Pengaruh Kalsium pada Ekspresi *Calmodulin* dan bFGF/FGF-2: Studi In Vitro pada Ligamentum Sakrouterina

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Abstrak
Tujuan: Mengevaluasi efek kalsium pada Ligamen Sakrouterina dengan evaluasi ekspresi calmodulin dan bFGF/FGF-2,
Metode: Sebuah penelitian true eksperimental dilakukan dengan menilai kultur sel ligamentum sakrouterurina dari pasien wanita yang menjalani histerektomi. Kultur sel dibagi menjadi beberapa kelompok yang diberi paparan kalsium pada konsentrasi yang berbeda yaitu 100 nM, 300 nM, 500 nM, 700 nM, dan kontrol tanpa paparan. Ekspresi calmodulin dan bFGF/FGF-2 selanjutnya dianalisis menggunakan metode ELISA dan imunofluoresensi.
Hasil: Penelitian ini menunjukkan bahwa paparan kalsium berpengaruh nyata terhadap ekspresi calmodulin (p-value <0,05). Konsentrasi yang paling efektif untuk menginduksi ekspresi calmodulin adalah pada 500 nM. Kalsium juga berpengaruh nyata terhadap ekspresi bFGF/FGF-2 (p-value <0,05), dengan konsentrasi yang paling efektif untuk menginduksi ekspresi bFGF/FGF-2 adalah pada 500 nM.
Kesimpulan: Penelitian ini menunjukkan bahwa kalsium memiliki efek positif yang signifikan terhadap peningkatan ekspresi matriks ekstraseluler. Penelitian in vivo lebih lanjut perlu dilakukan untuk meningkatkan bukti potensi efek sebagai agen pencegahan prolaps organ panggul. Kalsium banyak tersedia di negara tropis seperti Indonesia, sehingga olahan ini dinilai sangat mudah diterapkan untuk wanita Indonesia.

Kata kunci: Kalsium, *Calmodulin*, bFGF/FGF-2, Matriks Ekstraseluler

Effects of Calcium on Calmodulin and bFGF/FGF-2 Expression: In Vitro Study on the Sacrouterine Ligament

Abstract
Objective: *To evaluate the effects of calcium on the Sacrouterine Ligament by evaluating the expression of calmodulin and bFGF/FGF-2.*
Method: *A true experimental study was carried out by assessing the cell cultures of sacrouterine ligament from female patients who underwent hysterectomy. The cell cultures were divided into groups that were exposed to calcium at different concentrations of 100 nM, 300 nM, 500 nM, 700 nM, and control group without any exposure. The expression of calmodulin and bFGF/FGF-2 was subsequently analyzed using immunofluorescence and ELISA method.*
Results: *This study showed that exposure to calcium significantly affected calmodulin expression (p-value <0.05). The concentration found to be the most effective to induce calmodulin expression was at 500 nM. Calsium also significantly affected the bFGF/FGF-2 expression (p-value <0.05) with the concentration found to be the most effective to induce bFGF/FGF-2 expression at 500 nM.*
Conclusion: *This study suggested that calcium had a significant positive effect of increasing extracellular matrix expression. A further in vivo study needs to be conducted in order to enhance the evidence of the potential effects to become a preventive agent for pelvic organ prolapse. Calcium is widely available in tropical countries like Indonesia, so this preparation is considered very easy for Indonesian women to apply.*

Key words: *Calcium, calmodulin, bFGF/FGF-2, Extracellular Matrix*

Introduction

Pelvic organ prolapse is a condition that shows the protrusion or descent of one or more pelvic organs into or out of the vagina. Pelvic organ prolapse occurs because the muscles, ligaments, and fascia (woven connective tissue) that support these organs in the correct position become weak. Prolapse affects 1 in 3 women who have 1 or more children. Specific conditions, both symptoms and signs, refer to damage to normal pelvic organ function and decreased quality of life function.[1] Pregnancy and childbirth are considered to be the main factor that causes weakness of the vaginal wall and its supports.[2,16]

The extracellular matrix plays an important role because accelerated remodeling in POP patients is caused by biochemical changes in the extracellular matrix such as collagen, elastin, and stromal cells. Myofibroblasts play an important role in the remodeling of the extracellular matrix and its regulation by matrix cell regulators such as matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteinases (TIMP).[3]

