	SAEW
Total WBC	5.14 ± 0.95	4.04 ± 0.40*	3.88 ± 0.58**	4.02 ± 0.63*	4.07 ± 0.74*
Neutrophil	1.50 ± 0.28	1.66 ± 0.13	1.54 ± 0.18	1.77 ± 0.39	1.72 ± 0.16
Lymphocyte	3.24 ± 0.80	2.12 ± 0.34**	1.90 ± 0.42***	2.04 ± 0.39**	2.09 ± 0.59**
Monocyte	0.41 ± 0.09	0.26 ± 0.07***	0.30 ± 0.07*	0.19 ± 0.05***	0.22 ± 0.05***

Data were expressed as mean ± SD, n=10. NC: normal control, SAEW: slightly acidic electrolyzed water. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with NC group, indicates significant differences with ANOVA. Tukey's test was used for post hoc tests.

3.4.2. Cytokine analysis

In line, the production of cytokines is also important in immune response and wound healing. To check their activity and function upon treatment, a multiplex assay was done to test different inflammatory cytokines in serum and was compared among the groups. IL-1β showed a significant reduction in alcohol and SAEW-treated groups ($p < 0.001$) as compared with NC group and also the saline-treated group (Fig. 7A). A similar trend was seen in the expression of IL-6, wherein alcohol and SAEW- treated groups were also significantly reduced ($p < 0.05$ and $p < 0.001$) versus NC group and IL-6 expression was also significantly lower in SAEW-treated group than that of saline-treated group ($p < 0.01$, Fig. 7B). KC levels of all treatment groups were also reduced significantly especially upon alcohol and SAEW treatment ($p < 0.001$) as compared to NC group but no statistical difference was observed when compared to saline-treated group (Fig. 7C). Consistently, TNF-α (Fig. 7D) showed significant reduction in alcohol and SAEW-treated groups ($p < 0.01$) versus NC group but no significant difference observed versus saline-treated group. However,

IL-10 and IL-17 did not show any difference among all normal and treated groups (Figs. 7E and 7F).

