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Effect of Vitamin C Exposure to Fibroblast Cells on Woman Uterosacral Ligament Culture

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Abstract: Pelvic Organ Prolapse (POP) occurs when the uterosacral ligaments weaken. Vitamin C plays a major role in forming the extracellular matrix (collagen) and stimulates fibroblast cell proliferation. This study aimed to examine how a woman's uterosacral ligament culture was affected by vitamin C exposure to fibroblast cells. A straightforward experimental design investigation that is non-blind and includes analysis after exposure (Post-test control group design). This study's sample used uterosacral ligament from a woman who underwent at least two vaginal deliveries through a hysterectomy procedure. Several doses of vitamin C were given and repeated for 72 hours, followed by examining the number of fibroblast cells; then, the data was analyzed starting with the parametric prerequisite test and the one-way ANOVA test. There was a significant effect of vitamin C exposure on fibroblast cells of the uterosacral ligament at a dose of 50 µg/ml and 100 µg/ml. In contrast, at doses of 150 µg/ml and 200 µg/ml, there was no significant effect. From this study, it is hoped that the administration of vitamin C according to the optimal dose found in the study (significant impact at doses of 50 µg/ml and 100 µg/ml, can increase the proliferation of fibroblast matrix supporting the sacrouterine ligament in women, to prevent prolapse of the pelvic organs in women, and can maintain the quality of life of women.
Keywords: Fibroblast; pelvic organ prolapse; uterosacral ligament culture; vitamin C.

INTRODUCTION

The uterosacral ligament consists of a collection of collagen fibers, smooth muscle, elastin and nerve fibers collected as the main uterine supporting structure and upper vagina. In pelvic Organ Prolapse (POP), the uterosacral ligament weakens (Su et al., 2021). This problem has occurred in many women, but few studies still provide information about molecular mechanisms in POP (Connell et al., 2009).

In the previous study, Jackson et al. reported their hypothesis on supporting tissue and pelvic binding and tried to explain the molecular mechanism of POP(Jin et al., 2020). The extracellular matrix, which contains components like collagen, elastin, and stromal cells made by fibroblasts, and which is frequently altered in POP patients, is shown in recent investigations of women with POP to be responsible for several abnormalities in supporting tissues (Adnyana et al., 2018).

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Fibroblast is a cell that is most commonly found in connective tissue that is responsible for the synthesis of extracellular matrix components consisting of fibers and basic materials (Junqueira & Carneiro, 2005). Connective tissue fibers consist of collagen, reticular, and elastic fibers (Mubarak et al., 2023). Fibroblast cells divide relatively slowly in normal tissue but proliferate rapidly in response to tissue injury. This proliferation process is generally influenced by growth factors produced by platelets during injury (Junqueira & Carneiro, 2005).

Fibroblast is the first cell that was successfully cultured in vitro. According to Prowse et al., in vitro, cultures of fibroblast cells can synthesize about 175 types of protein (Hutabarat et al., 2020). Some of these proteins are important in maintaining the pluripotency of human embryonic stem cells. Some proteins produced by fibroblast also play a role as growth factors regulated by chemokines and cytokines; Vascular endothelial growth factor (VEGF), connective tissue growth factor, platelet-derived growth factor (PDGF), and transforming growth factor beta (CTGF),interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) which is expression determines the results of the wound healing process(Mohammed et al., 2016).

The role of vitamin C on fibroblasts is known, where fibroblasts are cells that synthesize collagen from proline and lysine, then hydroxylated with vitamin C involvement (Sugiritama, 2023). Vitamin C is very important in activating the prolyl hydroxylase enzyme, which converts proline residues in collagen to hydroxyproline (Carr & Maggini, 2017). Without hydroxyproline, collagen polypeptides will not form a triple helix structure under physiological conditions, and collagen fibers will not be produced in the extracellular space. The lysyl hydroxylase enzyme plays a role in the hydroxylation process of lysine to hydroxylysine and requires vitamin C as a cofactor. Hydroxilisin plays a role as an attachment place for sugar residues and important for forming cross-links that stabilize the extracellular collagen matrix (Ronchetti et al., 1996).