In normal tissues, bFGF is present in the basement membrane and in the subendothelial extracellular matrix of blood vessels. Various angiogenic factors are known to cause migration and proliferation of EC (Endothelial Cells). BFGF is associated with tumor angiogenesis and tissue healing. BFGF will bind to the receptor tyrosine kinase on the cell surface of FGFR, an interaction that is modulated in the presence of heparin-heparan sulfate and which increases the stability of the FGF-FGFR complex and its signaling effectiveness. BFGF helps signaling proliferation and developmental differentiation of various types of cells.[4]

Calcium ions are essential for the regulation of various cellular functions. Their role as a second messenger is based on their low cytosolic concentration of free Ca2+ ions under basal conditions (10 nm) and their transient increase (0.1–1 lm) after cellular activation. Calmodulin (CaM) plays an important role as Ca2+ mediation for cellular function, is a multifunctional regulator located in various eukaryotic cells, acts as an intracellular Ca2+ sensor, and decodes Ca2+ signals to control cellular responses (15). Calmodulin is involved in transactivation of epidermal growth factor receptor, while Ca2+ CaM complex is involved in angiotensin II type 1 receptor mediated transactivation of epidermal growth factor receptor and activation of signaling pathways.[5,15]

The current research focuses on the changes in the support network, where the Extracellular Matrix (ECM) plays a vital role in accelerating connective tissue remodeling in POP. Together, interstitial bFGF/FGF-2 and the Ca2+ CaM complex comprise the key important ECM constituents in the uterine connective tissue (5, 6). Our study aims to evaluate the effects of calcium to increase the ECM expression such as bFGF/FGF-2 and expression of calmodulin intracellular, so that it can be basic research for the prevention of POP.

Method

The authors conducted a true experimental study, and the samples were collected using the consecutive sampling technique. Samples were taken from the biopsy and cell culture of sacrouterine ligament in patients who underwent hysterectomy. Cell cultures with adequate confluency were included in the study. On the contrary, the sampling was not performed on patients with any history of adhesion in pelvic organs and suspicion of gynecological cancer, tumor, or mass potentially stretching the sacrouterine ligament. The samples were divided into case groups exposed to specific concentrations of calcium, i.e., 100 nM, 300 nM, 500 nM, 700 nM, and the control group which was not

exposed to calcium at all.

BFGF/FGF-2 expression was examined by immunofluorescence study, whereas calmodulin expression was examined using the ELISA method. All data were recorded and analyzed using ANOVA, Kruskal-Wallis, and regression analysis to find the predictive relationship between different calcium concentrations with the bFGF/FGF-2 and calmodulin expressions. The Research Ethics Committee has approved ethical research eligibility in the Health Division of Dr. Saiful Anwar Regional General Hospital in Malang.

Results

A total of 25 replicate samples were divided into five groups consisting of the case groups given exposure to calcium with a concentration of 100 nM, 300 nM, 500 nM, and 700 nM as well as a control group without any exposure. BFGF/FGF-2 and calmodulin expression variables were observed quantitatively and analyzed for their distribution normality using the Logarithm Natural Ln (Y) test. The variables were also analyzed for homogeneity with Levene's test.

Effects of Calcium on bFGF/FGF-2 Expression

BFGF/FGF-2 expression was observed from samples that had been tested using an immunofluorescence study (Fig. 1). Subsequently, the analysis was carried out with the results showing the influence of calcium exposure on bFGF/FGF-2 expression (Fig. 2). The ANOVA test showed a probability value of 0.000 (p-value < 0.05), meaning that calcium exposure had a significant effect on the bFGF/FGF-2 expression. Furthermore, another analytical test was carried out to determine the value of calcium concentration that most effectively influenced bFGF/FGF-2 expression. Furthermore, another analytical test was carried out to determine the value of calcium concentration that most effectively influenced bFGF/FGF-2 expression.

