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Experimental Analysis of Vaginal Laxity in Rats Treated With a Combination of Er:YAG Fractional Lasers and AMSC-MP

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Abstract

Introduction: Vaginal laxity, a symptom of pelvic floor dysfunction observed in women, has many negative biological and psychological impacts. Laser treatments and stem cell-based therapies are emerging therapeutic methods for treating this condition. This study aimed to determine changes in vaginal laxity in model rats using a combination therapy of erbium-doped yttrium aluminium garnet (Er:YAG) fractional lasers and topical treatment with amniotic membrane stem cell metabolite products (AMSC-MP).

Methods: The experimental animal population comprised 36 female white rats (*Rattus norvegicus*; 2-day-post-vaginal-delivery rats) allocated into the following four groups (n=9): K1, untreated two-day-post-vaginal-delivery rats; K2, two-day-post-vaginal-delivery rats treated with topical gel without AMSC-MP; P1, two-day-post-vaginal-delivery rats treated with Er:YAG fractional lasers and topical gel without AMSC-MP; P2, two-day-post-vaginal-delivery rats treated with Er: YAG fractional lasers and topical gel containing AMSC-MP. Immunohistochemical (IHC) examination was carried out for the expression and activity of heat shock protein 70 (HSP-70), collagen-1, tissue inhibitors of metalloproteinase 1 (TIMP-1) and matrix metalloproteinase 1 (MMP-1), as well as vaginal mucosal thickness.

Results: There was a significant difference ($P<0.05$) in the expression of HSP-70 among all groups except K2 and P1 ($P>0.05$); there was no significant difference in type I collagen and TIMP-1 expression between the groups ($P>0.05$); there was a significant difference ($P<0.05$) in MMP-1 activity, with the activity in the K2 group (5.79 ± 0.83) being higher than that in the P1 group (4.44 ± 1.82) and that in the K1 group (5.74 ± 1.03) being higher than that in the P2 group (4.24 ± 1.55). Also, there was a significant difference in the thickness of the vaginal mucosa in all groups except K2 and P1 ($P>0.05$).

Conclusion: Er:YAG fractional laser and AMSC-MP combination therapy improved vaginal laxity in model rats by increasing Hsp70 expression and vaginal mucosal thickness and decreasing MMP-1 activity.

Keywords: Vaginal laxity; Er:YAG fractional laser; AMSC-MP; Collagen; Reproductive health.

Introduction

Vaginal laxity, a symptom of pelvic floor dysfunction observed in women, especially after childbirth, increases with age.[1] Its biological and psychological impacts include reduced self-confidence, feeling unable to satisfy one's partner (sexually), loss of vaginal sensation, and decreased sexual satisfaction.[1] It is self-reported by 38% of women with symptoms of postpartum pelvic prolapse, stress urinary incontinence (SUI), overactive bladder, reduced vaginal sensation, and worsening sexual life.[2]

Therapies for vaginal laxity include invasive surgical procedures, behavioral/habitual therapies, hormone therapy, and pharmacotherapy with firming creams or sprays.[3] Despite the high satisfaction rate, surgical procedures involve considerable risks associated with vaginal scarring, nerve damage, and decreased sensory function. Additionally, the long recovery period associated with surgical procedures poses significant difficulties for patients, prompting researchers to devise treatments for improving vaginal laxity with minimally

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invasive procedures that are safe and effective and have a short recovery period.[4-6]

The erbium-doped yttrium aluminium garnet (Er:YAG) fractional laser with a wavelength of 2940 nm is one of the therapeutic methods for treating vaginal laxity. It induces neocollagenesis and vascularization to restore elasticity and moisture to the vaginal mucosa. The mechanism underlying its effect is associated with those of therapeutic methods using fractional ablative lasers known as photothermolysis: microscopic areas of necrosis are generated in the tissue, leading to the induction of wound healing, with the formation of new collagen and elastin fibres.[6] The Er:YAG fractional laser treatment is suitable for vaginal mucosa due to its appropriate penetration depth, resulting in a shorter healing time and almost no side effects. This can be attributed to the Er:YAG laser having a higher water absorption coefficient than carbon dioxide (CO_2) lasers.[7]

Histologic changes after ablative skin resurfacing with a CO_2 laser or an Er:YAG laser include neocollagenesis 4–6 weeks post-treatment. Reverse-transcriptase polymerase chain reaction and immunohistochemical (IHC) evaluation of the facial skin of 28 patients after CO_2 laser-induced resurfacing showed the upregulation of procollagens 1 and 3, interleukin (IL)1-β, tumor necrosis factor-α, transforming growth factor (TGF)-β1, and matrix metalloproteinases (MMPs).[8,9] The MMPs involved in this process, including collagenase (MMP-1 and MMP-3) and gelatinase (MMP-9), reportedly increase after CO_2 laser resurfacing therapy.[10] TGF-ß1 induces increased expression of collagen type I, MMP-1, and other cell cycle regulatory proteins in fibroblasts. Skin fibroblasts reportedly secrete increased amounts of collagen type I and alter gene expression in response to treatment.[11]