Several experimental studies that have been conducted on exposure to vitamin C with certain doses on human fibroblast cell cultures have been directly shown to increase the proliferation of fibroblasts, collagen, elastin and other extracellular matrix compilers, but study the effect of vitamin C exposure on fibroblast uterosacral ligaments itself has not yet been done, this is the base for researchers to conduct this research. This study aimed to determine the effect of exposure to vitamin C on cultured fibroblast cells' sacrouterine ligament in women. This research is also expected to be developed for further research related to the prevention of POP.

MATERIALS AND METHODS

This was a straightforward non-blinded experiment with analysis following treatment (post-test control group design). The study was designed in vitro using uterosacral ligament fibroblast cell cultures from women who underwent hysterectomy (Total Abdominal Hysterectomy).

Patients underwent surgery, a total abdominal hysterectomy procedure. The sample was taken from ± one-third center of the uterosacral ligament. It was washed in the cold containing liquid Ringer Lactate 1,2 and 3 for 1 minute, then put in a tube containing growth media/The tube has the sample transport media Dulbecco's Modified Eagle Medium (DMEM) transport. Media got labeled according to the specimen (name, register number, and sample number) on the tube at a temperature of 20-25^{o}C and delivered to the Biomedical Laboratory of the Faculty of Medicine, University of Brawijaya Malang.

The tissue sample was divided into the smallest part using tissue/scissors. Washed the uterosacral ligament tissue sample in buffer solution (pH ~ 7.4) commonly used in biological research. It is a water-based salt solution containing disodium hydrogen phosphate, sodium chloride and, in some formulations, potassium chloride and potassium dihydrogen phosphate. The buffer helps to maintain a constant pH. The osmolarity and ion concentrations of the solutions match those of the human body (isotonic) Phosphate-buffered saline (PBS) by gently vibrating in a 50-ml polypropylene tube until free of erythrocytes. Transfer the sample to a 15 ml centrifuge tube containing 0.9% collagenase type 1 (in 5 ml serum-free medium). Incubate at 37ºC overnight (18-20 hours).

Then the media was added to 5 ml Complete Medium suspended—centrifuge 800 rpm or 300 g for 8 minutes. The supernatant was discarded, and the pellet was resuspended with 1 ml Complete Medium DMEM using a sterile pipette. Implanted in a tissue culture flask of 25 cm^2 and added 8 ml of Complete Medium-DMEM was then incubated for 2-3 days with a 5% CO_2 incubator at a temperature of 37ºC. The old medium was removed and washed with Serum Free-DMEM 2 times, and each of the 2 ml samples was added 5 ml of Complete Medium-DMEM. Incubate with 5% CO_2 in an incubator with a temperature of 37ºC to confluent for approximately seven days by replacing the media every three days.

Confluent uterosacral ligament fibroblast cell culture in the growth medium was divided into the 96 tissue culture grade plate with 24 replications (4 controls and 20 treatments) incubated at 37ºC for 24 hours, then added a new vitamin C supplement was added to the culture daily, as ascorbic acid was found to be unstable in the culture medium when incubated at 37ºC, within 24 hours 88% to 98% of the ascorbate had either disappeared or had been oxidized.at a dose of 10 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, and 200 µg/ml, and four culture plates were not exposed to vitamin C as a control. The proliferation index of fibroblast cells was examined by 3-[4,5-dimethylthiazole-2-yl]-2,5- a colorimetric examination system that measures the absorbance of tetrazolium components that undergo reduction to formazan products in the mitochondria of metabolically active cells. This reduction process is influenced by the activity of dehydrogenase enzymes, such as NADH, NADPH and GDPH. This method widely used to examine cell viability and proliferation is called the diphenyl tetrazolium bromide (MTT) method.

Proliferation index

The proliferation index was measured by calorimetric examination with a microplate reader, which is called the method of MTT proliferation index. It uses a microplate reader at a wavelength of 570 nm, where a linear relationship was obtained between the number of proliferating cells and the absorbance of formazan. This method measured formazan's absorbance, which is produced by proliferative cells. The higher absorbance value means more formazan is made, indicating a growing number of proliferating cells. Ethical clearance was obtained from the Health Research Ethics Committee of Saiful Anwar General Hospital.