Based on the results of the regression analysis of bFGF/FGF-2 expression, the regression coefficient was 0.053 with a p-value of 0.000. The coefficient of determination (R-square) of 50.97% indicates that the diversity of data explained by the effect of calcium administration on bFGF/FGF-2 expression is only 50.97%. The R-square value is quite high, but it has not shown that the linear model explains the effect of calcium on bFGF/FGF-2 expression accurately. This happened because at a certain dose, there was an increase in the expression of bFGF/FGF-2 to the maximum point at a dose of 500 nM, then it decreased at a dose of 700 nM. Thus, a more complex regression model is needed, namely polynomial regression.

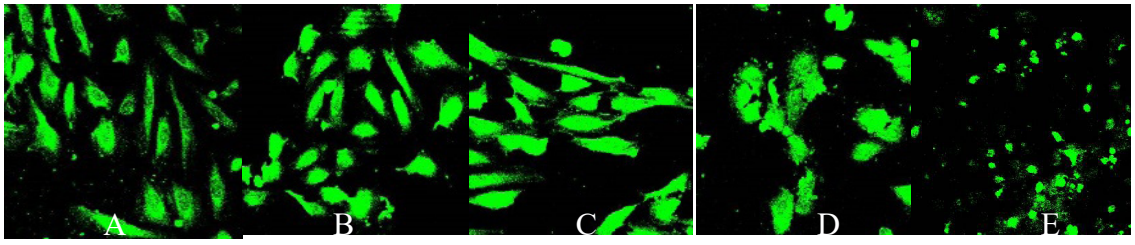

Figure 1 Photomicrograph of bFGF/FGF-2 Expression on Immunofluorescence Assay, Respectively (A) Without Calcium Exposure and Exposed with Calcium with Specific Concentrations of (B) 100 nM, (C) 300 nM, (D) 500 nM, and (E) 700 nM

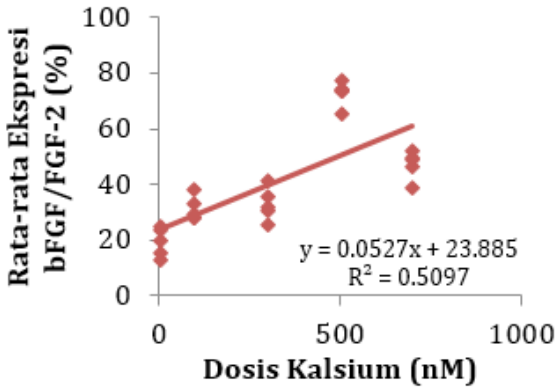

Figure 2 Scatter Plot Effect of Calcium on bFGF/FGF-2 Expression

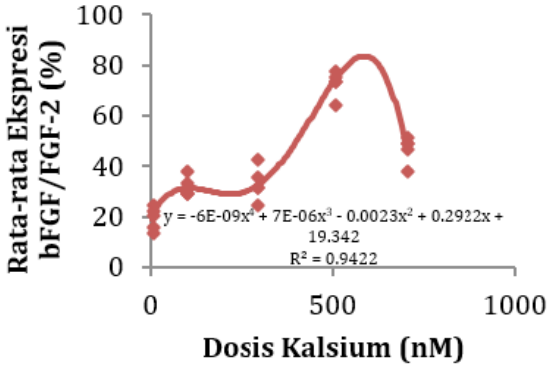

Figure 3 Scatter Plot Effect of Calcium on bFGF/FGF-2 Expression with Polynomial Regression Showing the Quantitative Differences of bFGF/FGF-2 Expression Based on The Concentration of Calcium Exposure

Table 1 Comparative Test of bFGF/ FGF-2 Expression with ANOVA Test and LSD 5%

Mean ± SD		p-value
19.34 ± 5.31	a	
31.38 ± 4.32	b	
32.96 ± 5.97	b	0.000
72.95 ± 4.71	d	
47.13 ± 4.97	c	

Based on the results of the polynomial regression analysis above, it is shown that the R-square value is 0.9422 or 94.22%. Calcium administration was able to affect changes in bFGF/FGF-2 expression by 94.22%. The remaining 5.78% is explained by other factors not involved in the study. Using Wolfram Alpha software, the peak point of the curve was predicted to be at a dose of 571.56 nM with bFGF/FGF-2 expression of 89.71%. The comparison based on the results of the 5% LSD test between the K0 and K1 groups showed that there was a significant difference between the K0 and K1 groups. This is indicated by the average value of ± SD in K1 which is higher and contains different letters. The significant difference proves that calcium exposure has been shown to significantly increase bFGF/FGF-2 expression. Likewise, the comparison of K0 with K2, K3, and K4 obtained different letter notations which proves that there was a significant increase in bFGF/FGF-2 expression.

