Regulation of Sex Steroid Sex Hormones on Calcitonin Gene-Related Peptide (Cgrp)'s Mrna Expression in Vaginal Mucosa Epitel of Bilateral Ovarectomized Wistar Rats

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http://dx.doi.org/10.13005/bpj/1885

(Submitted: 27 August 2019; accepted: 02 February 2020)

During menopause, there is a decrease in the level of synthesis of sex steroid hormones such as estrogen, progesterone and androgen. Dramatic changes in sex steroid hormone levels cause vulvovaginal atrophy which is characterized by vaginal dryness, atrophy, dyspareunia, and sexual arousal disorders. This mechanism is thought to be mediated by changes in the expression of calcitonin gene related peptide (CGRP), a neuropeptide which is a potent vasodilator. This study aims to determine how sex steroid hormones regulate the expression of Calcitonin Gene-RelatedPeptide (CGRP) mRNA in the vaginal mucosal epithelium of wistar rats with bilateral ovarectomy. A post-test only control group design research was conducted on 30 female rats. Menopausal state was induced by ovarectomy on bilateral ovaries. After two weeks the rats were divided into 3 groups: the control group (given 1 ml aquadest), the treatment group I (given a combination of estrogen (11 µg / day) + progesterone (180 µg / day) and the treatment group II were given a combination of estrogen (11 µg / day) + progesterone (180 µg / day) + testosterone (360 µg / day). After 30 days the mice were terminated and the vaginal tissue was taken from 2/3 part of posterior vagina. CGRP’s mRNA expression was calculated using the real time Polymerase Chain Reaction (rtPCR) method. The average CGRP gene mRNA levels in the control, treatment I and treatment II group were 1.60 ± 0.24 fg / ul, 4.77 ± 1.39 fg /ul, 7.09 ± 1.63 fg /ul respectively. There were significant differences in the mean mRNA levels of the CGRP gene between each group. This result suggests that the administration of sex steroid hormones increase CGRP mRNA expression.

Keywords: CGRP; mRNA; Ovarectomized Rats; Sex Steroid Hormone; Vaginal Mucosa Epithelium.

The topic of menopause has been a worldwide concern since the International Menopause Congress was first held in the South of France in 1976. Menopause is one of the processes in the natural reproductive cycle that every woman will experience besides puberty, pregnancy, and menstruation. In menopausal women various health problems arise due to decreased secretion of ovarian hormones such as estrogen, progesterone and testosterone. Decreasing the amount of hormone secretion and receptor protein synthesis causes health problems which include vasomotor, somatic, urogenital, psychological, osteoporosis and sexual function disorders.

More than half of menopausal women complain about vulvovaginal atrophy symptoms such as vaginal dryness, dyspareunia, pain during urination, spotting bleeding during intercourse, and decrease of vaginal lubrication (Naumova, 2018). Vulvovaginal atrophy can occur at any time in a
The Condition of the vagina during menopause plays an important role in maintaining sexual quality. Estrogen hormone plays a role in regulating hemodynamics in the sexual response cycle. When a woman experiences vaginal atrophy, the woman will experience vaginal dryness, which will cause dyspareunia in women who are still sexually active. These changes are also followed by lubrication disorders, which eventually refer to sexual disorders such as decreased desire to have intercourse, decreased sexual arousal, decreased sensation of stimulation, and difficult to reach orgasm (Sturdee and Panay, 2010). Women who experience sexual complaints due to vaginal atrophy should be diagnosed and treated as soon as possible to prevent the symptoms getting worse.

Testosterone is a hormone that is commonly found in men, but is also produced in smaller amounts in women. Hormone testosterone plays a role in the physiological response to sexual stimuli that are important in sexual function, which is characterized by vaginal vasoconstriction. In women with hypothalamic amenorrhoea, the administration of the hormone testosterone can increase vaginal response to sexual stimulation (Tuiten A et al., 2000). According to Davis (1999) androgen insufficiency is associated with sexual dysfunction. Arlt et al (2000) stated that giving DHEA to women with adrenal insufficiency improves sexual function and quality of life.