Statistical Analysis

Data are analyzed using a one-way ANOVA test followed by the Tukey test to identify group differences. Post hoc test was used if the ANOVA was significant. $P < 0,05$ was considered statistically significant.

RESULTS AND DISCUSSION

The proliferation index in fibroblast cells can be seen in Table 1 and Figure 1. Table 1 shows the mean data of the proliferation index according to varying doses of vitamin C.

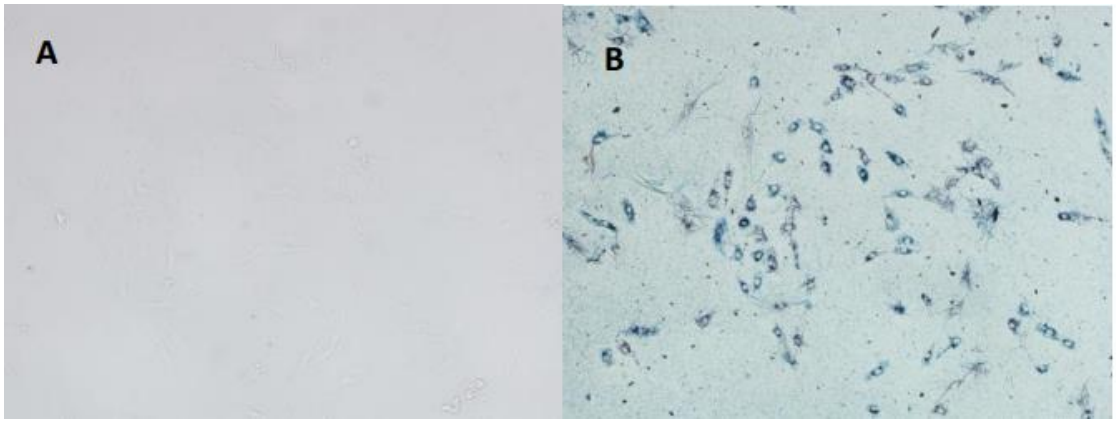

Figure 1. Observation Microscope Magnification 100 X at; (A) Control Fibroblast Cells, (B) MTT Method

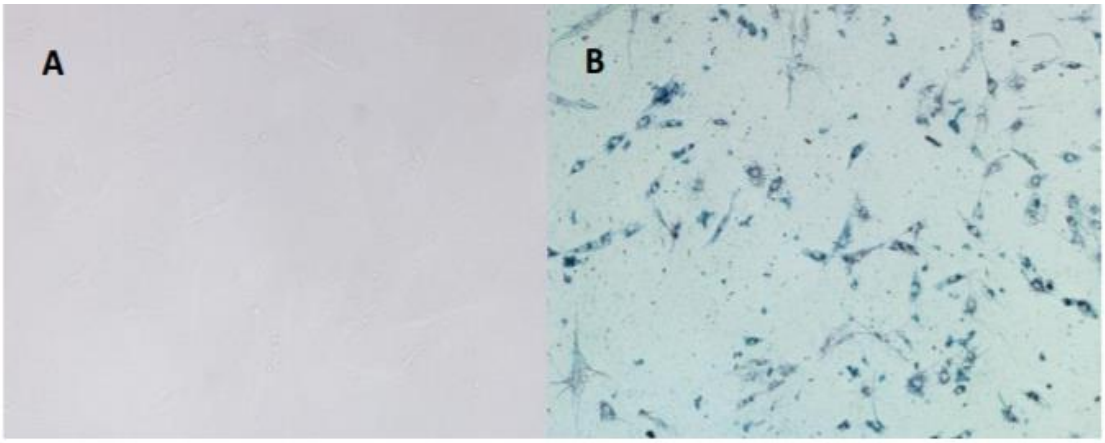

Figure 2. Observation Microscope Magnification 100 X at; (A) Fibroblast Cells are Exposed Vitamin C 10 µg/ml, (B) MTT Method

