TRANSFORMING GROWTH FACTOR BETA 1 (TGF-BETA 1) IN VAGINAL INCISIONAL WOUND HEALING IN RATS: RESPONSES TO *PEGAGAN* (*CENTELLA ASIATICA*) LEAVES EXTRACT

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ABSTRACT:

Background: Of the many molecular substances known to assist in wound repair, the *Transforming Growth Factor Beta 1* molecule (TGF-Beta 1) is known to play a crucial role in various stages of wound healing. Recently, researchers found the role of TGF-Beta 1 acting as a regulator of vaginal tropoelatin production in the vaginal wall. In this study, we assessed the potency of TGF-Beta 1 expression in epithelial vaginal cells to help with increasing vaginal wall thickness and collagen production, as well as its responses to *Centella asiatica* leaves extract as a potential postmenopausal hormone therapy for vaginal atrophy healing in a rat model.

Methods: We explored the effect of *Centella asiatica* leaves extract to TGF-Beta 1 expression in epithelial vaginal cells of *Rattus norvegicus* strain Wistar. The extract was administered orally for 40 days after the animals declared experiencing atrophy in doses of 0, 30, 60, and 120 mg/Kbw/day. A modified Avidin-Biotin Complex (ABC) staining method was employed to observe the TGF-Beta 1 expression in the epithelial vaginal cells of the animal models. Non parametric tests were used to compare the mean difference of the observed parameters.

Results: The rise of percentage cells indicated that TGF-Beta 1 is not dependent on the dose of the *Centella asiatica* leaves extract. The positive trend in this observed parameter was assessed only at a dose group of 60 mg per Kbw. TGF-Beta 1 results in a positive trend to an increase in cell proliferation (rs = 0.075) and collagen production (rs = 0.142).

Conclusion: The findings of the study carry an expectation that *Centella asiatica* leaves extract has a prevailing medical potency in postmenopausal hormone therapy for vaginal incisional wound repair. However, considerations in applying the results to clinical practice in human should be underlined. Regardless of this limitation, the data suggests that a positive independent-concentration manner of *Centella asiatica* leaves extract may claim a mediation of normal wound healing in the vagina, and avoid recurrence of prolapse and symptoms in postmenopausal women.

Keywords: Vaginal incisional wound healing; Transforming growth factor beta 1 (TGF-Beta 1); Centella asiatica leaves extract; Postmenopausal hormone therapy

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INTRODUCTION

Recent study highlights that 25% of 470 million women over the world experience severe

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postmenopausal symptoms which seriously determine their quality of life [1]. Researchers revealed among the post-menopausal women the anatomic, hormonal, psychosocial and psychosexual changes escalate the individual prevalence of distressing sexual problems [2, 3]. The symptoms appeared as a result of estrogen level drop among the mid-aged women which leads to collagen deficiency and atrophy at vaginal cell wall [4, 5]. To highlight the results of estrogen scarcity, postmenopausal hormone therapy may stand as priority for treatment allaying the climacteric symptoms and complaints during sex, and prevent some chronic diseases, such as osteoporosis and colorectal cancer [6].

Vaginal atrophy is one of the potential postmenopausal problems that leads menopausal women to dyspareunia (coital pain) and vaginal dryness – resulted with less frequent sexual activity [7, 8]. Through a microscopic observation, atrophy can be characterized by lowered epithelial thickness and decreased vaginal maturation index [9]. Wound healing occurs through several overlapping processes and involves numerous growth factors in those events by exhibiting complex intercellular interactions [10].

Our previous work revealed that histological and physical observation of vaginal incisional wound healing in ovariectomised rats declares the potency of *Pegagan* (*Centella asiatica*) leaves extract to increase vaginal wall thickness via a complex mechanism of rising estrogen receptor beta and collagen in epithelial vaginal cells [11, 12]. *Centella asiatica* is an important medicinal herb that is found in most tropical and subtropical regions, called *Pegagan* in Indonesia. The primary active constituents of *Centella asiatica* are triterpene saponins, asiaticosides, and sapogenins which believed to be responsible for its wide therapeutic actions. Apart from wound healing, it is recommended for the treatment of various vascular effects such as uterorelaxant actions, keratinization, collagen formation, and angiogenesis [13]. However, information regarding to growth factors orchestrating a process of vaginal wall improvement after atrophy is still lacking.