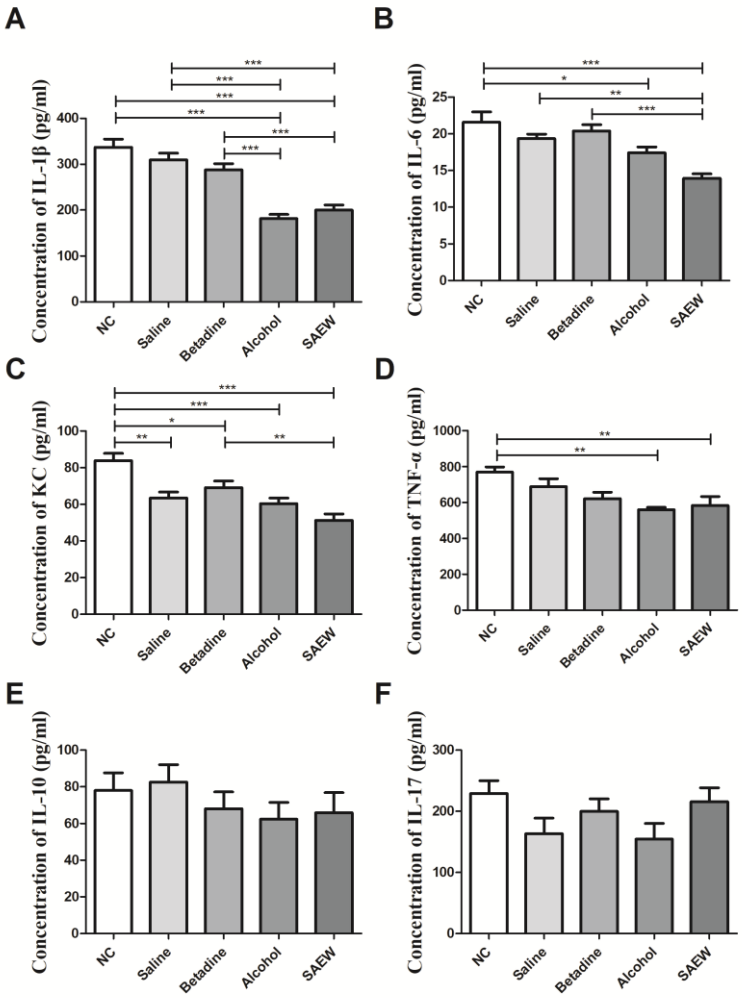

Figure 7. Effect of the treatment groups on IL-1β (A), IL-6 (B), KC (C), TNF-α (D), IL-10 (E) and IL-17 (F).

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate significant differences when tested with ANOVA.

3.5. NO production

NO also plays a role in both oxidative stress and inflammation related pathway. NO assay was done to check the effect of the treatment groups on its activity. The results show that NO levels in serum were high in saline and Betadine treated groups, where in alcohol treated group was significantly lower than saline-treated group ($p < 0.001$) but SAEW-treated groups were not statistically different (Fig. 8A). In addition, NO in skin lysate showed a trend of a slight increase in SAEW-treated group as compared to saline-treated group but generally did not show any significant difference among all treatment groups (Fig. 8B).

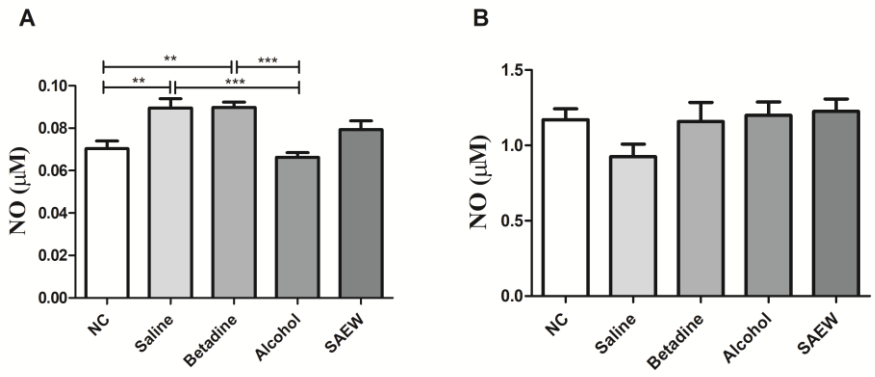

Figure 8. Nitric oxide levels in serum (A) and lysate (B) among all treatment groups.

** $p < 0.01$, and *** $p < 0.001$ indicate significant differences when tested with ANOVA. Tukey's test was used for post-hoc tests. NC: normal control, SAEW: slightly acidic electrolyzed water.

3.6. Intracellular calcium concentration

Calcium is a central regulator of skin homeostasis and is also involved in wound healing through ROS production. Calcium acts as an initial damage signal for tissue repair through activation of dual oxidase (DUOX) which produces H_2O_2 important in wound healing. To find out how SAEW could have an effect on its role in wound repair, intracellular calcium were assayed in both serum and skin lysate. Intracellular calcium concentration in serum showed significant reduction among all treated groups ($p < 0.001$) versus NC group, and alcohol treated groups showed significant reduction as compared to Betadine treated group ($p < 0.05$) while there was no observed significant difference between saline-treated and SAEW-treated groups (Fig. 9A). Calcium concentration on skin lysate, however, showed an increase among all groups but with no observed stastistical significance. However, it is also noted that SAEW had the lowest calcium concentration among all treatment groups (Fig. 9B).