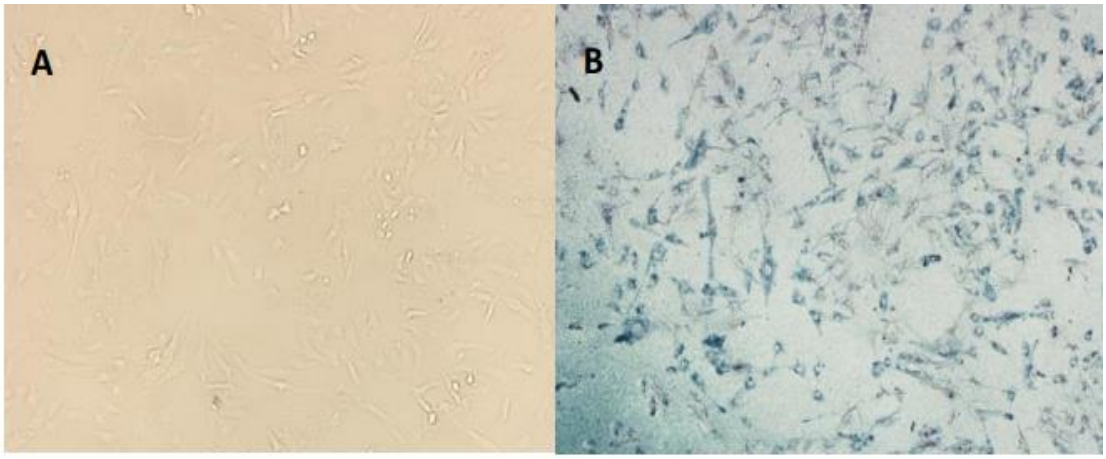

Figure 3. Observation Microscope Magnification 100 X at; (A) Fibroblast Cells are Exposed Vitamin C 50 µg/ml, (B) MTT Method

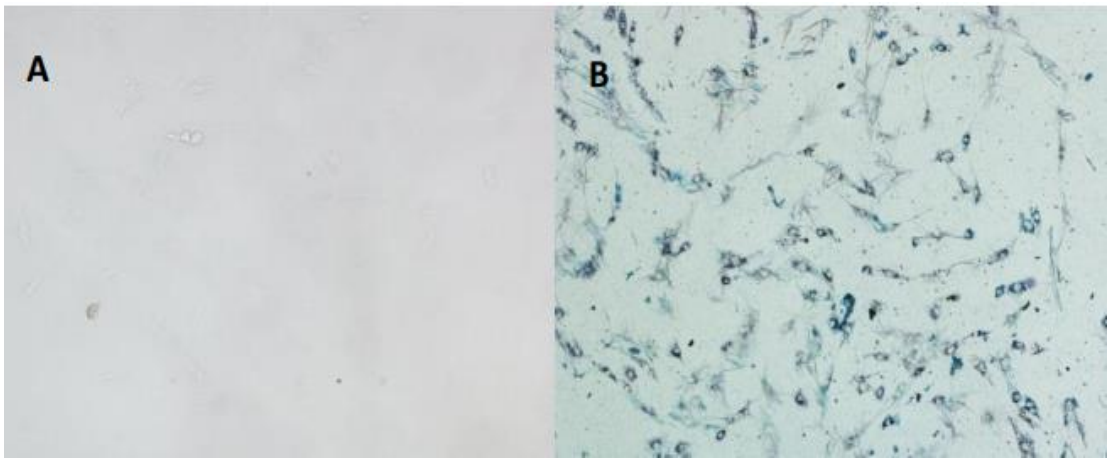

Figure 4. Observation Microscope Magnification 100 X at; (A) Fibroblast Cells are Exposed Vitamin C 100 µg/ml, (B) MTT Method

