Increase in Bcl\(_2\) expression of penile and prostate cells of Sprague Dawley male rats following treatment with buceng (combination of Pimpinella alpina molk with Eurycoma longifolia Jack)

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**ABSTRACT**

**Background:** Treatment with buceng combination of Eurycoma longifolia Jack and Pimpinella alpina Molk has been proven to increase testosterone level, decrease apoptosis and caspase expression. Bcl\(_2\) is an antiapoptotic protein found in cytoplasm which inhibits cells apoptosis. This study was aimed to investigate the effect of buceng on Bcl\(_2\) expression on penile and prostate tissues of the rats.

**Methods:** In this experimental study, 24 male Sprague Dawley rats of 90 days old, weighing ± 300 grams, were randomly assigned into four groups. Group A, normal rats. Group B, castrated rats and treated with buceng 100 mg/day, per oral (Cast-Bcg); Group C, castrated rats and treated with 2 ml of water as placebo against buceng (Cast-Plac). Group D, castrated rats, treated with mesterolone 6.75 mg/day, per oral, as exogenous testosterone (Cast-Mest). All rats were treated for 30 days. Manova test was used to analyze the different expression of Bcl\(_2\) among groups with significance level at p ≤ 0.05.

**Results:** Castration was associated with significant decrease of Bcl\(_2\) expression in the penile and prostate tissues (53.0 and 50.9%, respectively) compared to normal rats (82.6 and 84.2%, p < 0.001). Treatment with mesterolone reversed Bcl\(_2\) expression (77.1 and 78.1%) to a near normal level. The same level of Bcl\(_2\) expression was also observed with buceng treatment (73.8 and 78.2%).

**Conclusion:** The treatment with buceng could enhance Bcl\(_2\) expression in penile and prostate tissues, comparable to normal rats and mesterolone treated rats.

**Keywords:** apoptosis, Bcl\(_2\), buceng, testosterone
Pasak bumi (Eurycoma longifolia Jack) and purwoceng (Pimpinella alpina Molk) both are Indonesian indigenous plants\textsuperscript{1,2} combination of them so called buceng. Treatment of buceng extract has been proven to increase serum testosterone level, suppress apoptosis, and decrease caspase\textsubscript{3} expression.\textsuperscript{3-5} There are growing evidences that apoptosis process has strong correlation with decrease in antiapoptotic protein, on one hand, and on the other hand increase in proapoptotic protein.\textsuperscript{6} Caspase\textsubscript{3} is a proapoptotic protein that serves as an executor of apoptosis,\textsuperscript{7} whereas Bcl\textsubscript{2} is an antiapoptotic protein preventing apoptosis.\textsuperscript{8} Based on these facts, the decrease in both apoptosis and caspase\textsubscript{3} in buceng treatment, physiobiologically will be followed by increase in Bcl\textsubscript{2} expression. However, the effect of buceng extract treatment on Bcl\textsubscript{2} expression is still unknown.

The influence of buceng on Bcl\textsubscript{2} expression is important to investigate, since many antiapoptotic proteins regulate the apoptosis process. When buceng is proven capable to increase in Bcl\textsubscript{2} expression, will clarify the mechanism by which buceng reduces apoptosis. To date, studies on buceng have been extended to the increase of testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), decrease in apoptosis and caspase\textsubscript{3} whereas the effect of buceng on Bcl\textsubscript{2} expression has not been elucidated yet. Decrease in apoptosis pathway after buceng treatment is necessary to be explored, since the apoptosis process can be regulated by either caspase\textsubscript{3} or Bcl\textsubscript{2} and by both caspase\textsubscript{3} and Bcl\textsubscript{2}.\textsuperscript{7,8} In line with those arguments, study on buceng effect on Bcl\textsubscript{2} expression and its role in the down-regulation of apoptosis in order to enhance male vitality is necessary.

Bcl\textsubscript{2} is an anti apoptosis protein, existing in cell cytoplasm and has role in preventing the release of Cyt C and diablo/Smac, a protein that binds inhibitor apoptosis protein (IAP) from mitochondria.\textsuperscript{9,10} When released into cytoplasm, diablo/Smac will bind IAP in order to activate apotosome and caspase and then induce cell to undergo apoptosis.\textsuperscript{9,10} In addition, Bcl\textsubscript{2} is an antiapoptototic protein whose activity is dependent on testosterone level.\textsuperscript{11} Buceng, has previously been proven to up-regulate Te, LH, FSH level, down-regulate apoptosis process and caspase\textsubscript{3} expression in Sprague Dawley male rats.\textsuperscript{4,5} However, its effect on Bcl\textsubscript{2} expression has not yet been explored. Thus, the aim of the present study was to elucidate the effect of buceng on Bcl\textsubscript{2} expression in penile and prostate tissues of Sprague Dawley male rats.

