JTL 11 (2) December 2022
Page: 95-104
Contents list available at Jurnal Teknologi Laboratorium
JURNAL TEKNOLOGI LABORATORIUM
Journal Homepage: www.teknolabjournal.com
ISSN 2580-0191(Online) I ISSN 2338 – 5634(Print)

Original Study

OPEN ACCESS

Effectiveness of temulawak (*Curcuma* xanthorrhiza) ointments *as wound healing agents in wistar rats*

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Abstract: Temulawak is known for its potential as a powerful antioxidant, anti-inflammatory, and anti-aging. Numerous studies have been reported on how temulawak extract components minimize wrinkles, boost collagen production, and prevent degradation. This study aims to determine whether temulawak ointment promotes wound healing in Wistar rats. Pure experimental laboratory research and only post-test control group design observations have been done. Our results showed changes in the macroscopic appearance of wounds, particularly in the look of closed, dry wounds, which were exclusively observed in patients who received ointments containing 15%, 20% and 25% temulawak ointments after 14 days. Based on the histopathology observation there was a significant difference in the mean comparison of the area of epithelialization, the number of fibroblasts, and the quantity of angiogenesis. We conclude that temulawak ointment benefited incision wound healing in Wistar rats.

Keyword: Temulawak; Wound healing; Epithelialization; Fibroblasts; Angiogenesis

INTRODUCTION

The skin is a complicated organ that shields the body from the environment. Approximately 15% of body weight is comprised of skin, making it the heaviest organ [1]. The skin has multiple roles, including providing a physical permeability barrier, protecting against pathogenic agents, thermoregulation, sensation, ultraviolet radiation protection, regeneration, and wound healing [2]. Various skin aging phenotypes are age, gender, ethnicity, air pollution, nutrition, smoking, and sun exposure [3]. Skin aging changes physiological functions, skin structure, and age. Signs of skin aging are reduced skin barrier function, slowed epidermal cell turnover, and reduced skin vascularity [4]. The decrease in keratinocytes and fibroblasts during the aging process will degrade the skin functions such as protection, excretion, secretion, absorption, thermoregulation, and decreased sensory perception [5]. In addition, a decrease in the number of *Langerhans* cells and melanocytes will cause a decrease in skin pigmentation, and a decreased number of collagen cells, elastic fibers, mast cells, and macrophages will cause changes in skin texture [5].

Recently, many antioxidant agents have been used, both synthetic and herbal antioxidant agents [6,7]. Anti-aging agents may help to prevent degenerative processes with visible symptoms such as wrinkles, rough skin, and dark spots [8]. One of Indonesian herbal widely used in traditional medicine is temulawak (*Curcuma xanthorrhiza*). Temulawak has been reported for its bioactive compound called *Curcumin* [9], which is efficacious as an antioxidant, anti-inflammatory, and anti-aging[10]. The bioactive components of temulawak extract can reduce wrinkles, increase collagen synthesis and suppress collagen degradation [11]. There is a relationship between temulawak extract and anti-aging on the skin by inducing a

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DOI: 10.29238/teknolabjournal.v11i2.376
Received 31 August 2022; Received in revised form 07 October 2022; Accepted 14 November 2022

cellular response in normal human skin fibroblasts, which causes the induction of peroxides and other antioxidant enzymes to modulate aging and improve cell function [12]. Oxidative stress and inflammation mostly contribute to aging and age-related conditions including skin aging [13,14].

To the best of our knowledge, the literature on the effect of temulawak ointments on wound healing in non-diabetic Wistar rats based on the comparison of the area of epithelialization, the number of fibroblasts, and the quantity of angiogenesis are still rarely reported. Recently, a study reported that temulawak extract has increased the number of fibroblasts, tissue granulation, blood vessel density and wound contraction in diabetic Wistar rat induced by Streptozotocin (STZ) [15]. The results of other studies showed that temulawak extract was able to accelerate wound closure by 15.262%/day compared to the untreated group by 13.54%/day [16]. Previous study on curcumin bioactivities have revealed that there were neovasculogenesis and expressions of CD31, VEGF, TGF-b1, hypoxia-inducible growth factor-1alpha, stromal cell-derived growth factor-1alpha, and heme oxygenase-1, and western blotting studies [17]. The wound healing potential of curcumin has been proposed regarding its antioxidant and antiinflammation bioactive compounds [18]. However, there is no report of the study on the tipical non-diabetic rats to date.