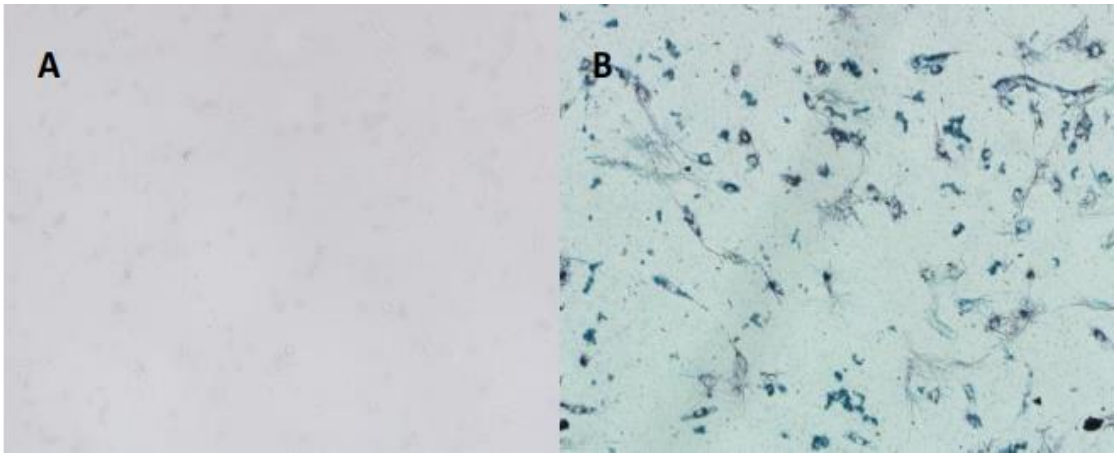

Figure 5. Observation Microscope Magnification 100 X at; (A) Fibroblast Cells are Exposed Vitamin C 150 µg/ml, (B) MTT Method

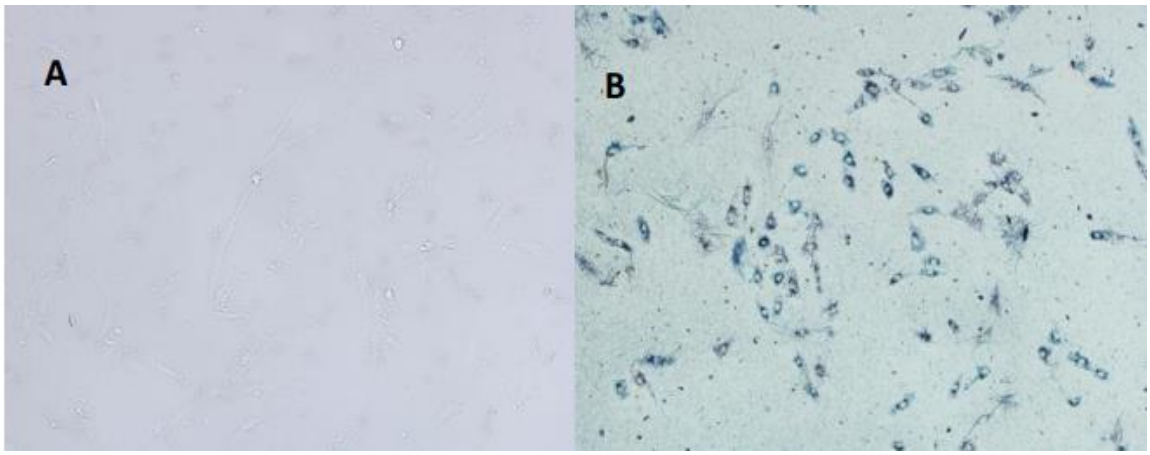

Figure 6. Observation Microscope Magnification 100 X at; (A) Fibroblast Cells are Exposed Vitamin C 200 µg/ml, (B) MTT Method

Table 1. Mean Data of Proliferation İndex According to Varrying Doses of Vitamin C Used One Way ANOVA and Tukey Test 5%

Treatment	Mean ± SD*	p-value**
K+	0.29 ± 0.006[a]	0.000
10 µg/ml	0.29 ± 0.008[ab]	
50 µg/ml	0.33 ± 0.004[c]	
100 µg/ml	0.30 ± 0.005[b]	
150 µg/ml	0.29 ± 0.004[ab]	
200 µg/ml	0.29 ± 0.004[ab]	

*at the mean ± SD, if it contains different letters, mean significant difference (p-value <0.05), and if it has the same letters, mean no significant difference (p-value>0.05).
** Significance (p< 0.05) with One Way ANOVA test.

Based on the results of the 5% Tukey test in Table 5.3, in the comparison between the K+ group and the treatment group given all doses of vitamin C, it was shown that there was a significant increase in fibroblast cell proliferation due to the administration of vitamin C at doses of 50 µg/ml and 100 µg/ml. This is shown from the mean ± SD value of the vitamin C group with a dose of 50 µg/ml and 100 µg/ml, which is higher and contains letters different from the K+ group. Meanwhile, at other doses of vitamin C, there was no significant increase where the mean ± SD of the vitamin C group with doses of 10, 150, and 200 µg/ml were relatively the same and contained the same letter as the K+ group. So from this test, it can be concluded that there is a significant influence significant effect of vitamin C on fibroblast cell proliferation. Or with In other words, there is a significant difference in the proliferation of fibroblast cells; as a result administration of vitamin C at different doses.