Stem cell-based therapies have been widely used in various medical fields, including skin tissue engineering and wound healing.[12] Amniotic membrane stem cells (AMSCs) can be obtained from the placenta post-delivery. The amniotic membrane exhibits low differentiation and immune reactivity, and thus it is hypoallergenic. Metabolite products of AMSCs (AMSC-MP) produced during culture include cytokines and growth factors. The anti-inflammatory cytokines secreted by AMSCs include prostaglandin E2, indoleamine 2,3 dioxygenase, hepatocyte growth factor, and TGF-β. AMSCs promote angiogenesis by the secretion of several angiogenic factors such as angiogenin, vascular endothelial growth factor, and platelet-derived growth factor. PM-AMSCs harbor growth hormones such as TGF-β, epidermal growth factor, essential fibroblast growth factor, and keratinocyte growth factor that function as anti-aging agents. TGF-β is a multifunctional growth factor that is essential in modulating cellular behaviour.[13-15]

Combined therapy with topical AMSC-MP and Er:YAG fractional lasers is expected to synergistically improve treatment for vaginal laxity. AMSC-MP treatment after ablative fractional laser treatment has been used in skin rejuvenation therapy and laser-assisted drug delivery (LADD). The use of laser treatment before topical therapy can increase permeability and facilitate deeper penetration of topical drugs and other molecules. Hydrophilic molecules with molecular weights greater than 500 Da do not easily penetrate the stratum corneum, and most growth factors have molecular weights greater than 20 kDa. Therefore, herein, we performed LADD using the Er:YAG fractional laser, which facilitated the penetration of topical AMSC-MP into the vaginal mucosa.

This study aimed to determine the improvement in vaginal laxity in vaginal laxity model rats using a combination therapy of Er:YAG fractional laser and AMSC-MP topical treatment. The analysis of heat shock protein 70 (Hsp70), collagen type I, tissue inhibitors of matrix metalloproteinase 1 (TIMP-1), and MMP-1 expression and activity as well as vaginal mucosa thickness corresponding to the improvement in vaginal laxity is presented herein. Using Er:YAG fractional laser therapy without any complementary treatment for vaginal laxity has yielded unsatisfactory results based on the data. Thus, combining PM-AMSC topical treatment with Er:YAG fractional laser therapy is expected to be a more efficient method for treating vaginal laxity.

Materials and Methods

Animals, Experimental Design, and Tissue Collection

A post-test-only control group design was implemented herein. The experimental groups comprised three-month-old, two-day-post-vaginal-delivery female Wistar strain rats (*Rattus norvegicus)* (n = 36) weighing 140–250 g each. The rats were categorized into four treatment groups (n = 9 individuals per group) as follows: Control group 1 (K1) comprised untreated two-day-post-vaginal-delivery rats that were terminated on day two after vaginal delivery; control group 2 (K2) contained two-day-post-vaginal-delivery rats that were treated with topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; treatment group 1 (P1) comprised two-day-post-vaginal-delivery rats that were treated with Er:YAG fractional laser (Table 1) exposure and topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; treatment group 2 (P2) rats were treated with Er: YAG fractional laser exposure and topically administered AMSC-MP gel on day two after vaginal delivery and terminated on day 21 post-treatment. Immediately post-termination, the vaginal tissue of each rat was fixed in 10% buffered formalin for histological and IHC analyses.

Table 1. Laser Parameters

Parameters	Specification
Type of laser	Laser Erbium Yttrium Alumunium Garnet (Er: YAG) 2940 nm ⟶ Laser Intimalase® XS Dynamis, Fotona, Slovenia
Emission mode	Fractional scanning
Time on/Time off (Fluence range)	250 ms
Delivery system	A collimated beam enables the precise delivery of laser energy
Energy distribution	3 J
Peak power	20 W
Average power	-
Spot diameter at the focus	2-7 mm
Focus spot area	7 mm
Spot diameter at the tissue	7 mm
Focus-to-tissue	Vaginal mucosa
Spot area to	Area dorsal of the vagina
Peak power density at spot area	3 J/cm^2
Peak power density at the tissue	3 J/cm^2
Average power density at spot area	0.1-95 J/cm^2
Beam divergence	Minimal
Water irrigation	No
Air and aspirating airflow	No

Mating and Pregnant Female Rats

Female rats were injected with pregnant mare serum gonadotropin (PMSG) for synchronization of the estrus cycle and human chorionic gonadotropin (HCG) for superovulation. PMSG (10 IU) was administered intraperitoneally, followed by HCG (10 IU) after 48 hours. After the HCG injection, the rats were monogamously mated with male rats. The occurrence of copulation in mating rats was confirmed by the presence of vaginal plugs that form approximately 17 hours after copulation and consist of clotted gelatin, which prevents leakage of spermatozoa. The time of observation of the vaginal plug was designated as day zero of pregnancy.

AMSC-MP Manufacturing Procedure

The AMSC culture medium that was 80% confluent was aspirated and poured into a 50 mL conical tube and centrifuged at 3000 rpm for 10 minutes. The supernatant was aspirated and poured into a 100 mL beaker. The dialysis tubing was pretreated by soaking in a phosphate-buffered saline (PBS) solution. The AMSC-MP supernatant was poured into the dialysis tubing membrane, which was then tied tightly and immersed in a cold PBS solution. The PBS solution was then subjected to mixing by rotation on a magnetic stirrer at 750 rpm and incubated for 24 hours at a constant temperature that enabled it to remain cold. On observing a color change in the PBS solution, the end of the dialysis tubing membrane was cut to extract the AMSC-MP solution, which was then re-centrifuged to extract the supernatant containing PM-AMSC. Sodium alginate was added to the prepared AMSC-MP solution to convert it to gel form, consisting of 98% AMSC-MP and 3% sodium alginate. The supernatant obtained after centrifugation was filtered and placed in a 15 mL conical tube, packaged in sterile Medipack pouches, and stored at cold temperature. AMSC-MP was prepared in the laboratory of Tissue Bank and Regenerative Medicine at Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, in liquid form, and then it was converted to gel form at the Pharmacy Installation laboratory of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.