This research is supposed to fill the gap in the literature on the wound healing effect of temulawak ointments based on microscopic observation, wound appearances, number of fibroblasts, neovascularization points, and epithelialization area of non-diabetic Wistar rats. Hence, this experimental research is highly recommended to fill the gap in this field of study.

MATERIAL AND METHOD

This study was an experimental laboratory with a post-test-only control group design of observations of male Wistar rats. Experimental research was conducted at the animal house laboratory of FMIPA USU Medan for approximately three months (September to November 2021). The protocol of animal research has been approved by Animal Research Ethics Committees/AREC FMIPA USU No. 0763/KEPH-FMIPA/2021.

Temulawak extract was obtained via maceration method using 96% ethanol to separate solid-liquid mixtures from temulawak simplicia. Maceration was followed by rotary evaporation to get the crude extract.

To get temulawak ointments, we have prepared by following the formulation described in Table 1 below:

Table 1. Ointment Formulation

Group Treatment	Temulawak Extract (g)	Vaseline Album (g)	Adeps Lanae (g)	Ointment (g)
15%	1.5	7.225	1.275	10
20%	2	6.8	1.2	10
25%	2.5	6.375	1.125	10
Ointment base	-	8.5	1.5	10

Wistar rats were placed in cages made of a plastic material covered with a cover made of wire mesh and placed separately according to each treatment. All test animals under the same conditions were acclimatized for one week and fed in an adlibitum way. The incision was made in the back area of the Wistar rats that had been disinfected with 70% alcohol. Anesthesia was performed using a lidocaine injection of 0.1-0.3 ml/kg BW intramuscularly. About 2 cm incision with a depth of 0.5 cm to the subcutis layer on the back using a sterile scalpel. Cuts in rats were smeared with ointment with a predetermined concentration twice/day, at 08.00 am and 05.00 pm, based on the group. The cuts in the negative control group (K(-)) were only cleaned with normal saline in the positive control group (K(+)). The

wound was smeared with povidone-iodine. Treatment group I (PI), treatment II (PII), and treatment III (PIII) were smeared with temulawak extract ointment with concentrations of 15%, 20%, and 25%, respectively.