Histologically CGRP is found in almost all parts of the human body including the vagina, clitoris and surroundings. CGRP in the vagina and clitoris act as a neurotransmitter, controlling blood flow, relaxing vaginal muscles and capillary permeability. CGRP vasoactive function is indispensable in the process of vaginal vasodilation during sexual stimulation, so that vaginal lubrication will occur (Goldstein, 2006).

Based on the background above, we interested to know the regulation of steroid sex hormones on the expression of calcitonin gene-related peptide (CGRP) mRNAs in the vaginal mucosal epithelium of wistar rats with bilateral ovariectomy.

Subjects and Methods

Subjects

Thirty female Wistar rats (Rattus
norvegicus) were included in this experimental study as subjects. Subjects were 3-4 month age and divided into 3 groups. The study protocol approved by the Ethics Committee of Udayana University of Medical Faculty / RSUP Sanglah Denpasar (No. 2709 / UN.14.2 / KEP / 2017). Subjects were excluded if getting sick, and die.

**Menopause Induction**

The mice were adapted for one week and feeded ad libitum. Ovariectomy carried out at Integrated Biomedical Laboratory of the Medicine Faculty, Udayana University. As much as 0.3 ml of ketamin and 0.35 ml Cefoperazone Sodium & Sulbactam Sodium were injected to the rats. Rat’s hair were shaved right in the flank area (between the last rib and above the pelvis). The skin area was disinfected by chlorhexidine solution and incisions was made on the left and right side. The ovary was removed by clamping the area under the ovary and binding using sterile threads. The incision wound was healed using dafilon 4.0 and catgut plain 4.0. Rats were adapted for 2 weeks for the recovery process and reduction of internal steroid hormones.

**Hormonal Treatment**

Two weeks after ovariectomy, rats divided into three treatment groups. The first group is a control group and was given 1 ml of distilled water/day. The second group was the treatment group who was given a combination injection of estrogen (11 µg/day) + progesterone (180 µg/day). The third group was given a combination of estrogen (11 µg/day) + progesterone (180 µg/day) + testosterone (360 µg/day). The dose was given orally with a syringe for 30 days.

**Design of Experiment**

This research was an experimental study using post test only control group design. Subjects were divided randomly into three treatment groups. CGRP mRNA expressions in each group were then compared to determine the differences in the regulation of sex steroid hormones on CGRP mRNA expression.

**CGRP mRNA Expression Assessment**

Samples were taken from 2/3 of the posterior part of the vagina, then cut with a thickness of ± 3 mm and 1 cm in diameter. The vaginal specimens obtained were then fixed with a 10% neutral buffer solution of formalin and left at room temperature for ± 48 hours. CGRP mRNA expressions were quantified using the rt-PCR method. The extraction and PCR protocol are mentioned as below:

Vagina was biopsied and immerse immediately in RNAlater® as preservative for 24 hours in 4oC. Then total RNA was isolated by using RNaseasy mini kit (Qiagen, Germany) according manufacture protocol. The RNA was amplified using absolute quantification one step qRT-PCR (SensiFAST SYBR No-ROX One-Step Kit, Bioline, UK) for CGRP quantification. Amplification was done by using MyGo mini realtime PCR (IT-IS Life Science, UK) to obtain the Ct value. Then the Ct values were interpolated by using standard curve. Standard curve was created from previously purified PCR product of CGRP which then diluted serially. The forward primer was 5'-TCTCTGCAACACTGCCACCTG-3' and reverse primer was 5'-GGTGGGCACAAAGTTGTCTT-3'. The PCR condition were provided in Table 1.

**Statistical Analysis**

Data were analyzed using SPSS 20.0. Differences in CGRP mRNA expression in each group is analyze using the One Way Anova test followed by the LSD test. Statistical Significance was considered when p<0.05.