Administration of Er:YAG Fractional Laser Treatment and AMSC-MP Topical Gel

Experimental rats were subjected to general anesthesia and disinfection of the dorsal vaginal canal with betadine fluid using a cotton swab, followed by exposure to the Er:YAG fractional laser (IntimaLase® XS Dynamis, Fotona, Slovenia) using a human vaginal introitus laser probe. The rat vagina was opened using two tweezers and irradiated on the dorsal vaginal mucosa by firing the laser (energy density, 3 J/cm^2; spot size, 7 mm; frequency, 1.6 Hz; the number of passes, 1; pulse duration, 250 ms). AMSC-MP gel (0.1 mL) was topically administered to the rats in the P2 group after the completion of the laser treatment. A syringe (1 cc) was used to smear the AMSC-MP gel on the dorsal vaginal mucosa of the rats along the laser site (Figure 1).

Vaginal Sampling

The entire vagina was surgically removed immediately after disinfection with 70% alcohol to prevent scattering the hair. The surgical procedure was initiated by sacrificing experimental animals using total anesthesia (0.3 mL/100 g body weight) comprising ketamine (2 mL), xylazine (1.25 mL), acepromazine (0.33 mL), and saline (6.41 mL). The excised vaginal tissue was then split and fixed in 10% formalin. The selected tissue samples were then subjected to IHC and histopathological examinations.

Immunohistochemical Analysis

IHC staining was performed to detect Hsp70, MMP-1, TIMP-1, and collagen type I. The following antibodies were used for IHC: anti-HSP 70 mouse monoclonal antibody (3A3) (sc-32239, Santa Cruz Biotechnology, USA), anti-MMP-1 antibody (HRP) (orb479375, Biorbyt Ltd., United Kingdom), anti-TIMP1 antibody (ab61224 Abcam, UK), and anti-COL1A1 antibody (GTX82721 GeneTex Inc, North America).

Vaginal tissue was sliced to 5 μm thickness, deparaffinized, and immersed in xylol for 5 minutes thrice, followed by immersion in alcohol solutions of

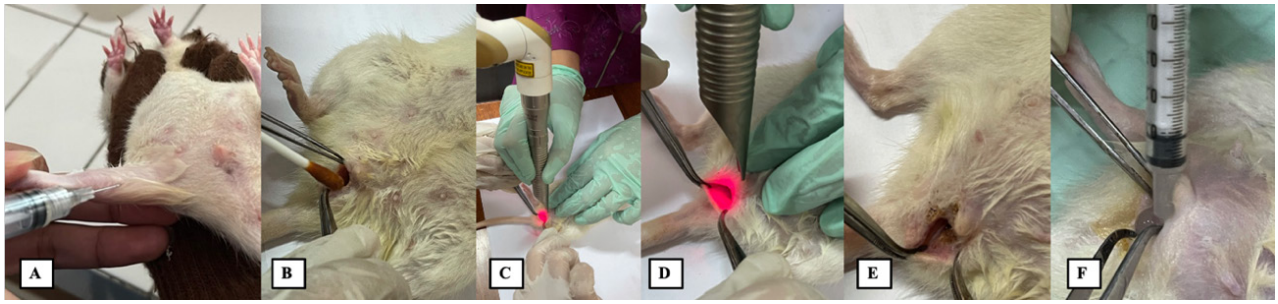

Figure 1. (A) General anesthesia used on experimental rats; (B) Vaginal disinfection of experimental rats with betadine solution using a cotton swab; (C) Administration of Er:YAG fractional laser therapy using a human vaginal introitus probe; (D) Firing of the Er:YAG fractional laser at the dorsal area of the vaginal mucosa of experimental rats; (E) Condition of the vaginal mucosa of experimental rats shortly after Er:YAG fractional laser treatment; (F) Administration of 0.1 cc AMSC-MP gel using a 1 mL injection syringe (without a needle)

varying concentrations (100%, 95%, 80%, 70%, and 30% alcohol). Each immersion was for 5 minutes, followed by washing with PBS (pH 7.4) for 5 minutes thrice. The samples were soaked in 3% hydrogen peroxide for 5–10 minutes and PBS containing 1% bovine serum albumin for 10–30 minutes at room temperature. The addition of the primary antibody was followed by incubation for 1 hour at room temperature and washing with PBS (pH 7.4) for 5 minutes thrice. Streptavidin-horseradish peroxidase was added, followed by incubation for 30–60 minutes at room temperature and washing with PBS (pH 7.4) for 5 minutes (repeated thrice). Next, 3,3′-diaminobenzidine tetrahydrochloride was added, followed by incubation for 10–20 minutes. Another three washes of 5-minute duration each were performed with distilled water at room temperature, followed by counterstaining with an aceto-orcein solution for 3 minutes. The samples were mounted with Entellan® rapid mounting medium for microscopic observations at magnifications of 40x, 100x, and 400x using an Olympus DP12 Digital Camera. The protein expression level corresponded to the excess brownish discoloration observed in the treated vaginal tissue compared to that in the control.