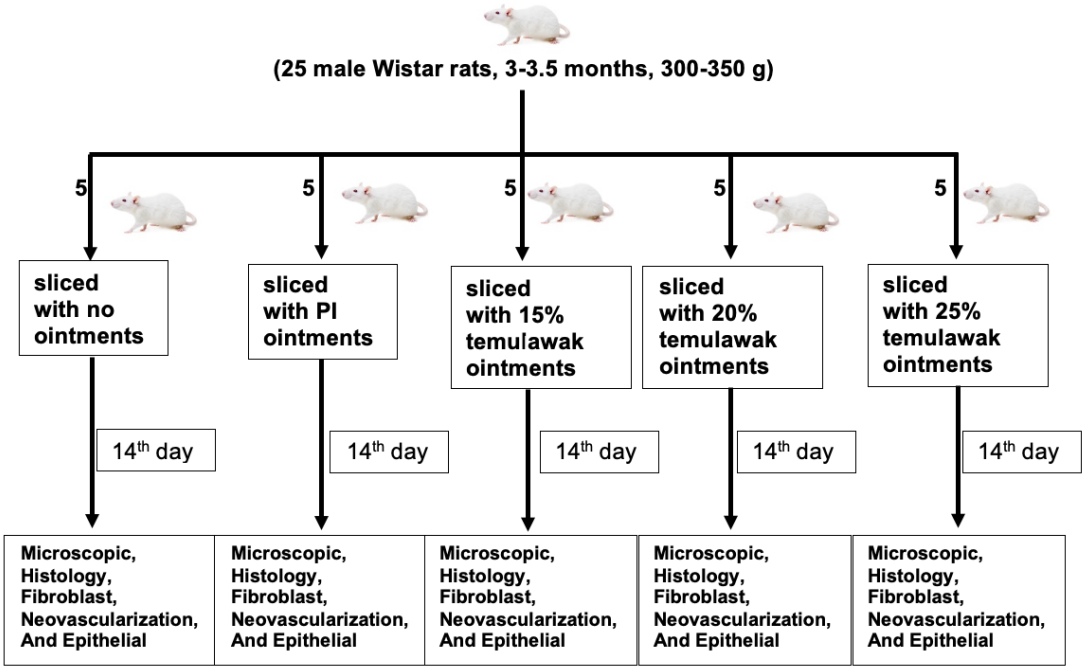

Fig.1 Scheme of animal experiments

After 14 days treatments, observations were carried out macroscopically and microscopically. The macroscopic observation parameters were Mb (red swelling), M (Red), Kt (dry open), and Km (dry closed, healed), and observe the length of the wound healing day between the control and the ginger extract ointment with each concentration. Microscopic observations were conducted by observing rats' skin on the 14th day. The histology preparations used the paraffin method. The number of fibroblast cells, neovascularization, and epidermal epithelial thickness was assessed by taking rat skin samples at the time of wound care and by *Hematoxylin-eosin* staining under a light microscope.

The normality test uses the *Shapiro-Wilk* test with α = 0.05 because the number of samples is less than 30. The data is normally distributed if the data shows the *p*-value > 0.05. *Levene Statistic test* used to determine homogeneity with α = 0.05. When the *p*-value > 0.05, the data is homogeneous and has the same variance, then a parametric test using one-way ANOVA is applied. If a *p*-value < 0.05 was obtained after statistical testing with one-way ANOVA, then there was a significant difference in the number of fibroblast cells, neovascularization, and epidermal thickness on day 14th. *Post-Hoc test* was applied to determine the most significant among the experimental groups with a *p*-value <0.05.

RESULTS AND DISCUSSION

The treatment groups with cuts and administration of temulawak ointments were 15%, 20%, and 25% showed no dead animals. There were no infected wounds in the experimental animals. All experimental animals were observed in a safe and clean environment and fed without distinction. During the surveillance period, the experimental animals were active, not in pain and suffering. Microscopic observations of fibroblast cells, neovascularization, and epidermal epithelial thickness on the 14th day were shown in Figure 2.

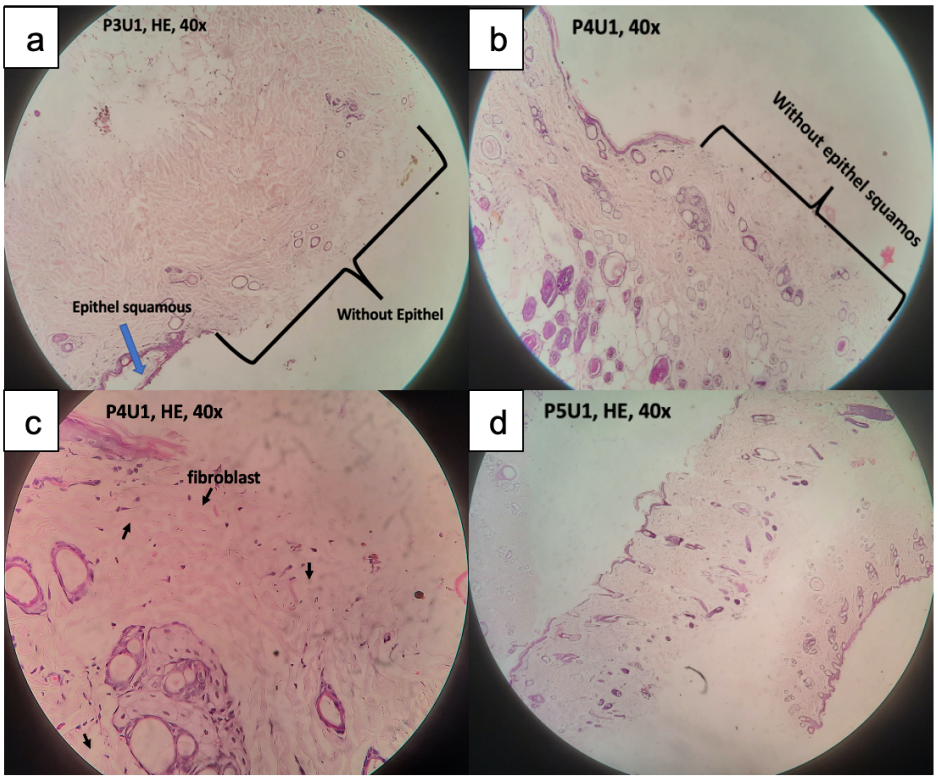

Fig. 2. Microscopic observation on the 14th d showed the epithel squamous (a), without epithel squamous (b) fibroblast cells (c), and neovascularization (d).