Of many molecular substances known to mediate wound repair, *Transforming Growth Factor Beta 1* (TGF-Beta 1) plays widest actions at various stages of wound healing via cell proliferation, differentiation, mitigation, and migration, and also extra cellular matrix (ECM) production, and other biological functions in cells [14]. Recently, researchers stressed to its potency as a regulator of vaginal tropoelatin production in the vaginal wall [15], and its responsibility of increasing surgical vaginal wound closure rate in rabbit [16]. This study aimed to assess the roles of TGF-Beta 1 expression in epithelial vaginal cells to an increasing of vaginal wall thickness and collagen production, and its responses to *Centella asiatica* leaves extract as a potential postmenopausal hormone therapy for vaginal atrophy healing in a rat model.

METHODS

Study design

It was an experimental study with a *post-test only in control group design* to explore the effect of *Centella asiatica* leaves extract to TGF-Beta 1 expression in epithelial vaginal cells of *Rattus norvegicus* strain Wistar with seven replications. Twenty eight ovariectomized and healthy 4-month female rats weighting 290 – 300 gr were used as menopausal animal models. An increase of TGF-Beta 1 expression in the epithelial vaginal cells of ovariectomized rat models after given *Centella asiatica* leaves extract compared to the control group with no exposed to the extract would confirm the link of *Centella asiatica* leaves extract to the TGF-Beta 1 activation.

Rats were housed individually in cages in the animal care facility at Faculty of Veterinary Medicine, Airlangga University, Indonesia. We provided free access to water and standard rat chow to the animals and maintained a 12-h light–dark cycle during the study. Total samples were equally distributed to 4 experimental groups, consisted of 1) Group 1, no *Centella asiatica* leaves extract given; 2) Group 2, received dose of 30 mg/Kbw per day; 3) Group 3, obtained 60 mg extract/Kbw per day, and; 4) Group 4, treated dose of 120 mg/Kbw per day. *Centella asiatica* leaves extract was given orally to the experimental models for 40 days after the animals declared experiencing atrophy. From a preliminary study, focusing on parabasal epithelial cells of vaginal wall through a histological examination using haematoxylin eosin staining, we found that atrophy in experimental animals were observed on day 21 after ovariectomy [11, 12]. After 40-day diet with *Centella asiatica* leaves extract, the animals were sacrificed sequentially using ether in a transparent chamber; and then tissue of animals' vaginal walls were observed.

***Centella asiatica* leaves extract preparation**

Extraction method of *Centella asiatica* leaves was performed from our previous study [12]. The dried leaves were sieved to obtain the simplisia powder; and it was macerated with 60% (v/v) n-hexane:isopropyl alcohol and stirred during the process. The residue was later macerated with 96% ethanol. We condensed the ethanolic filtrate using a rotator. The denser filtrate was added with 1% HCl until pH 3 or 4. Then, chloroform was mixed to the filtrate with a composition of 1:3. Later, we had a partition of chloroform and water in the media. The water phase was dried using freeze drying method to obtain the final powder.

Ovariectomized rat models

We modified a most common surgical method by removing both ovaries on the female animals [17]. In this present study, abdominal skin and vagina were rubbed with antiseptic cream, contained cetrimide and chlorhexidine gluconate, and betadine solution. Animals were anesthetized using intramuscular injection with ketamine HCl (40 mg/Kbw). Transabdominal incision was performed above the uterus, approximately 1.5 to 2 cm, through the abdominal wall to parietal peritoneum. After found uterus, the surgery continued to the oviducts which joined with the ovaries to each uterine tube; and then we removed the distal oviduct and the ovaries. The opening was then pulled together to sew up the wound. Gentamicin (60-80 mg/Kbw/day) was injected to the animals for 3 days after wounding as postoperative therapy.