The protein expression in each sample was assessed semi-quantitatively based on the modified Remmele method, where the Remmele scale index, also referred to as the immunoreactive score (IRS),[16] is defined as the product of the percentage score of immunoreactive cells or areas and the color intensity score of immunoreactive cells or regions. The data for each sample are the mean of IRS values observed at five different visual fields at 400x magnification.

The scoring of the Remmele scale index is defined as follows: Positive cell percentage score: score 0, no positive cells; score 1, positive cells < 10%; score 2, positive cells between 11% and 50%; score 3, positive cells between 51% and 80%; and score 4: positive cells > 80%. Color reaction intensity score: score 0, no color reaction; score 1, low color intensity; score 2, medium color intensity; and score 3, strong color intensity.[16]

Histopathological Examination of Vaginal Mucosa Thickness

Examination of the thickness of the vaginal mucosa was performed using hematoxylin and eosin (H&E) staining. The 5 µm vaginal mucosal tissue slices were deparaffinized on a glass surface. The tissue slices were immersed in xylol for 2 minutes thrice and then dipped in 100% ethanol for 1 minute thrice, followed by immersion in 95% ethanol for 1 minute twice and then in 90%, 80%, and 70% ethanol for 1 minute each. After the ethanol immersions, the samples were washed with water for 5 minutes, placed in hematoxylin for 6 minutes, rinsed with water, dipped in acid alcohol 3–5 times, and again rinsed with water. After rinsing, the tissues were immersed in liquid ammonia, in a solution of eosin and 95% ethanol, and in xylol, twice each, before observation under a microscope. The thickness of the vaginal mucosal epithelium was measured using Image Raster 3 software at ten times the field of view and 200x magnification.

Statistical Analysis

Data between treatment groups were analyzed using one-way ANOVA for normally distributed data with homogeneous variance between groups and the Kruskal-Wallis test for data that were not normally distributed. For comparisons showing a significant difference between the groups, further analysis was performed using the multiple comparisons least significant difference (LSD) test or the Mann-Whitney test to identify pairs of different groups. To determine the mechanism involved in improving vaginal laxity based on the observed variables, an analysis of the relationship between the variables was performed using path analysis.

Results

Analysis of Hsp70 Expression With Er:YAG Fractional Laser and AMSC-MP Treatment

The P1 treatment group had a lower average Hsp70 expression (5.41 ± 1.19) than the P2 treatment group (6.59 ± 0.94). These results demonstrate that the expression of Hsp70 in vaginal laxity model rats treated with the combined treatment of Er:YAG fractional laser and

topical AMSC-MP was higher than that in those treated with the Er:YAG fractional laser without the topical AMSC-MP treatment.

The normality test showed that the Hsp70 expression data for each group were normally distributed ($P>0.05$), and the variance of data between the groups was homogeneous ($P>0.05$). One-way ANOVA results showed a minimal significant difference between the pairs of groups ($P<0.05$). Further analysis using the multiple comparisons LSD test showed significant differences between all pairs of groups ($P<0.05$), excluding the pair comprising groups K2 and P1 ($P>0.05$) (Table 2).

Figure 2 shows IHC results indicating relative amounts of Hsp70 expression. The observed staining was used to calculate the IRS score, which corresponded to the results of the statistical analysis.

Analysis of Collagen Type I Expression and Activity With Er:YAG Fractional Laser and AMSC-MP Treatment

The P2 treatment group (7.12 ± 1.39) showed higher average collagen type I expression than the P1 treatment group (6.89 ± 1.81). These results demonstrate that the expression of collagen type I in vaginal laxity model rats treated with the combination treatment of Er:YAG fractional laser and topical AMSC-MP was higher than that in those treated with the Er:YAG fractional laser without the topical AMSC-MP treatment. The combined Er:YAG fractional laser and topical AMSC-MP treatment also resulted in more collagen type I expression compared with levels in control groups K1 and K2.

The normality test yielded statistically significant values for each group ($P>0.05$), indicating that normal collagen type I expression data distribution was associated with each treatment. The analysis of the homogeneity of variance also yielded significant values ($P>0.05$), indicating that the variance of the collagen type I expression data among all treatments was homogeneous.

One-way ANOVA yielded a P value of 0.799 ($P>0.05$), indicating no significant differences in collagen type I expression between the treatment groups (Table 3).

Figure 3 shows IHC results indicating the relative amounts of collagen type I expression. The observed staining was used to calculate the IRS score, which corresponded to the results of the statistical analysis.

Analysis of MMP-1 Expression and Activity With Er:YAG Fractional Laser and AMSC-MP Treatment

MMP-1 activity in the P2 treatment group (4.24 ± 1.55) was lower than that in the P1 treatment group (4.44 ± 1.82). These results demonstrate that the activity of MMP-1 in vaginal laxity model rats treated with the combination treatment of Er:YAG fractional laser and topical AMSC-MP was lower than that in those treated with the Er:YAG fractional laser without the topical AMSC-MP treatment.

The normality test yielded statistically significant values for each treatment group ($P>0.05$), indicating that the MMP-1 activity data for each treatment group were normally distributed. The analysis of the homogeneity of variance also yielded significant values ($P>0.05$), indicating that the variance of MMP-1 activity data among the groups was homogeneous.

The one-way ANOVA yielded significant differences in minimal MMP-1 activity between the pairs of treatment groups ($P<0.05$). Further analysis using multiple comparisons of LSD showed a significant difference in MMP-1 activity between groups K2 and P1 and between groups K1 and P2 ($P<0.05$), indicating that the treatments significantly decreased MMP-1 activity in the treatment groups as compared to that in the control groups (Table 4).