**RESULTS**

Based on the results of data analysis, it was found that the mean CGRP mRNA expression in the control group was 1.60 ± 0.24 fg/ul. The mean CGRP mRNA expression in the group given estrogen and progesterone was 4.77 ± 1.39 fg/ul and the mean CGRP mRNA expression in the group given estrogen progesterone and testosterone had the highest CGRP mRNA expression which was 7.09 ± 1.63 fg/ul (Table 1).

Shapiro Wilk Test was performed to analyzed the normality of the mRNA CGRP expression in each group. It was concluded that the data were normally distributed at p> 0.05 (Table 2). Levene’s Test was used to determine the homogeneity of mRNA CGRP expression and it was found that the data were homogeneous with p value> 0.05 (Table 3).

The mean differences of CGRP mRNA expression were analyzed using the One Way Anova test. Based on the data presented in Table 4, it could be concluded that there are significant differences in mean CGRP mRNA expression after
treatment (p <0.05). LSD test was performed to find out the differences in each group (Table 5).

**DISCUSSION**

Sex steroid hormones are an important factor for maintaining the integrity and function of female genital organs. Estrogen play an important role in maintain lubrication, thickness and rugae in the vaginal wall. Estrogen is also able to increase sensation and maintain blood flow in the vagina. Previous studies found that ovovectomized animals showed a decrease in the thickness of the vaginal epithelium and smooth muscle (Abdel-Aal et al., 2015).

Testosterone doesn’t have a significant effect on the thickness of smooth muscle and vaginal epithelium. Administration of testosterone increase level of nerve growth factor (NGF) in the vagina so that the density and myelination of adrenergic nerve fibers in the vagina are increase. While progesterone does not have a significant effect on the structure of the vagina, but progesterone acts to prevent the occurrence of endometrial hyperplasia due to estrogen administration (Pessina et al., 2006).

Many studies which was focused on the role of sex steroid hormones on the structure and integrity of the vagina has been done and it has been proven that sex steroid hormones play an important role in maintaining the integrity of the structure of the vagina including vaginal vascularization involved in the genital arousal process. However, research on how the molecular

**Table 1.** The one step qRT-PCR condition

<table>
<thead>
<tr>
<th>Description</th>
<th>T (°C)</th>
<th>Duration (second)</th>
<th>Cycle (times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcriptase</td>
<td>42</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Enzyme inactivation</td>
<td>95</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Annealing</td>
<td>63</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Melting</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Descriptive Test Result of CGRP mRNA Expressions After Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Samples (n)</th>
<th>CGRP mRNA Expressions (mean±SD fg/uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>1,60±0,24</td>
</tr>
<tr>
<td>E+P*</td>
<td>10</td>
<td>4,77±1,39</td>
</tr>
<tr>
<td>E+P+T**</td>
<td>10</td>
<td>7,09±1,63</td>
</tr>
</tbody>
</table>

Description: *=Estrogen+Progesteron
**= Estrogen+Progesteron+Testosteron

**Table 3.** Normality Test Results of CGRP mRNA Expressions After Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Samples(n)</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>0,266*</td>
</tr>
<tr>
<td>E+P</td>
<td>10</td>
<td>0,472*</td>
</tr>
<tr>
<td>E+P+T</td>
<td>10</td>
<td>0,787*</td>
</tr>
</tbody>
</table>

Description: *=Normal at p>0,05

**Table 4.** Homogeneity Test Results of CGRP mRNA Expressions After Treatment

<table>
<thead>
<tr>
<th>Data Variations</th>
<th>Levene Statistic(n)</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP mRNA Expressions</td>
<td>1,782</td>
<td>0,187*</td>
</tr>
</tbody>
</table>

Description: *=homogenous at p>0,05

**Table 5.** One Way Anova Test Result of CGRP mRNA Expressions After Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Samples (n)</th>
<th>Mean of CGRP mRNA Expressions (fg/ul)</th>
<th>SD</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kontrol</td>
<td>10</td>
<td>1,60</td>
<td>0,24</td>
<td>48,69</td>
<td>0,001</td>
</tr>
<tr>
<td>E+P</td>
<td>10</td>
<td>4,77</td>
<td>1,39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E+P+T</td>
<td>10</td>
<td>7,09</td>
<td>1,63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Description: *=significantly different at p<0,05
processes that mediate the function of these hormones in the vagina is not clearly known. Recent research suggests that the effects of female sex steroid hormones on the vascularization system are mediated by at least the vasodilation effect of CGRP (Gangula, et al., 1997).