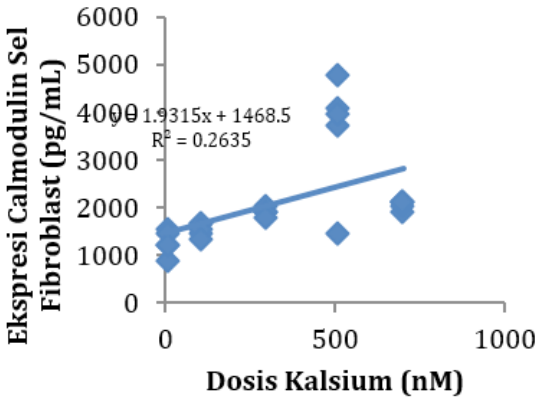

Figure 4 Scatter Plot Effect of Calcium on Calmodulin Expression

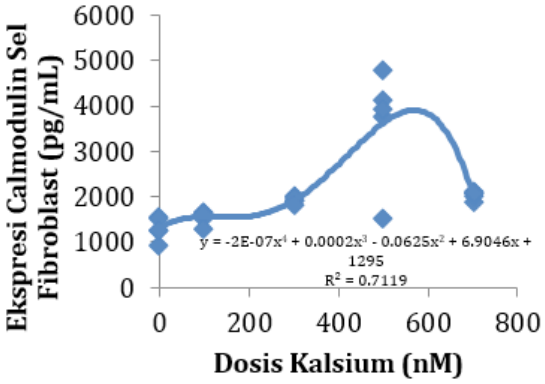

Figure 5 Scatter Plot Effect of Calcium on Calmodulin Expression with Polynomial Regression

Effects of Calcium on Calmodulin Expression

Calmodulin expression was observed from samples that had been tested based on the ELISA method. Subsequently, the analysis was carried out with the results showing the influence of calcium exposure on calmodulin expression (Fig. 4). The Kruskal-Wallis test showed a probability value of 0.002 (p-value <0.05), meaning that calcium exposure had a significant effect on the calmodulin expression.Based on the results of the regression analysis of calmodulin expression, the regression coefficient was 1.932 with a p-value of 0.009. The coefficient of determination (R-square) of 26.35% indicates that the diversity of data explained by the effect of calcium administration on calmodulin expression is only 26.35%. The low R-square value indicates that the linear model has not accurately explained the effect of calcium on calmodulin expression which indicates that the diversity of data.

Table 2 Comparative Test of Calmodulin Expression with Kruskal-Wallis Test and 5% Dunn Test

Variables	Mean ± SD		*p-value*
K0	1,295.0 ± 245.22	a	0.002
K1	1,554.0 ± 155.68	b	
K2	1,918.0 ± 70.79	c	
K3	3,624.5 ± 1,254.72	c	
K4	2,041.5 ± 94.50	c	

The results of polynomial regression analysis shows that the R-Square value is 0.7119 or 71.19%. Giving calcium was able to affect the change in the expression of calmodulin by 71.19%. The remaining 28.81% is explained by other factors not involved in the study. Using Wolfram Alpha software, the peak point of the curve was predicted to be at a dose of 537.47 nM with a calmodulin expression of 3306.38 pg/mL.

The comparison between the K0 and K1 groups based on the Dunn 5% test results in table 2 above shows that there is a significant difference between the K0 and K1 groups. This is indicated by the average value of ± SD in K1 which is higher and contains different letters. The significant difference proves that calcium exposure at a dose of 100 nM has been shown to significantly increase the expression of calmodulin. Likewise, in the comparison of K0 with K2, K3, and K4, different letter notations which proved a significant increase in calmodulin expression were found.