Figure 4 shows IHC results indicating the relative amounts of MMP-1 activity. The observed staining was used to calculate the IRS score, which corresponded to the results of the statistical analysis results.

Table 2. Differentiation Test of Hsp70 Expression in Each Group

Group	n	HSP-70 (IRS Score)		*P* Value
		Mean ± SD	Min-Max	
K1	9	4.53 ± 0.75[a]	3.30-5.40	0.000*
K2	9	5.59 ± 0.56[b]	4.70-6.30	
P1	9	5.41 ± 1.19[b]	3.20-6.90	
P2	9	6.59 ± 0.94[c]	5.50-8.40	

* Significance level $\alpha=0.05$ (One-way ANOVA), $P<0.05$; Superscripts letters indicate significant differences between groups (multiple comparisons least significant difference (LSD).
IRS, immunoreactive score; SD, standard deviation; n (number of samples); K1, two-day-post vaginal delivery rats; K2, two-day-post vaginal delivery rats treated with topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P1, two-day-post vaginal delivery rats treated with the Er:YAG fractional laser and topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P2, rats treated with the Er: YAG fractional laser and AMSC-MP gel on day two after vaginal delivery and terminated on day 21 post-treatment.

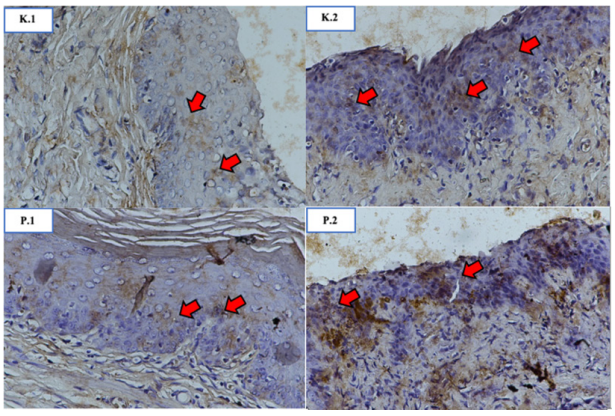

Figure 2. Comparison of Hsp70 Expression in the Vaginal Epithelium of the Rats Belonging to the Four Groups (upper panel: control groups K1 and K2, lower panel: treatment groups P1 and P2). The red arrows mark the presence of Hsp70 expression in the vaginal epithelium indicated by the presence of brown chromogenic color (IHC, 400×)

Table 3. Differentiation Test of Collagen Type I Expression in Each Group

Group	n	Collagen Type I (IRS Score)		*P* Value
		Mean ± SD	Min-Max	
K1	9	6.41 ± 1.67	3.00-8.20	0.799
K2	9	6.54 ± 1.80	3.90-9.00	
P1	9	6.89 ± 1.81	4.50-9.00	
P2	9	7.12 ± 1.39	5.60-9.00	

Significant at $P<0.05$.
IRS, immunoreactive score; SD, standard deviation; n (number of samples); K1, two-day-post vaginal delivery rats; K2, two-day-post vaginal delivery rats treated with topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P1, two-day-post vaginal delivery rats treated with the Er:YAG fractional laser and topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P2, rats treated with the Er: YAG fractional laser and AMSC-MP gel on day two after vaginal delivery and terminated on day 21 post-treatment.

Table 4. Differentiation Test of MMP-1 Activity in Each Group

Group	n	MMP-1 (IRS Score)		*P* Value
		Mean ± SD	Min-Max	
K1	9	5.74 ± 1.03^{bc}	4.50-7.50	0.033*
K2	9	5.79 ± 0.83^{c}	4.70-7.20	
P1	9	4.44 ± 1.82^{ab}	1.40-6.90	
P2	9	4.24 ± 1.55^{a}	1.60-6.00	

* Significance level $\alpha = 0.05$ (One-way ANOVA), $P<0.05$; Superscripts letters indicate significant differences between groups (multiple comparisons least significant difference (LSD).
IRS, immunoreactive score; SD, standard deviation; n (number of samples); K1, two-day-post vaginal delivery rats; K2, two-day-post vaginal delivery rats treated with topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P1, two-day-post vaginal delivery rats treated with the Er:YAG fractional laser and topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P2, rats treated with the Er: YAG fractional laser and AMSC-MP gel on day two after vaginal delivery and terminated on day 21 post-treatment.

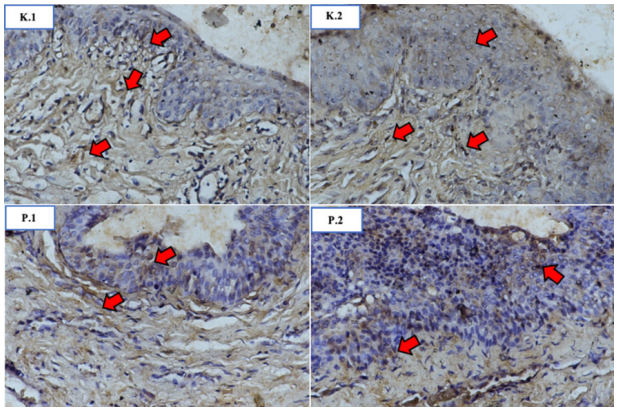

Figure 3. Comparison of Collagen Type I Expression in the Vaginal Epithelium and Lamina Propria of the Rats Belonging to the Four Groups (upper panel: control groups K1 and K2, lower panel: treatment groups P1 and P2). The red arrows mark the collagen type I expression in the vaginal epithelium and lamina propria indicated by the brown chromogenic color (IHC, 400×)