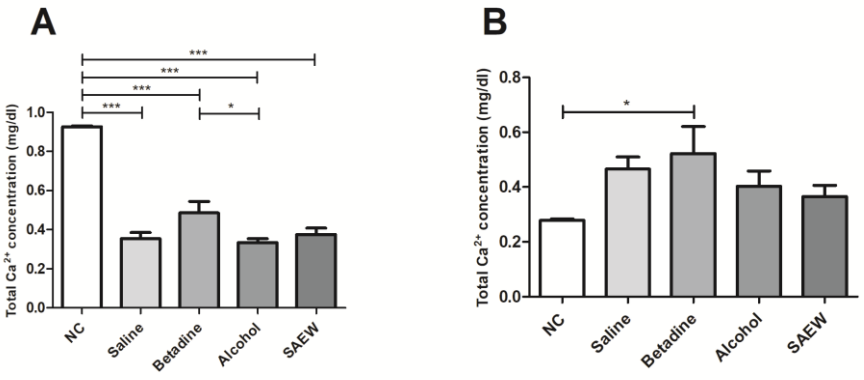

Figure 9. Intracellular calcium concentration of serum (A) and skin lysate (B) among all the treatment groups.

All values are presented as mean ± SD., n=10. * $p < 0.05$ and *** $p < 0.001$ indicate significant difference when tested with ANOVA. Tukey's test was used for post-hoc tests. NC: normal control, SAEW: slightly acidic electrolyzed water.

3.7. MMP Production

MMPs, important markers in the remodeling stage of wound healing, were also checked. Western blot results showed that MMP1 and MMP9 were upregulated in all groups (Fig. 10) as compared to unwounded mice. In comparison to saline-treated group, MMP1 showed more protein expression while MMP9 showed similar or less expression but not statistically significant.

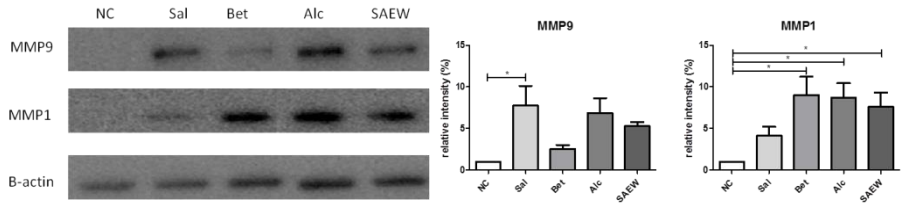

Figure 10. Expressions of MMP1 and MMP9 in all treatment groups.

* $p < 0.05$ indicates significant difference when tested with ANOVA. Tukey's test was used for post-hoc tests. NC: normal control, Sal:saline, Bet: Betadine, Alc: alcohol, SAEW: slightly acidic electrolyzed water.

IV. DISCUSSION

Our study indicates that SAEW is effective in wound healing of hairless mice via immune-redox modulation, and heals better/faster than conventional agents such as alcohol and Betadine. This was evidenced by the wound size reduction and gross morphology. Wound healing stages such as hemostasis, inflammation, proliferation and remodeling are vital processes to understand the mechanism of restoring the skin. These processes are influenced by different factors, one of which is the difference in pH by affecting angiogenesis, collagen formation, macrophage and fibroblast activity, keratinocyte proliferation and effects on enzyme activities.[18] Taken together, SAEW appear to have a favourable condition that affects effective healing of cutaneous wounds via immune-redox modulation.

One factor essential to wound healing is redox homeostasis. It might be achieved by controlling the balance between ROS production and antioxidant scavenging. The balance of ROS levels is required to escape damaging effects as well as to execute its beneficial functions.[4] ROS' role in the inflammatory phase is well known wherein inflammatory cells migrate to the wound and secretes ROS to help in

attacking pathogens, and moderate amount of ROS could facilitate angiogenesis and re-epithelialization.[3] Based on these, we can infer the potential intervention of SAEW in ROS production from SAEW-treated group having the lowest ROS concentration in both serum and lysate. It shows that upon treatment of SAEW, ROS in the whole body and the wound area are in a controlled amount. Therefore, SAEW produced a desired amount of ROS as compared to alcohol and saline treated groups for its functional roles. These roles can be attributed to the involvement of a low level of ROS in migration and proliferation[19] and angiogenesis.[20]