From this study, it was found that mean of CGRP mRNA expressions in the control group was $1.60 \pm 0.24$ fg / ul. Mean of CGRP mRNA expressions in estrogen and progesterone group was $4.77 \pm 1.39$ fg / ul and mean of CGRP mRNA expressions in estrogen progesterone and testosterone group were $7.09 \pm 1.63$ fg / ul. One Way ANOVA test result showed the $p$ value $= 0.001$. From these results it can be concluded that the treatment group given the combination of estrogen, progesterone and testosterone have the highest CGRP mRNA expression among other groups.

This result indicate that the administration of hormonal therapy to ovariectomized rats increase the mRNA levels of the CGRP gene in the vagina. Combination of estrogen and progesterone increase CGRP levels up to 198% compared to the control group. Combination of estrogen, progesterone and testosterone increase CGRP gene mRNA levels by 343% compared to the control group and by 48.63% compared with the group given estrogen and progesterone.

CGRP is a very potent microvascular vasodilator, 10 times stronger than prostaglandins and 10-100 times stronger than ACh and SP. Recently, CGRP is known as the most potent vasodilator (Brain, et.al., 2004). The vasodilation effect of CGRP is also more enduring compared to other vasodilators. CGRP vasoactive compression is indispensable in the process of vaginal vasodilation during sexual stimulation, so that vaginal lubrication will occur (Goldstein, 2006).

Pessina et al. (2006) stated that there were no significant changes in the density of adrenergic nerve fibers after administering estradiol in ovariectomized animals. This shows that estrogen is not the main mediator for adrenergic nerve density in the vagina. Whereas both PGP and TH immunoreactive density were found to be significantly increased in the vaginal tissues of animals given testosterone compared to the control group. There are several studies that report that administration of testosterone in female mice results in a 20-fold increase in the level of mRNA nerve growth factor (NGF) (Black et al. 1992). The administration of testosterone stimulates the formation of nerve fibers and myelination and structure of male erectile tissue.

In accordance with the above explanation, it is clear that the group given testosterone therapy had higher CGRP levels compared to the group given only estrogen and progesterone. This is due to the fact that testosterone can increase NGF levels. Moreover, NGF is enhanced by upregulation of CGRP synthesis in the dorsal root ganglia (DRG) and stimulates CGRP expression in hypertensive rats (Supowit, et.al., 2005) and is currently considered to be involved in influencing both the sensory nervous system and the central nervous system in a manner that is both complex during cardiovascular dysfunction (Hashikawa-Hobara, et.al., 2012). Increased levels of NGF and CGRP are also found in plasma and saliva of migraine patients. Other potential factors such as brain-derived neutropic factor (BDNF) also influence the release and activity of CGRP (Salio, et.al., 2007).

**CONCLUSION**

The administration of steroid sex hormones was able to increase the expression of CGRP mRNA in ovariectomized vaginal epithelium where groups given estrogen progesterone and testosterone produced the highest levels of mRNA CGRP genes. Further research is needed for the minimum dose, side effects and research in humans.

The treatment of phytoestrogen can produce mild estrogenic effects in the postmenopausal woman, including estrogen-like effects on vaginal cytology and reduction in hot
flushes (Alice, et al., 1997). However the findings are inconsistent. But, is the phytoestrogen can improve CGRP's level is still unknown.

ACKNOWLEDGEMENT

The researcher would like to thank the staff of the Integrated Biomedical Laboratory at the Faculty of Medicine, Udayana University, who have helped in completing this research. This research is approved by Institute Technology and Health Bali, we would like to thank them for funding this project. The authors also gratefully acknowledge this study have no conflict of interest.

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