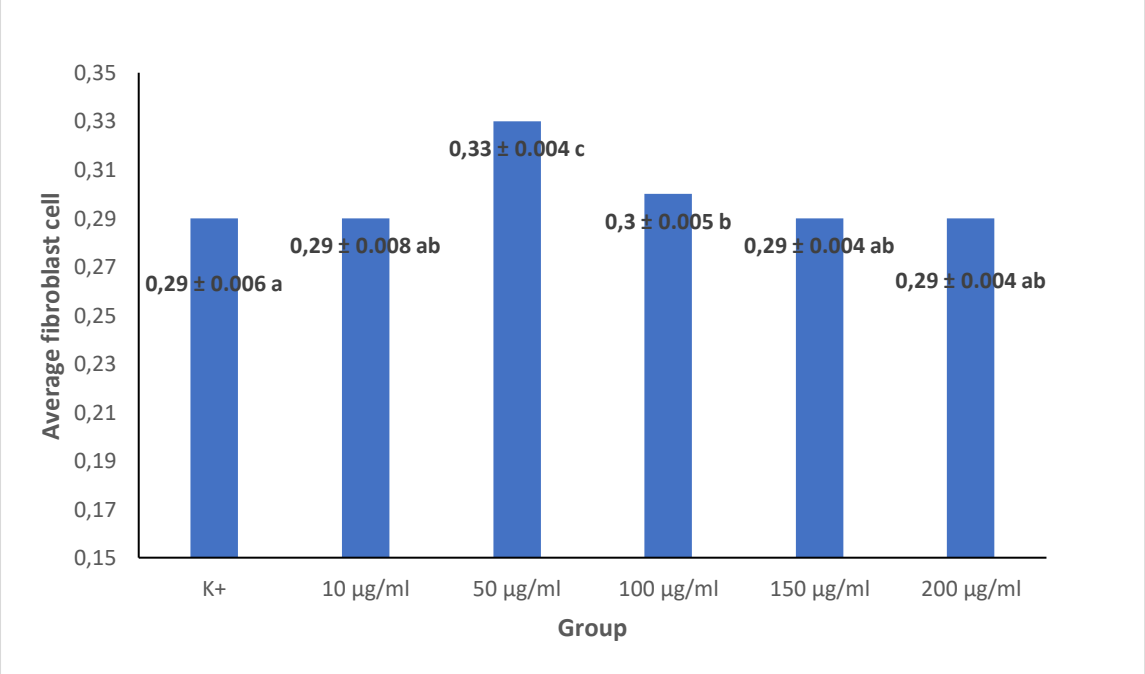

Figure 7. Histogram of the Average Proliferation of Fibroblast Cells in the Sacrouterine Ligament Culture in the Control Group and the Vitamin C Treated Group

Based on the one-way ANOVA analysis results, the study obtained a p-value of 0.000, smaller than α = 0.05 (p <0.05). So from this test, it can be concluded that there is a significant influence significant effect of vitamin C on fibroblast cell proliferation. Or with In other words, there is a significant difference in the proliferation of fibroblast cells; as a result administration of vitamin C at different doses. The analysis results using the 5% Tukey test, in the K+ ratio with the group of varying doses of vitamin C, using one-way ANOVA, obtained a p-value of 0.000, smaller than α = 0.05 (p <0.05). So from this test, it can be concluded that there is a significant effect of giving vitamin C on the proliferation of fibroblast cells in the comparison between the K+ group and the treatment group given vitamin C with a dose of 50 µg/ml and 100 µg/ml. Based on this comparison, it was shown that there was a significant increase in fibroblast cell proliferation due to the exposure to vitamin C with a dose of 50 µg/ml and 100 µg/ml. Whereas in the other doses, it was shown that there was no significant increase where the mean ± SD value of the vitamin C group with doses of 10, 150 and 200 µg/ml was relatively the same (p> 0.05).