In addition, antioxidant enzymes play a role in detoxification of ROS in the skin to avoid oxidative stress and to maximize the healing effect.[4] In this study, SOD did not have any significant change which is evidence that the step toward the dismutation of superoxide ion into H_2O_2 was already done. This result confirms that the wounds were past initial inflammatory phase of wound healing. CAT and GPx, on the other hand, showed some significant differences because they work to reduce H_2O_2 to oxygen and water. The decreased CAT expression directly compensates for the decreased ROS activity. While GPx increased levels may mean that, at this wound state, GPx still continues

to work for a complete recovery. Therefore, it is important to note that antioxidant enzymes work in a temporal and spatial expression pattern.[21] MPO, an antioxidant enzyme which is abundant in neutrophils and a marker of inflammatory infiltration,[22] showing decrease levels proves that SAEW is competent in neutrophil infiltration inhibition. Moreover, SAEW mediates the expression of Nrf2, a main regulator of the antioxidant response,[23] and AHR, another important signalling for the oxidation and antioxidation which has a power to switch as oxidant through cytochrome P450 (CYP1A1) or an antioxidant through Nrf2 by producing antioxidant enzymes.[24] Taken together, antioxidant enzymes seem to regulate the produced ROS, producing oxidative stress balance, leading to wound healing.

NO also plays an important role in wound healing by its influence on inflammation, proliferation, matrix deposition, angiogenesis and remodelling, and on oxidative and nitroxidative stress balance.[25,26] Therefore, the higher concentration of NO in SAEW-treated group may indicate its help in facilitating faster wound healing either through phagocytosis or inflammation related mechanism. It can also be possible to be involved in the oxidative stress mechanism as observed in the activities of the antioxidant enzymes.

The involvement of immune cells in wound healing has been known for the migration of neutrophils, macrophages and lymphocytes into the wound site.[10] For instance, macrophages move to the wound through the chemical messengers from the damage cells and platelets wherein they can thrive in the more acidic wound environment.[11] Similarly, immune cells in wounded mice might move to the wound area, which might be supported by our previous finding about the correlation between circulating WBC, and its differential counts and the damaged histopathology. This might explain the reduction of WBC count in the circulating serum. However, the absence of histological confirmation is the limitation of this study. To explain the mechanism further,[27] the blood's neutrophil count, one of the important markers of inflammation stage, and functions to remove foreign materials such as bacteria, and non-functional host cells from the wound site,[27] shows no significant difference among all groups and it might be because the wounds were past the initial inflammatory phase. Other WBC components are lymphocytes, which migrate into the wounds in proliferative stage, peaking on day 7,[28] and monocytes, which mature into macrophages, and good sources of cytokines and stimulates fibroblast activity, collagen synthesis and angiogenesis.[29] Both

lymphocytes and monocytes showed a significant decrease compared to the normal groups proving its work in late inflammation to remodelling phase. It is also observed that Betadine and alcohol and the experimental group, SAEW, did not show any significant difference with the negative control, saline, which may be due to innate ability of wounds to heal according to time. In addition, pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, produced by neutrophils, macrophages and keratinocytes and are known to be upregulated during the inflammatory phase[30] while chemokines, such as KC, is also known to have roles in epithelialization, tissue remodelling, and angiogenesis[31]. The observed lower serum IL-1β, IL-6, TNF-α and KC concentration may mean that alcohol and SAEW may potentially mediate the activity of these cytokines more in response to wound healing earlier than Betadine and saline treatment. However, IL-10 and IL-17 have no observed changes which might be due to either very low detected signal due to its lower activity on the remodelling phase of wound healing. It is therefore important to note that the spatio-temporal factor of these cytokines and growth factors is vital in wound healing. Taken together, there seems to be a quick

immune response modulation upon treatment of SAEW causing the wound healing efficacy.

Calcium is identified as a potential central regulator in wound healing in the skin which can be through maintaining normal homeostasis or helps in keratinocyte proliferation and differentiation.[32)] Calcium also plays an important role in the wound healing process. In one review paper, several studies have concluded that calcium is an important factor especially in early phase of wound healing by producing H_2O_2 which promotes healing through the MAPK kinase or directly through the transcription genes.[33] The reduced amount of calcium concentration in serum can be due to its action to the wound site. Because the wounds were in remodelling phase and towards complete healing, the amount of calcium concentration in skin lysate was back to normal state. Taken together, SAEW-treated group shows to be favourable in mediating intracellular calcium also related to ROS production.