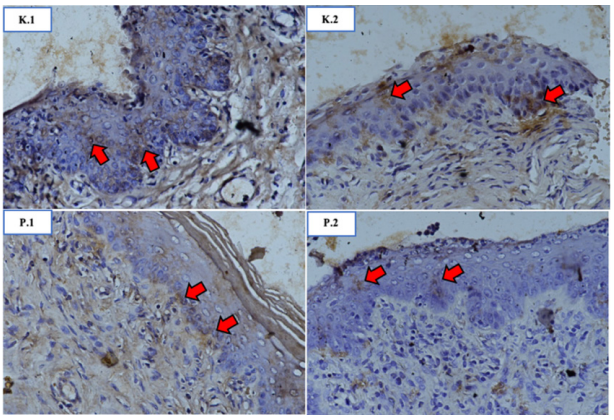

Figure 4. Comparison of MMP-1 Activity in the Vaginal Epithelium of the Rats Belonging to the Four Groups (upper panel: control groups K1 and K2, lower panel: treatment groups P1 and P2). The red arrows mark the presence of MMP-1 activity in the vaginal epithelium, indicated by the brown chromogenic color (IHC, 400×)

Analysis of TIMP-1 Expression With Er:YAG Fractional Laser and AMSC-MP Treatment

The average expression of TIMP-1 in the P2 treatment group (5.22 ± 1.64) was higher than that in the P1 treatment group (5.03 ± 1.39). These results demonstrate that the expression of TIMP-1 in vaginal laxity model rats treated with the combination treatment of Er:YAG fractional laser and topical AMSC-MP was higher than that in those treated with Er:YAG fractional laser without the topical AMSC-MP treatment.

The normality test yielded statistically significant values for each treatment group ($P>0.05$), indicating that the TIMP-1 expression data for each group were normally distributed. The analysis of the homogeneity of variance yielded significant values ($P>0.05$), indicating that the variance of the TIMP-1 expression data among treatments was homogeneous.

One-way ANOVA showed no significant differences in TIMP-1 expression between the treatment groups ($P>0.05$) (Table 5).

Figure 5 shows IHC results indicating the relative amounts of TIMP-1 expression. The observed staining was used to calculate the IRS score, which corresponded to the statistical analysis results.

Analysis of Vaginal Mucosa Thickness With Er:YAG Fractional Laser and AMSC-MP Treatment

The vaginal mucosal thickness in rats of the P2 treatment group (751.62 ± 84.13) was greater than that of those in the P1 treatment group (626.53 ± 413.08). These results demonstrate that the vaginal mucosal thickness of vaginal laxity model rats treated with the combination therapy of Er:YAG fractional laser and topical AMSC-MP was higher than that of the rats treated with the Er:YAG fractional laser without the topical AMSC-MP treatment and was also considerably higher than that of the rats in both control groups, K1 and K2. This indicated that the treatment had a significant effect on vaginal mucosal thickness.

Table 5. Differentiation test of TIMP-1 Expression in Each Group

Group	n	TIMP-1 (IRS Score)		*P* Value
		Mean ± SD	Min-Max	
K1	9	4.87 ± 1.09	2.70-6.00	0.951
K2	9	4.98 ± 1.07	2.80-6.00	
P1	9	5.03 ± 1.39	2.60-7.50	
P2	9	5.22 ± 1.64	2.90-7.50	

Significant at $P < 0.05$.
IRS, immunoreactive score; SD, standard deviation; n (number of samples); K1, two-day-post-vaginal delivery rats; K2, two-day-post-vaginal delivery rats treated with topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P1, two-day-post-vaginal delivery rats treated with the Er:YAG fractional laser and topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P2, rats treated with the Er:YAG fractional laser and AMSC-MP gel on day two after vaginal delivery and terminated on day 21 post-treatment.

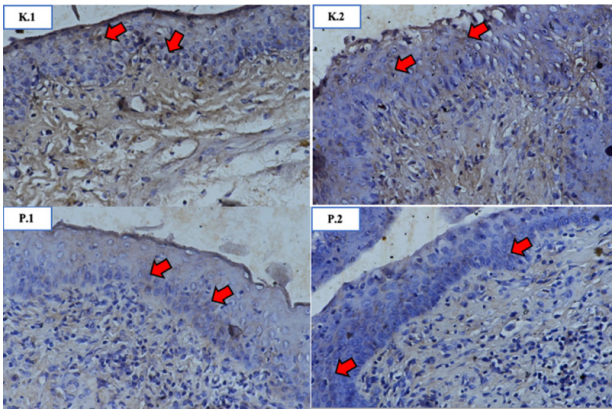

Figure 5. Comparison of TIMP-1 Expression in the Vaginal Epithelium of the Rats Belonging to the Four Groups (upper panel: control groups K1 and K2, lower panel: treatment groups P1 and P2). The red arrows mark the presence of TIMP-1 expression in the vaginal epithelium, indicated by the brown chromogenic color (IHC, 400×)