Several studies have examined the wound healing ability of temulawak extract regarding the macroscopic appearance of wounds. Previous studies have shown that wounds in experimental animals treated with temulawak extract experienced faster-wound healing or wound closure [15]. Temulawak was also reported to inhibit *methicillin-resistant staphilococcus aureus* (MRSA) [19]. The area of erythema in rats given 20% temulawak extract was much smaller than standard treatment with saline and vaseline, namely 81.87±8.01. The results of the one-way ANOVA test in this study showed a significant value (p = 0.000). A bioactive compound, namely *Xanthorrhizol*, which has bioactivity as antidiabetic, anti-inflammatory, and antioxidant that can counteract free radicals so that the inflammatory phase does not expand and the wound healing process improves better [19]. Another study also reported that temulawak extract could accelerate wound closure in white rats by 15,262% per-day compared to the group that was not treated [20]. Pieces of literature reveal that the treatment with antioxidants strengthens cellular antioxidant defense mechanisms, can reduce free radical-mediated damage and minimize tissue damage during burn injury, and can accelerate the wound healing process [21,22].

In the experimental group with normal saline (NS); 2 out of 5 rats (40%) had swollen red wounds (MB), 1 out of 5 (20%) had red sores (M), 2 out of 5 (40%)) had an open dry wound (KT). In the experimental group with povidone-iodine, 1 out of 5 rats (20%) had a swollen red wound (MB), and 4 out of 5 (80%) had an open dry wound (KT).

In the experimental group treated with 15% ointment; 1out of 5 mice (20%) had red sores (M), and 4 out of 5 (80%) had open dry results (KT). In the experimental group of animals that were treated with 20% ointment; 2 out of 5 (40%) had an open dry wound (KT), and the remaining 3 out of 5 (60%) had a closed dry wound (KM). In the group of experimental animals treated with 25% ointment; 3 out of 5 (60%) had an open dry (KT), and the remaining 2 out of 5 (40%) had a closed dry wound (KM). The *p-value* shows the result of 0.126 under

the *Fisher-Exact test,* as shown in Table 2, indicating no statistical significance between groups.

Table 2. Macroscopic Appearance of Wounds in Experimental Animals

Group Treatment	Macroscopic Appearance					*p-value*
	MB	M	KT	KM	Total	
NS	2 (40%)	1 (20%)	2 (40%)	0 (0%)	5 (100%)	
P. Iodine	1 (20%)	0 (0%)	4 (80%)	0 (0%)	5 (100%)	
S15%	0 (0%)	1 (20%)	4 (80%)	0 (0%)	5 (100%)	0.126
S20%	0 (0%)	0 (0%)	2 (40%)	3 (60%)	5 (100%)	
S25%	0 (0%)	0 (0%)	3 (60%)	2 (40%)	5 (100%)	

**Fisher-Exact test*

We further analyze the dry wound closure (KM) only found in the experimental group of animals treated with temulawak ointment, either with a concentration of 20% or 25%. The *Fisher-Exact test* showed that $p = 0.04$ indicated statistical significance, as shown in Table 3.

Table 3. Closed-Dry Macroscopic Appearance (KM) in Experimental Animals

Group Treatment	Dry Close (KM)			*p-value*
	Yes	No	Total	
NS	0 (0%)	5 (100%)	5 (100%)	
P. Iodine	0 (0%)	5 (100%)	5 (100%)	
S15%	0 (0%)	5 (100%)	5 (100%)	0.04*
S20%	3 (60%)	2 (40%)	5 (100%)	
S25%	2 (40%)	3 (60%)	5 (100%)	

**Fisher-Exact test*

There was no significant difference between the wound sizes in each group on day 14^{th,} as shown in Table 4. Significant differences were found in the other three microscopic parameters. Epidermal area and total area were calculated using IC Measure® software. Here, we compare the epidermis area against the total area of each experimental treatment.