In the comparison between groups K1 and K2, K3, and K4, it was shown that there were significant differences between groups K1 and K2, K3, and K4. This is indicated by the average values of ± SD in K2, K3, and K4 which are higher and contain different letters. Significant differences prove that calcium exposure at a dose of 300 nM (K2), a dose of 500 nM (K3), and a dose of 700 nM (K4) proved to have an impact on increasing calmodulin expression higher than a dose of 100 nM (K1). In the comparison between groups K2 and K3 and K4, it was shown that there was no significant difference between groups K2, K3, and K4. This is indicated by the average values of ± SD in K2, K3, and K4 containing the same letters. From this test, it was proved that the administration of calcium with a dose of more than 300 nM had the same effect on the expression of calmodulin.

Discussion

The sacrouterine ligaments are an essential part of the pelvic support system [7-9]. An in vitro study by Gabriel and colleagues revealed that the cervical portions of the sacrouterine ligament was responsible for bearing the weight of up to 17 kg before prolapsing. The most important supporting element of this structure is the ECM. However, several risk factors, including aging, multiple parity, or frequent vaginal deliveries may cause a greater chance for women to develop POP [10]. A prospective study from The Oxford Family Planning, which involved 17,000 women, found that hospital admission due to POP increased fourfold in women having one child, eightfold in those having two children, and tenfold for those having multiple parities as compared with nulliparous women.[11]

Effects of Calcium on Calmodulin

Calmodulin is a Ca2+ receptor protein that mediates a large number of intracellular signaling processes in eukaryotic cells (12). Calmodulin is a center that plays a role in regulating various cellular functions through interactions with various target proteins. Calmodulin acts through the signaling system to control vertebral cell proliferation, cell death programs, and autophagy processes (12). Calmodulin is considered to be the main regulator of Ca2+ in eukaryotic cell signaling function and plays a role in cellular physiology. Based on data, calmodulin plays a role in the control of a large number of cellular physiological processes, namely cellular motility, cytoskeleton function and architecture, cell proliferation, apoptosis, autophagia, metabolic homeostasis, phospholipid turnover, protein folding, protein phosphorylation and dephosphorylation, ion transport, osmotic control, reproductive processes, muscle contraction, and gene expression.[5, 12]

The aim of this study was to prove that calcium increased the expression of calmodulin in sacrouterine ligament fibroblast cultures. In this study, the results showed that calcium had a significant effect on calmodulin expression. In other words, there is a significant difference in the expression of calmodulin due to the administration of calcium with different doses. Through the Kruskal-Wallis test and the Dunn 5% test, there was a significant difference in the average calmodulin expression in the comparison of the control group with the groups exposed to calcium with doses of 100 nM, 300 nM, 500 nM, and 700 nM because the p-value was less than 0.05 ($p < 0.05$). From this test, it was proved that there was a significant difference in the mean expression of calmodulin in the 100 nM, 300 nM, 500 nM, and 700 nM calcium administration groups compared to the control group or the group without calcium administration. The results of this study are in line with the results of research conducted by Misárková, namely an increase in Ca2+ ion levels will cause Vascular Smooth Muscle Cell (VSMC) contraction through elevation of Myosin Light Chain Kinase (MLCK) via calcium–calmodulin complex (13). The results of this study are also in line with Ringer's research, which found that the experimental heart ventricles given distilled water stopped contracting, and then when NaCl and CaCl2 were added to the distilled water, the ventricular contractions would return spontaneously even for a long time, so it was concluded that contractions of muscles need calcium salts.[14]

Calcium administration was able to affect the change in calmodulin expression by 71.19%. Using Wolfram Alpha software, the peak point of the curve was predicted to be at a dose of 537.47 nM with a calmodulin expression of 3,306.38 pg/mL. The results of this study are also in line with Gonzalez's research, namely calcium is an ion that has an important role in the regulation of cellular multiple functions. Calcium is a second messenger and based on the concentration of calcium in the free cytosol, the calcium level in basal cellular conditions is about ~10 nM and the calcium level will increase by ~0.1-1mM upon cellular activation. In carrying out its role, calcium (Ca2+) is mediated by intracellular calmodulin. Calmodulin acts as an intracellular calcium sensor (15). Changes in extracellular calcium concentration affect various cellular processes including cell secretion and proliferation. Intracellular calcium acts as a signaling system that bridges the response of a cell to specific stimuli (physiology textbooks 883; 4:29-49). In many cell systems, calcium ions and cyclic nucleotide act as messenger pairs.[14]