TGF-Beta 1 assay

A modified Avidin-Biotin Complex (ABC) staining method was employed to observe the TGF-Beta 1 expression in epithelial vaginal cells of the animal models. Multi clearing and rehydrating slices tissue of animals' vaginal wall initiated the staining process. These samples were washed with distillation water (DW) for 3 times subsequently before soaking the samples to 3% hydrogen peroxide (in DW) for 15 minutes at room temperature. After, the samples were washed consecutively with DW for 3 times and phosphate buffer saline (PBS; $NaH_2P0_4.2H_20$: $NaH_2P0_{41}.2H_20$: NaCl 0.45:3.23:8, pH 7.4) for 3 times; then, the samples were incubated in TGF-Beta 1 antibody monoclonal (as primary antibody) for 30 minutes in room temperature and washed with PBS for 3 times. The biotinylated secondary antibody was performed for second incubation (30 minutes, room temperature), and then rinsed with PBS for 3 times. The third incubation was done in ABC mixture (1:1 in 1 cc PBS) for 30 minutes at room temperature. Finally, we rinsed the samples with PBS for 3 times before the fourth incubation in diaminobenzidine solution (DAB; 2.5 gr of 3,3-diaminobenzidine tetrahydrochloride in 100 ml TBS, pH 7,6). Counter staining was performed using coomassie brilliant blue to count the number of epithelial cells which recognised as fibroblast – due to its ability to generate extra cellular matrix (ECM) and collagen, and stroma – under 400 times magnification in 10 different sections. Peroxide was added when applied DAB solution.

Statistics

We provided range, median, and standard deviation in this assessment; and the results were reported as mean ± standard error. Non parametric tests were used to compare the mean difference where p values < 0.05 were considered statistically significant. We also compared the data observed in this present study (% cells expressed TGF-Beta 1) with our previous results (thickness of vaginal wall and % cells expressed Collagen Type 1). Spearman test was performed to find correlation among two related parameters where p values < 0.05 exhibits the significant correlation between two parameters.

Ethical consideration

The treatment and animal handling procedures were approved by Animal Care and Committee of Faculty of Veterinary Medicine, Airlangga University, Indonesia, at May 1st, 2009, with reference number: 056-KE.

RESULTS

All rats survived the ovariectomy operation without complications. The twenty eight models were eligible for TGF-Beta 1 analysis (7 replications each dose group). The percentages of cell expressed TGF-Beta 1 are exhibited in Figure 1. With no treatment given, ovariectomy to the animal models followed with averagely 0.74% (Std. error at 0.18%) of epithelial cells at vaginal wall expressed TGF-Beta 1 while one of 7 replications was assessed with no cells, at 10 observation sides, expressed the subjected parameter. Providing *Centella asiatica* leaves extract orally to the menopausal rats at dose of 30, 60, or 120 mg per kg of body weight (Kbw) per day stimulated the epithelial cells to express TGF-Beta 1 at minimum of 0.60%. Data shows a link between an increase dose of *Centella asiatica* leaves extract given to the menopausal rats and percentages of cells expressed TGF-Beta 1. When

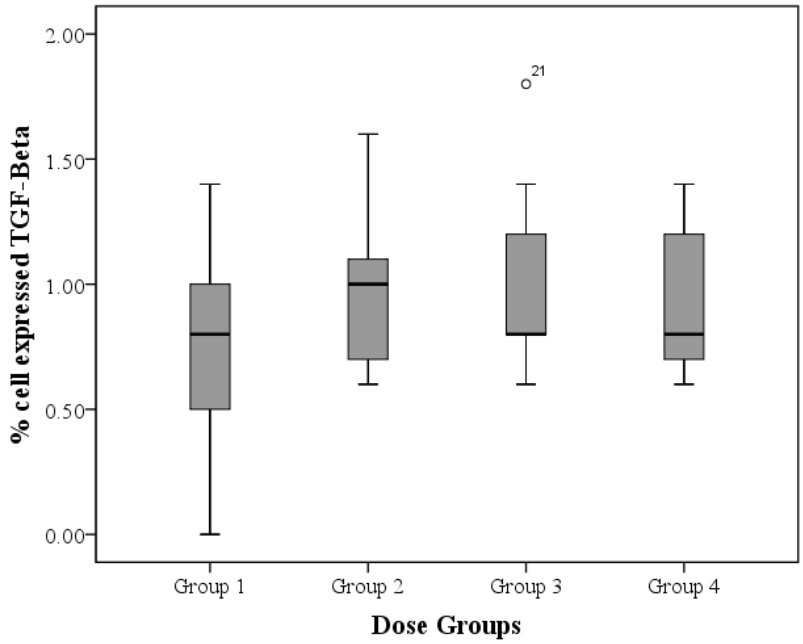

Figure 1 Percentage of cell expressed TGF-Beta 1 among the four experimental dose groups: 1) Group 1 (0 mg/Kbw); 2) Group 2 (30 mg/Kbw); 3) Group 3 (60 mg/Kbw); 4) Group 4 (120 mg/Kbw)