Matrix metalloproteinases and their inhibitors are enzymes which also play a critical role in wound healing by mainly degrading substances in extracellular matrix but is also recently known to be also

responsible in inflammation, epithelial repair and resolution, wherein MMP1 and MMP9 were studied to be the major regulators.[34] In addition, MMPs activity is controlled by specific locations and stages of wound healing.[35] MMP1 facilitates keratinocyte migration and MMP9 promotes cell migration.[34,36] Towards complete re-epithelialization, MMP1 and MMP9 should decrease, which was shown in our results upon SAEW treatment, as high levels are evident in chronic wounds. However, this result shows that saline treatment mediates MMP1 expression sooner than the SAEW treatment, while SAEW mediates MMP9 more. Taken together, SAEW seems to be regulating MMP1 and MMP9, at this wound stage, which facilitates faster healing through keratinocyte and cell migration.

Since wound healing is a complex process, it is possible that different factors interact and affect one another to help in faster and effective healing. First, ROS production is influenced by calcium signalling through H_2O_2 production leading to immune cell recruitment.[28] In addition, calcium and oxidative stress have an effect on ERK-1/2 in the production of MMP-9 for vascular remodelling.[37] This cross linking of different factors, plus the earlier mentioned oxidative stress and immune modulation all together can influence

wound healing. On the wound healing effect of SAEW, which components would execute the healing mechanism needs to be speculated. Our product is mainly composed of hypochlorous acid (HOCI) wherein we can attribute its effects. Several line of evidences have shown HOCl as an ideal wound care agent thanks to its powerful microbicidal, anti-biofilm, and wound healing potency.[38, 39]

As a plausible mechanism involving acidic water, acidic water has been known to induce hBD2, irrespective of nuclear factor NF-kB.[13, 14] Strategically, acidic water inhibits NF-kB activity by attenuating nuclear–cytoplasmic shuttling of p65 and p50 subunits.[14] Further, increased NF-kB expression would induce cutaneous inflammation[40], consequently hBD might improve wound healing.[41] These potential mechanisms implicated in wound healing remain to be elucidated in our on-going acidic water study. In summary, our results show that SAEW show favourable results in wound healing via immune-redox modulation and the cross linking of all factors leading to not only its potency in healing wounds, but most especially on its quick action toward wound healing.

V. CONCLUSION

Knowing the properties of SAEW having pH 5.0 to 6.5, ORP around +800 mV and chlorine concentration of 10 to 30 mg/L and learning how and what electrolysis of the water produces, this study shows that SAEW can mediate and is helpful in wound healing. It is shown that comparable to potent medicine in wound healing such as betadine and alcohol, SAEW is similarly effective in wound healing. SAEW group, with pH ranging from 5 to 6.5 is believed to have better effect on skin barrier because of its favourable condition adapting the normal skin pH which is from 4-6. So far, acidic water is proven to be effective in wound healing through immune-redox modulation, and heals better/faster than conventional agents such as alcohol and Betadine. More studies can be done to identify the specific signalling pathway via immune-redox modulation to fully elucidate its exact and complete mechanism.

Importantly, this finding might imply the potential use of SAEW as a wound healing agent with more benefit of cost-effectiveness and safety than conventional antiseptics.