Table 6. Differentiation Test of Vaginal Mucosa Thickness in Each Group

Group	n	Vaginal Mucosa Thickness (μm)			*P* Value
		Mean ± SD	Median	Min-Max	
K1	9	284.48 ± 69.56	260.84[a]	194.94-411.62	0.000*
K2	9	405.45 ± 88.46	383.00[b]	318.03-604.32	
P1	9	626.53 ± 413.08	426.66[b]	295.23-1465.47	
P2	9	751.62 ± 84.13	740.53[c]	613.34 – 878.36	

* Significance level $\alpha = 0.05$ (Kruskal-Wallis test); Superscripts letters indicate significant differences between groups (Mann-Whitney test).
IRS, immunoreactive score; SD, standard deviation; n (number of samples); K1, two-day-post-vaginal delivery rats; K2, two-day-post-vaginal delivery rats treated with topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P1, two-day- post-vaginal delivery rats treated with the Er:YAG fractional laser and topical gel without AMSC-MP on day two after vaginal delivery and terminated on day 21 post-treatment; P2, rats treated with the Er: YAG fractional laser and AMSC-MP gel on day two after vaginal delivery and terminated on day 21 post-treatment.

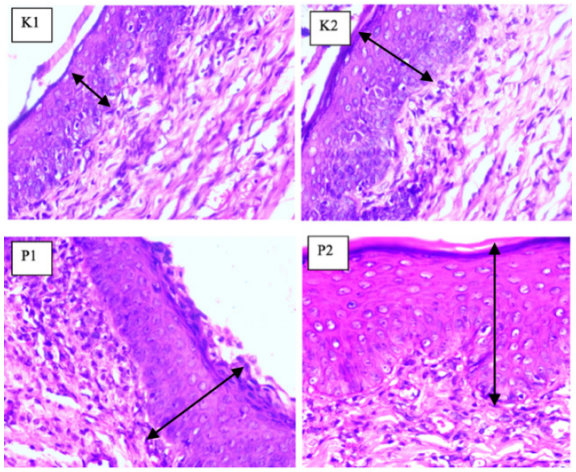

Figure 6. Comparison of the Vaginal Mucosal Thickness of the Rats in Each Group (upper panel: control groups K1 and K2, lower panel: treatment groups P1 and P2) (Hematoxylin-Eosin: 400×Magnification)

The normality test showed that the vaginal mucosal thickness data of the K2 and P1 treatment groups were not normally distributed ($P < 0.05$). Further analysis using the Mann-Whitney test showed significant differences in vaginal mucosal thickness for all pairs of groups ($P < 0.05$), excluding the pair comprising groups K2 and P1 ($P > 0.05$). The Kruskal-Wallis test showed a minimal significant difference between the two treatment groups (Table 6).

Figure 6 depicts the histopathological examination results, showing greater relative vaginal mucosal thickness in rats in the treated groups than in those in the control groups.

Analysis of the Relationship Between Variables

The path analysis results showed that the combination of Er:YAG fractional laser and topical AMSC-MP treatment directly affected the thickness of the vaginal mucosa (B = 0.904; $P < 0.05$), where the combined treatment increased the thickness of the vaginal mucosa. Other variables that were directly affected by the combined treatment were Hsp70 expression (B = 0.566; $P < 0.05$) and MMP-1 activity (B = -0.558; $P < 0.05$). The combined treatment increased the expression of Hsp70 and decreased that of MMP-1. Hsp70 and MMP-1 had no direct or indirect influence on vaginal mucosal thickness. Other variables showing significant correlations were TGF-β and IL-1 (B = 0.382; $P < 0.05$). However, neither of these variables was directly or indirectly related to the thickness of the vaginal mucosa.

Discussion

Hsp70 expression in the K2 group (5.59 ± 0.56) was higher than that in the P1 group (5.41 ± 1.19), but the values were not significantly different ($P > 0.05$). This observation may be attributed to damage induced by the high temperature of the laser to the cellular mechanisms involved in producing HSPs, thus resulting in a decline in Hsp70 expression.[17] Hsp70 expression in the P1 group (5.41 ± 1.19) was significantly higher than that in the K1 group (4.53 ± 0.75) ($P < 0.05$). This observation may be due to the P1 group receiving Er:YAG fractional

laser therapy, which could have led to thermal damage, consequently resulting in Hsp70 expression during the repair process. This is consistent with a study by Kwon et al. who demonstrated a significant increase in Hsp70 expression in the vaginal mucosa of pigs treated with the CO_2 fractional laser on day 0 and day 30 after vaginal delivery compared to that in untreated pigs.[18] Increased Hsp70 expression occurs after laser therapy due to the heat shock response experienced by cells. This is a characteristic response of cells undergoing changes in their microenvironment, such as those induced by the high temperatures of the laser beams utilized in our study. We observed that Hsp70 continued to be expressed up to 21 days post-treatment.