Table 1 Effects of *Pegagan* (*Centella asiatica*) leaves extract among the four experimental dose groups

Parameters	Group 1 (N=7)	Group 2 (N=7)	Group 3 (N=7)	Group 4 (N=7)	*p* value
Thickness of vaginal wall (µm) [11]					
Range	9.40 - 11.00	16.40-20.00	15.80-33.00	22.00-36.20	**
Mean ± Std. error	10.29±0.22[1)3)4)]	18.23±0.45[1)5)6)]	25.94±2.15[3)5)]	30.03±2.11[4)6)]	
Median	10.60	18.20	27.60	29.80	
Std. Deviation	0.59	1.19	5.68	5.58	
% cells expressed collagen type 1 [11]					
Range	0.80-1.60	0.80-2.00	1.60-2.00	2.40-3.40	**
Mean ± Std. error	1.14±0.11[3)4)]	1.11±0.16[5)6)]	1.77±0.07[3)5)7)]	2.83±0.14[4)6)7)]	
Median	1.2000	1.00	1.80	2.60	
Std. Deviation	0.30	0.43	0.18	0.37	
% cells expressed TGF-Beta 1					
Range	0-1.40	0.60-1.60	0.60-1.40	0.60-1.40	#
Mean ± Std. error	0.74±0.18	0.97±0.13	1.03±0.16	0.94±0.12	
Median	0.80	1.00	0.80	0.80	
Std. Deviation	0.49	0.35	0.42	0.32	

From Kruskal Wallis Test: #) No significant difference; *) Significant at *p value* < 0.01; **) Significant at *p value* < 0.001. From Mann-Whitney Test: 1) Significant at *p value* < 0.01 in Group 1 and 2; 2) Significant at *p value* < 0.05 in Group 1 and 3; 3) Significant at *p value* < 0.01 in Group 1 and 3; 4) Significant at value < 0.01 in Group 1 and 4; 5) Significant at *p value* < 0.05 in Group 2 and 3; 6) Significant at *p value* < 0.01 in Group 2 and 4

Centella asiatica leaves extract applied to animals' diet at dose of 30 and 60 mg/Kbw, we observed the number of cells expressed the protein higher from 1.40%, respectively, to 1.60 and 1.80%. However the positive trend in this observed parameter was assessed only at dose group of 60 mg per Kbw, and it changed at dose group of 120 mg per Kbw.

We compared the present result with data from previous works [11] in Table 1 and Table 2, regarding to the effects of *Centella asiatica* leaves extract for vaginal atrophy healing in a rat model. Mean differences among the four experimental groups were analyzed by Kruskal Wallis test whilst the comparison between two experimental groups

Table 2 Spearman correlation coefficient (rs) in the observed parameters (N=28)

	% cells expressed TGF-Beta 1	% cells expressed collagen type 1
Thickness of vaginal wall	0.075	0.687**
% cells expressed Collagen Type 1	0.142	

** Correlation is significant at the 0.01 level (2-tailed).

was done through a Mann-Whitney Test. We revealed an increasing dose of *Centella asiatica* leaves extract to dose of 120 mg/Kbw per day delivered significant improvement to the thickness of vaginal wall and % cells expressed collagen type 1. However, the rise of the % cells expressed TGF-Beta 1 is not dependent on dose of *Centella asiatica* leaves extract. Neither mean difference of the % cells expressed TGF-Beta 1 among the four experimental groups nor between two experimental groups noticed a significant difference.

The correlations among the three observed parameters were presented in Table 2. No adverse effect was observed in these parameters. TGF-Beta 1 results a positive trend to an increasing of cell proliferation (rs = 0.075) and collagen production (rs = 0.142). By Spearman test, we found a positive correlation between the % cells expressed collagen type 1 and the thickness of vaginal wall (*p* value < 0.001).