VI. REFERENCES

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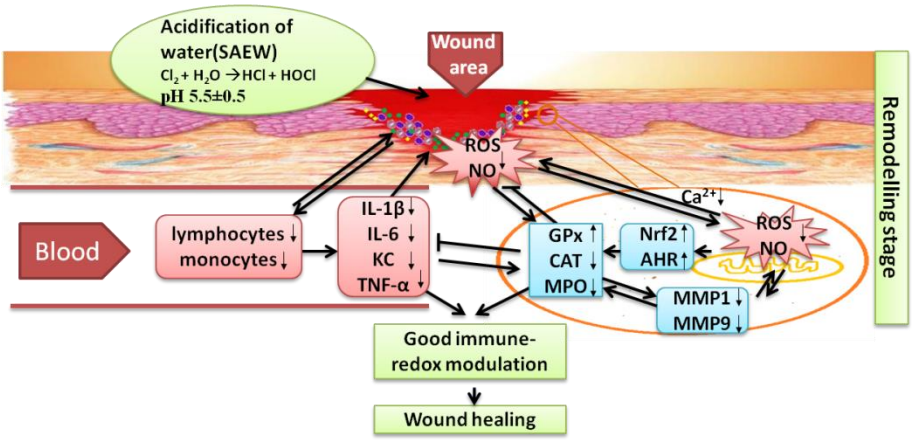
VII. GRAPHICAL ABSTRACT



VIII. KOREAN ABSTRACT

초록

헤어리스 마우스에서 면역-레독스 조절을 통한 약산성전해수의 피부 상처치유 효과

유해선

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<지도교수 임 현 교>

전해산성수는 보건위생에 사용될 수 있는 신개념의 기능수로 식품산업, 농업, 보건 및 건강산업 분야에 널리 활용되고 있다. 의료분야에서도 유효성이 확인되어 사용되고 있으나 상처 치유에 대한 기전은 명확히 밝혀지지 않은 상태이다. 본 연구는 산도 5.0-6.5, 산화환원전위 +800mV,

유효염소농도 25ppm 의 약산성전해수(SAEW)를 7 일 동안 무모쥐의 피부 상처에 처리하여 상처치유 과정을 관찰하였다. 상처치유의 효과를 확인하기 위해 상처의 크기 변화, 산화적 스트레스 지표, 칼슘 농도, 면역반응관련 지표, 신호전달체계 등을 관찰하였으며 일반적으로 상처소독에 사용되는 알코올과 요오드용액(베타딘)을 함께 비교하여 약산성전해수의 살균제로서의 활용 가능성을 평가하였다. 그 결과, 약산성전해수군에서 다른 살균제군에 비하여 실험 2 일에서 6 일까지 유의한 상처의 크기 감소가 관찰되었다($p < 0.01$). 항산화 활성 실험에서 약산성전해수군은 글루타치온 페록시다제, 카탈라제, 마이엘로 페록시다제의 활성에도 영향을 미침을 알 수 있었고 피부 샘플에서 전체적인 활성산소 감소를 나타내었다. 피부재생 반응 중 산화적 스트레스에 관계되는 nuclear factor erythroid-2-related-factor-2 와 aryl hydrocarbon 수용체의 수치가 증가함으로써 상처 치유에 필요한 활성산소의 조절 경로에 영향을 미침을 알 수 있었다. 또한 약산성수 처리군은 세포 내 칼슘 농도가 증가하였으며

이것이 상처치유 과정을 촉진한 것으로 판단된다. 또한 상처치유에 필요한 염증반응 관련 면역조절 인자인 IL-1β, IL-6, keratinocyte chemoattractant 와 tumor necrosis factor-α 의 증가가 관찰되었다. 피부세포와 cell migration 에 관련되어 있는 matrix metalloproteinases (MMP1 와 MMP9)의 활성 또한 증가된 것으로 확인되었다. 결과적으로 약산성전해수는 무모쥐에서 피부의 상처를 일반적인 살균제보다 빠르게 회복시킴을 확인하였고 이는 약산성전해수에 포함된 차아염소산이 빠른 염증반응을 유발하여 상처치유단계의 면역반응에 영향을 미쳤기 때문으로 판단된다.

핵심단어: 약산성전해수, 상처치유, 산화적 스트레스, 면역반응

IX. PUBLICATION LIST

1. You HS, Fadriquela A, Sajo ME, Bajgai J, Ara J, Kim CS, Kim SK, Oh JR, Shim KY, Lim HK, Lee KJ. Wound healing effect of slightly acidic electrolyzed water on cutaneous wounds in hairless mice via immune-redox modulation. *Biol. Pharm. Bull.*, **40**, 1423-1431, (2017). doi: 10.1248/bpb.b17-00219.