The results in Figure 7 found that administering vitamin C with a dose of 50 µg/ml and 100 µg/ml was the most optimal dose in increasing fibroblast cell proliferation in *the invitro* culture of fibroblast cells uterosacral ligament. In several studies conducted in humans or animals, vitamin C also affects fibroblast cells by supporting proliferation and migration, remodeling, and wound healing processes (Carr & Maggini, 2017). The wound healing process has a regular process including hemostasis, inflammation, proliferation and remodeling involving several other cells; PMN, and involves several growth factors; Platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), interleukin-6 (IL-6), interleukin-1β (IL- 1β) and tumor necrosis factor-alpha (TNF-α) (De la Fuente et al., 2020).

This study showed an increase in fibroblast proliferation uterosacral ligament, which was exposed to various doses of vitamin C, by measuring the proliferation index (Pack et al., 2020). According to a study conducted by Mohammed et al., supplementation of vitamin C can induce the expression of self-renewal genes and the proliferation of fibroblasts in wound healing. Vitamin C supplementation increases the expression of IL-6 in mRNA levels in wound tissue environments resulting in fibroblast motility and tissue remodeling. A study by Munira proved the increase of fibroblast proliferation in the preputium fibromas cell culture of donors exposed to vitamin C (Uehara et al., 2023).

On the results of this study conducted, fibroblast cell proliferation increased significantly in the group 50 µg/ml and 100 µg/ml dose of vitamin C. Then there was a decrease in fibroblast cell proliferation to the administration of vitamin C at a dose of 200 µg/ml. The authors made this assumption in light of the following factors that may have an impact on the culture or exposure process:(1) changes in the growth environment of each network, including the type of substrate (where the cell grows), (2) the level of cell contact with other cells,(3) the psychophysical and physiological conditions of the medium,(4) the incubator temperature, and (5) the availability of tissue oxygen (Malole, 1990). The primary culture's cell population's heterogeneous composition may cause variations in proliferative rates. Moreover, the primary culture's cells have not adapted to the in vitro environment, which has hindered their ability to expand (Vishniakova et al., 2011).

A cell density level influences fibroblast cell proliferation level. The dermal fibroblastic cells will uniformly develop relatively in low-density culture (approximately 104 cells/ml), and vice versa, will not develop uniformly in high-density culture

(approximately 105 cells/ml); the cell is inducted to differentiate and delay in cell proliferation (Freshney, 2010). Delay in cell proliferation can be initiated by each cell contact which is shown by density, a change in cell formation, and a decrease in cell spread (Dixon, 2007).

Munira's study showed an increase in fibroblast proliferation until 300 µg/ml dose of vitamin C exposure. The author considers a decrease in fibroblast count at 150 and 200 µg/ml dose of vitamin C exposure as a homeostasis mechanism of fibroblast to fibroblast cell proliferation. There is a delayed cell proliferation that's caused by cell contact, which is shown by its cell density (Vasanth et al., 2020). Stress metabolism mechanism and normal cell response are also said as one of the causes why apoptosis and cell necrosis happened (Di Tano et al., 2020). Vishniakova et al. stated that an increase in cell culture density with a cell area decrease can cause cell mobility and agonistic activity of a cell (Vishniakova et al., 2011).

The limitation of this study is that we did not provide exposure to vitamin C with a dose of 300 µg/ml when compared with previous studies using these doses. Further research is needed regarding the effect of exposure to vitamin C on the culture of sacrouterine ligament cells to observe Other connective tissue components that are concerned with preventing their occurrence of POPs.

CONCLUSION

Based on the findings and analysis in this study, it can be said that there was an increase in fibroblast proliferation index cells in vitamin C exposure to uterosacral ligament cell culture in women. In comparison, administering vitamin C doses of 50 µg/ml and 100 µg/ml were statistically significant in increasing the proliferation of uterosacral ligament fibroblast cells in women. In future research, it is hoped that in vitro cultures will be able to close to cell characteristics resembling those in vivo. It is necessary to carry out clinical trials on the effect of vitamin C on sacrouterine ligaments both in vitro and in vivo.

CONFLICT OF INTEREST

All authors state that there is no conflict or problem with any party in the writing of this journal publication.

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