There was no significant difference in collagen I expression ($P > 0.05$) in all the groups. This is possibly due to the fact that vaginal collagen after vaginal delivery (in the control group) increases physiologically, and this is consistent with the research by Daucher et al who reported an increase in collagen in virgin rats 3 weeks after giving birth.[19]

To the best of our knowledge, there are no previous reports on MMP-1 expression in the vaginal mucosa at 21 days postpartum. Physiologically, at 21 days postpartum, the vagina is still in a state of laxity, where an increase in MMP-1 activity is expected. This was corroborated by our finding that there was no significant difference in MMP-1 activity in control groups K1 and K2. The significant difference observed between groups K1 and P2 indicates that the combined treatment with the Er:YAG fractional laser and topical AMSC-MP decreased MMP-1 expression and activity significantly on day 21, which corresponds to the remodeling phase of wound healing, which is associated with a decrease in MMP-1 activity. AMSC-MP is also expected to affect MMP-1/TIMP-1 regulation during the remodeling process. The significant difference observed between groups K1 and P2 indicates that MMP-1 activity decreased due to the photothermal action of the ablative laser treatment, which causes tissue damage, leading to wound healing. Gantsetseg et al compared MMP-1 expression in nulliparous and postpartum rats and found an increase in MMP-1 at 1 day postpartum.[20] Herein, the decrease in MMP-1 and the increase in TIMP-1 levels observed in groups P1 and P2 compared to levels in groups K1 and K2 led us to infer that the decrease in MMP-1 is associated with a reduction in tissue degradation and a subsequent increase in collagen and elastin content.

Further, at two days postpartum, vaginal laxity model rats experienced vaginal laxity, with decreased TIMP-1 expression and increased MMP-1 activity. The Er:YAG fractional laser treatment caused photodamage to the vaginal mucosal surface via a photothermal effect, thereby triggering wound healing. In the remodeling phase of the wound healing process, TIMP activity gradually increased, accompanied by decreased MMP-1 activity and accumulation of a new collagen matrix.[21]

Our findings demonstrated a significant difference in the average thickness of the vaginal mucosa ($P < 0.05$) between different pairs of treatment groups, excluding the pair comprising groups K2 and P1 ($P > 0.05$). This is consistent with the observed increase in collagen on day 21 post-vaginal delivery, which is also in agreement with the results of a study that showed an increase in collagen in 3-week-postpartum rats to a level that approaches that observed in the vagina of virgin rats.[22] A systematic review stated that Er:YAG laser treatment can cause an increase in epithelial thickness, inflammatory response, fibroblast proliferation, and vascularization, as well as an increase in the amount of collagen.[23] Herein, the topical AMSC-MP gel treatment combined with laser therapy significantly increased vaginal mucosal thickness compared to that observed in treatment by fractional laser therapy without AMSC-MP treatment. This agrees with the results of Gaspar et al. who analyzed atrophic or hypotrophic vaginal mucosa in patients with SUI and rectovaginal fascia damage using platelet-rich plasma controls and fractional CO_2 laser treatment.[24]

The termination of rats on day 21 post-treatment led to our results being representative of physiological activity in the remodeling phase in this period. The non-significance of TIMP-1 and collagen type 1 expression could be explained by day 21 post-treatment being the beginning of the remodeling phase. Alperin et al proved the presence of vaginal distensibility in experimental rats by comparing virgin rats, pregnant rats, and 4-week-post-vaginal-delivery rats. Vaginal distensibility increased in pregnant rats compared to that in nulliparous virgin rats ($P < 0.001$), and vaginal laxity occurred in 4-week-postpartum rats and did not recover to virgin levels ($P < 0.001$), so it was concluded that the increase in vaginal distensibility at the end of the postpartum period was a continuous change due to vaginal biomechanical factors related to pregnancy and vaginal delivery.[25]

The combination therapy of Er:YAG fractional laser and AMSC-MP increased vaginal mucosal thickness and also affected Hsp70 expression as well as MMP-1 activity. However, this increase in Hsp70 expression and the decrease in MMP-1 activity did not affect vaginal mucosal thickness. The increase in the thickness of the vaginal mucosa observed herein was not caused by an increase in collagen type I resulting from an increase in Hsp70 expression and a decrease in MMP-1 activity. It was possibly due to other factors, such as an increase in collagen type III, collagen type V, glycoprotein, hyaluronan, and proteoglycans. Although collagen type V may not be considered necessary in the remodeling process since it produces small fibers with low tensile strength, the copolymerization of collagen types I, III, and V leads to the formation of fibrils that affect tissue

biomechanics.[26]

Conclusion

This study presents the novel combination therapy of Er:YAG fractional laser and topical AMSC-MP for vaginal laxity. Our results show that this combined treatment increases vaginal mucosal thickness concomitantly with a correlated increase in Hsp70 expression and a decrease in MMP-1 activity. However, these changes related to Hsp70 and MMP1 did not directly affect the thickness of the vaginal mucosa. Additionally, the analysis of TIMP-1 and collagen type I expression in this combination therapy did not demonstrate any direct effect of these proteins on vaginal mucosal thickness in vaginal laxity model rats on day 21 post-treatment.

One limitation of this study was that the expression of collagen types III and V, glycoproteins, hyaluronan, and proteoglycans was not examined. Further, the termination time of 21 days post-treatment led to the results only being representative of the remodeling phase.

In conclusion, we present the combination therapy of Er:YAG fractional lasers and topical AMSC-MP as a potential new treatment method for vaginal laxity, as it demonstrated increased vaginal mucosal thickness and Hsp70 expression along with decreased MMP1 activity in vaginal laxity model rats. Further studies regarding the efficacy of this method in humans are still warranted.

Competing Interests

The authors declare no conflict of interest.

Ethical Approval

All procedures were performed following the guidelines for animal experiments provided by the Clinical and Ethical Committee, Veterinary Faculty, Universitas Airlangga, Surabaya (Certificate number: No: 2.KE.116.12.2020, December 22, 2020), and the Ethical Committee of the Dr Soetomo General Academic Hospital, Surabaya, Indonesia (Certificate number: No: 0258/LOE/301.4.2/XII/2020).

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