Effects of Calcium Administration on bFGF/FGF-2

FGF-2, also known as basic fibroblast growth factor, is a signaling control that plays a role in fibroblast differentiation. Fibroblast growth factor plays a role in the healing process of bone damagebecause the healing process requires cells to form new bones. The proliferative phase, or the repair phase, begins when the inflammatory phase releases cytokines and growth factors resulting in fibroblast proliferation to form extracellular matrix, and fibroblast cell proliferation requires calcium for the formation of calcium salts through an attachment, thus forming new bones/woven bones (17).

This study proved that calcium increased the expression of bFGF/FGF2 in sacrouterine ligament fibroblast culture. The data from this study indicate that there is a significant effect of calcium administration on the expression of bFGF/FGF2. Or in other words, there is a significant difference in bFGF/FGF2 expression due to the administration of calcium at different doses. Based on the results of this study, from the results of the ANOVA test and the 5% LSD

test, the comparison between the control group and the treatment groups with various doses of calcium showed that after giving calcium with doses of 100 nM, 300 nM, 500 nM, and 700 nM, the p-value was less than 0.05. ($p < 0.05$). From this test, it was proved that there was a significant difference in the mean expression of bFGF/FGF2 between the groups given calcium with doses of 100 nM, 300 nM, 500 nM, and 700 nM and the control group.

Based on the data from this study, data of the average bFGF/FGF2 expression statistically showed that calcium administration with all doses was proven to be able to increase bFGF/FGF-2 expression until the highest average value of 72.95 was obtained at 500 nM (K3) calcium administration. This occurred because there was an increase in the expression of bFGF/FGF-2 to a maximum point at a dose of 500 nM with a scatter plot polynomial regression analysis of the effect of calcium on bFGF/FGF-2 showing that the value of R-Square was 0.9422 or 94.22%. This means that calcium can affect changes in bFGF/FGF-2 expression by 94.22%. The remaining 5.78% is explained by other factors not involved in the study. Using Wolfram Alpha software, the peak point of the curve was predicted to be at a dose of 571.56 nM with bFGF/FGF-2 expression of 89.71%. The results of this study are in line with research conducted by Yavatrisna (2016) stating that calcium sulfate can act as a stimulus for osteoblast differentiation. Stimulation of mechanoreceptors on osteoblast cells (integrins and calcium channels) along with growth factors (TGF-β, IGF, bFGF, VEGF, PDGF, and BMP) will induce several genes/transcription factors that regulate the formation and differentiation of osteoblasts.[18]

The results of this study are in line with the results of research conducted by Kresnoadi U (2012) stating that calcium is a biogenic stimulator to stimulate and accelerate the formation of alveolar bone after tooth extraction. The use of a combination of Aloe vera gel and xenografts can increase the proliferation of fibroblast cells, increase the expression of Fibroblast Growth Factor-2 (FGF-2) and osteocalcin in the healing of tooth socket bones (Kresnoaadi U, 2012; Rahmitasari, 2018). One of the natural ingredients that have the potential to heal wounds is the aloe vera plant, which is rich in calcium. Aloe vera has more than 75 active compounds that play a role in the healing process, namely calcium, protein (aloctin), amino acids, enzymes, alkaloids, flavonoids, saponins, collagen, vitamins, potassium, and polysaccharides including pectin, cellulose, hemicellulose, fructans, and mannan.[19, 20]

Conclusion

Calcium exposure has a significant positive effect on bFGF/FGF-2 and calmodulin expressions performed by in vitro studies using sacrouterine tissue. A further in vivo study needs to be conducted to determine the calcium functions for the ultrastructural components in the female genital organs, so it may enhance the evidence of the potential effects for maintaining and preventing gynecological health, mainly to prevent POP. Calcium is widely available in tropical countries like Indonesia, so this preparation is considered very easy for Indonesian women to apply.

Research Funding and Ethics

The study was funded by the Faculty of Medicine, University of Brawijaya, and received ethical approval from The Ethics Committee of Dr. Saiful Anwar General Hospital in Malang (Letter Number: 400/237/K.3/302/2020).

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

Advice and Thanks

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