Table 4. Comparison of Wound Size on Day 14th in Each Treatment Group

Treatment (n=25)	Mean ± SD (cm)	Min-Max	95%CI	*p-value*
NS	1.86±0.11	1.70-1.98	1.72-2.00	
P. Iodine	1.77±0.12	1.60-1.88	1.62-1.77	
S15%	1.76±0.39	1.70-1.80	1.71-1.81	0.91*
S20%	1.66±0.08	1.58-1.78	1.56-1.76	
S25%	1.68±0.17	1.40-1.85	1.68-1.90	

**One-way ANOVA*

The variable number of fibroblasts found that the highest mean number of fibroblasts was 65.20 ± 6.38, as shown in Table 5. The highest mean value was found in the experimental group of animals that received ointment treatment with

a concentration of 20% temulawak extract. The lowest mean number of fibroblasts was obtained from the experimental group that received normal saline, which was 55.40 ± 3.21. The smallest number of fibroblasts was found in the normal saline (NS) group. After performing the one-way ANOVA test, it was found that the *p-value* = 0.008 indicated a significant difference in the number of fibroblasts.

Table 5. Comparison of the number of fibroblasts in each treatment group

Treatment (n=25)	Mean ± SD	Min-Max	95%CI	*p-value*
NS	55.40 ± 3.21	52 – 60	51.42 – 59.38	
P. Iodine	58.60 ± 5.13	52 – 65	52.23 – 64.97	
S15%	61.60 ± 2.70	59 – 65	58.25 – 64.95	0.008*
S20%	65.20±6.38	58 – 73	57.28 – 73.12	
S25%	64.60±2.51	61 – 67	58.84 – 63.32	

**One-way ANOVA*

The highest mean value of neovascularization points was 39.00 ± 5.52, as shown in Table 6. It was found in the experimental group of animals that received ointment treatment with a concentration of 20% temulawak extract. The lowest mean neovascularization was obtained from the experimental group that received normal saline (NS), which was 31.80 ± 1.48. The distribution normality test was carried out using the *Shapiro–Wilk test*. It was found that the *p-value* > 0.05 means the data was normally distributed, so the multivariate analysis was then carried out using the one-way ANOVA test. It was found that the value of *p* = 0.003 indicated a significant difference in the amount of neovascularization.

Table 6. Comparison of Neovascularization Points in Each Treatment Group

Treatment (n=25)	Mean ± SD	Min-Max	95%CI	*p-value*
NS	31.80±1.48	30 – 34	29.96 – 33.64	
P. Iodine	34.80 ± 3.11	32 – 40	30.93 – 38.67	
S15%	30.60±3.36	27 – 35	26.43 – 34.77	0.003*
S20%	39.00 ± 5.52	30 – 45	32.14 – 45.86	
S25%	39.20±4.09	34 – 45	34.13 – 44.27	

**One-way ANOVA*

The highest mean area ratio of epithelialization area against total skin area was 23.90 ± 2.52 $mm^{2,}$ as shown in Table 7. The highest mean value was found in the experimental group of animals that received ointment treatment with 25% temulawak extract. The experimental animal group receiving normal saline obtained the lowest mean area ratio, 18.12 ± 1.13 mm^2. The distribution normality test was carried out using the *Shapiro–Wilk test*. It was found that the *p*-value > 0.05 means the data was normally distributed, so the multivariate analysis was carried out using the one-way ANOVA test. The *p*-value = 0.001 indicated a significant difference in the number of fibroblasts.

Table 7. Comparison of New Epithelialization Area with Total Skin Thickness

Treatment (n=25)	Mean ± SD	Min-Max	95%CI	*p-value*
NS	18.12 ± 1.13	16.88 – 19.67	16.71 – 19.52	
P. Iodine	18.31 ± 1.33	16.86 – 20.31	16.67 – 19.97	
S15%	19.78 ± 2.28	17.56 – 23.57	16.94 – 22.61	0.001*
S20%	21.88 ± 2.65	17.88 – 24.59	18.59 – 25.17	
S25%	23.90 ± 2.52	20.77 – 26.48	20.77 – 27.03	

**One-way ANOVA*

The homogeneity test showed a *p*-value > 0.05 for the three variables, so the *Tukey-LSD post-hoc test* was carried out. In the fibroblast, it was found that there were significant differences in the mean between the normal saline (NS), povidone-iodine treatment groups, S15%, S20%, and S25% treatment groups. There was a mean difference of -6,200 (p = 0.032) between NS and S15%; -9,800 (p = 0.002) between NS and S20%; -9,200 (p = 0.003) between NS and S25%; -6,600 p = 0.024) between povidone-iodine and S20%; and -6,000 (p = 0.038) between povidone-iodine and S25%.