DISCUSSION

TGF-Beta 1 is a complex protein responsible for proliferation, cell differentiation, cell migration, extra cellular matrix (ECM) production, and other biological functions in cells [14]. Researchers have acknowledged that TGF-Beta 1 receptor plays important role on fibroblast transcription and collagen synthesis in healing process [18, 19]. Our last work revealed how this mechanism rolled for vaginal atrophy healing in rat model [11]. In this present study, the observation focused on the roles of *Transforming Growth Factor β 1* (TGF-Beta 1) expression in epithelial vaginal cells to an increasing of vaginal wall thickness. A research in 2012 confirmed that TGF-Beta 1 gene expression increased significantly during vaginal wound healing in a rabbit menopause model [20].

Scholars stressed the potency of active components on *Pegagan* (*Centella asiatica*) leaves as an alternative healing agent through an induction to collagen and cell proliferation [11, 19, 21, 22]. Data from this present work provides more information orchestrating benefits of *Centella asiatica* leaves extract to vaginal wall improvement after atrophy. From the results, we revealed *Centella asiatica* leaves extract evokes expression of TGF-Beta 1 protein at the epithelial cells of the vaginal wall. This data suggests *Centella asiatica* leaves extract responses a prevailing medical potency in postmenopausal hormone therapy for vaginal atrophy healing. By Spearman test, we found a positive correlation between the % cells expressed collagen type 1 and the thickness of vaginal wall (*p* value < 0.001). The data supports the role of *Centella asiatica* leaves extract to higher % cell expressed TGF-Beta 1 linking to improvement of collagen deficiency and atrophy at vaginal cell wall among the menopausal rats. Triterpenoid components of *Centella asiatica* leaves titrated extract activate angiogenesis and stimulate human fibroblast in wound healing [23]. Through TGF-Beta receptor 1 kinase (TbetaRI kinase), asiaticoside, in *Centella asiatica* leaves extract, induces synthesis of collagen type 1 [18]. The statistical analysis in this work exhibits that TGF-Beta 1 plays larger magnification to the collagen type 1 production than cell generation in vaginal wounding healing. It is important since a drop in quantity of collagen I in pelvis during menopause significantly weaken the tensile strength and escalate the susceptibility to anterior vaginal wall prolapse [24].

The findings of this study can be used as a foundation for future study in developing evidence-based surgical and hormone replacement therapy using *Pegagan* (*Centella asiatica*) leaves as a natural medication for treatment of vaginal incisional wound repair. Nevertheless, future research should cover the efficacy of *Centella asiatica* leaves extract for vaginal incisional wound healing in different solvents (hexane, ethyl acetate, methanol, water) and various formulations (ointment, cream, and gel). Previous study revealed that wound healing activities of *Centella asiatica* leaves extract in skin incision and burn exhibited that the asiatic acid facilitates the wound healing process and tends to be more active in ethyl acetate extract [25]; and also the healing was more remarkable with the gel product [26].

Although the present study showed a promising result, precaution should be taken when it's intended

for human application. As well as previous similar studies on rats [16, 20], the present one also did not include an analysis of the histology of the wound. Yet, the type of wound healing may be associated with a more intensive inflammatory reaction, more fibrin and necrotic debris, and incomplete reconstitution of the original tissue architecture. However, the positive independent-concentration manner of *Centella asiatica* leaves extract in the present study may claim a mediation of wound healing in the vagina, and perhaps lower a recurrence of prolapse in postmenopausal woman.

CONCLUSION

This present study remarks the level of *Transforming Growth Factor β 1* (TGF-Beta 1) higher in vaginal incisional wound healing process. Orchestrating a process of vaginal wall improvement after atrophy, TGF-Beta 1 results a positive trend to an increasing of cell proliferation and collagen production. As an alternative healing agent, *Centella asiatica* leaves extract evokes expression of TGF-Beta 1 protein at the epithelial cells of the vaginal wall. The rise of % cells expressed TGF-Beta 1 is not dependent on dose of *Centella asiatica* leaves extract. The positive trend in this observed parameter was assessed only at dose group of 60 mg per Kbw. The findings of the study carry an expectation that *Centella asiatica* leaves extract has a prevailing medical potency in postmenopausal hormone therapy for vaginal incisional wound repair. Besides a claim of wound healing in the vagina, *Centella asiatica* leaves extract perhaps is lower a recurrence of prolapse in postmenopausal woman.

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