In the neovascularization variable, it was found that there were significant differences in the mean between several variables. There was a mean difference of -7.20 (p = 0.007) between NS and S20%; -7.40 (p = 0.005) between NS and S25%; -8.40 (p = 0.002) between S15% and S20%; - 8.60 (p = 0.002) between S15% and S25%; and -6,000 (p = 0.038) between povidone-iodine and S25%.

In the comparison between the area of epithelialization and the total skin area, it was found that there were significant differences in the mean between several variables. There was a mean difference of -3.7640 (p = 0.01) between NS and S20%; -5.7880 (p = 0.00) between NS and S25%; -4.1260 (p=0.005) between NS and S25%; -3, 5620 (p = 0.014) between povidone-iodine and S20%; and -5.5860 (p = 0.0001) between povidone-iodine and S25%.

The wound healing process may be explained by the number of fibroblasts, neovascularization, and epithelialization. A study showed that a mouse model that had been intentionally injured, when treated with turmeric extract, experienced a much more significant increase in fibroblasts [23]. When a *Post-Hoc* analysis was performed, the mean difference between the control group and the topical curcumin extract group was found to be -20.11 (p = 0.021). Epithelialization has been reported in an animal model treated with 5% temulawak gel. It has produced the highest re-epithelialization among the other modeling groups (1.67 ± 0.36 mm). The 1% and 3% temulawak gels also produced good re-epithelialization compared to the control (1.49 ± 0.4 mm and 1.24 ± 0.47 mm, respectively) [19]. *Curcumin* has been reported to accelerate wound healing by triggering more TGF-β1 formation [23]. Elevated levels of TGF-β1 and growth factors in the region of a wound stimulate fibroblast proliferation and migration and induce extracellular matrix creation. The TGF-β1 can also trigger the proliferation of epithelial cells, thereby accelerating re-epithelialization [24].

However, *Curcumin* inhibits the production of *Tumor Necrosis Factor-alpha* (TNF-α) and Interleukin-1 (IL-1) as the two central cytokines released by monocytes and macrophages, thereby inducing an inflammatory response [24]. Inflammatory cells, such as polymorphonuclear cells, will reduce the wound area by inhibiting the production of proinflammatory cytokines, thereby shortening the length of the inflammatory phase and accelerating the wound healing process to enter the next healing phase, namely the proliferation phase with the formation of fibroblasts [25]. Angiogenesis will allow the delivery of oxygen and nutrients to the wound area resulting in suppression of ROS activity, facilitating local collagen synthesis and repelling processes. In this study, we found angiogenesis in the form of capillary dots or endothelium in the microscopy significantly increased, especially in the treatment group that received 20% and 25% extract. This is also in line with the previous studies that curcumin application caused markedly fast wound closure with well formed granulation tissue dominated by fibroblast proliferation, collagen deposition, and complete early regenerated epithelial layer [17].

CONCLUSION

Temulawak ointments was found to be effective for incision wound healing in non-diabetic Wistar rats during the 14th day of observation. The treated groups showed significant differences based on microscopic observation, wound

appearances, number of fibroblasts, neovascularization points, and epithelialization area compared to the untreated groups with normal saline and povidone-iodine. Based on literature studies, the wound healing activity was proposed due to the curcumin compound involved in the temulawak ointmentst, which may induce the TGF-β1 formation and inhibit the TNF-α and IL-1, respectively.

ACKNOWLEDGEMENT
We would like to thank Arlen H.J, head of the animal house laboratory of FMIPA USU Medan, for all the support regarding this study.

AUTHOR'S CONTRIBUTION STATEMENT
JP has conducted the experiment, data analysis, and wrote the manuscript. EG dan LC have supervised the experiment, and revised the manuscript.

FUNDING INFORMATION
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DATA AVAILABILITY STATEMENT
The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT
The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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