This was an experimental study on 24 Sprague Dawley male rats, 90 days old, and body weight of 300 grams. All animals were randomly allocated into 4 groups, consisting of 6 rats in each group. Group A, normal rats. Group B, castrated rats and treated with buceng of 100 mg/day in 2 ml water, per oral (Cast-Bcg). Group C, castrated rats and treated with 2 ml of water as placebo against buceng (Cast-Plac). Group D, castrated rats, treated with mesterolone 6.75 mg/day, per oral, as exogenous testosterone (Cast-Mest). All rats were treated for 30 days.

Buceng extract was obtained from soxhlet extraction method with water as a solvent. The effective dose of buceng 100 mg/day has been determined according to the buceng previous study.\textsuperscript{5,5} All rats underwent acclimatization for a week in individual cage. All rats got daily chow and ad libitum access to tap water. After a week acclimatization, rats in group A were sacrificed at 7 a.m. After treated for consecutive 30 days, rats in group B, C, and D were sacrificed and their tissues of penis and prostate for Bcl\textsubscript{2} expression examination as normal reference. Rats were given buceng in group B, aquadest in group C, mesterolone in group D orally for 30 days at 7 a.m. After treated for consecutive 30 days, rats in group B, C, and D were sacrificed and their penile and prostate tissues taken for measurement of Bcl\textsubscript{2} expression. Measurements of Bcl\textsubscript{2} expression were performed by immunohistochemical method (kit: Bcl-2/100/ D5 from novocasta) and then observed by light microscope in 400x magnification in each 5 fields. The Bcl\textsubscript{2} expression was calculated as percentage of total Bcl\textsubscript{2} expressing cells to total cells (total Bcl\textsubscript{2} expressing cells/total cells x 100). Before examination, all penile and prostate tissues were frozen in nitrogen solution and kept in temperature of -20°C.\textsuperscript{12}

The experimental study has taken place at the Animal Experimental Development Unit and Pathological Anatomy Sardjito Hospital/School of Medicine Gadjah Mada University. Manova test
was used to examine the difference among groups, and HSD Tukey was implemented to evaluate the difference among groups. The significant level of all examinations was set at \( p \leq 0.05 \).

**RESULTS**

Genetic and age variability of rats in the present study can be neglected, since all rats were bred in the same growth condition. The 30 days treatment resulting \( \text{Bcl}_2 \) expression both in penile and prostate, shown in table 1.

As shown in table 1 both \( \text{Bcl}_2 \) expression in penis and prostate were significantly different among 4 groups, \( p < 0.001 \).

**Difference of \( \text{Bcl}_2 \) expression of penile**

Observation with 400x magnification of light microscope on immunohistochemical staining of penile tissues can be seen in figure 1.

The numbers of cells (cytoplasm), in which \( \text{Bcl}_2 \) positive expression (brown) and \( \text{Bcl}_2 \) negative expression (blue) in each five observation fields were counted as shown in table 1. That table indicates that the highest percentage mean of \( \text{Bcl}_2 \) positive expression was in normal group, followed by Cast-Bcg group, Cast-Mest group, and the lowest was in Cast-Plac group (figure 2).

**Difference of \( \text{Bcl}_2 \) expression of prostate**

Light microscopic scanning with 400x magnification on immunohistochemically stained prostate tissues preparation provides results as shown in figure 3.

All data were collected from these observation, and the numbers of cells (cytoplasm) in which \( \text{Bcl}_2 \) expression was positive (brown) and \( \text{Bcl}_2 \) negative expression (blue) in each five observation fields were counted as shown in table 1. That table indicates that the highest percentage mean of \( \text{Bcl}_2 \) positive expression was in normal group, followed by Cast-Bcg group, Cast-Mest group, and the lowest was in Cast-Plac group (figure 2).

![Figure 1. Bcl2 expression in penile tissue. a) normal, b) castrated-buceng group, c) castrated-placebo group, d) castrated-mesterolone group.](http://mji.ui.ac.id)

![Figure 2. Bcl2 expression in prostat tissue. a) normal, b) castrated-buceng group, c) castrated-placebo group, d) castrated-mesterolone group.](http://mji.ui.ac.id)

![Figure 3. Body weight and Bcl2 expression in penile and prostate cells](http://mji.ui.ac.id)

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<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
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<tr>
<td></td>
<td>A (Normal) (n = 6)</td>
<td>B (Cast-Bcg) (n = 6)</td>
<td>C (Cast-Plac) (n = 6)</td>
<td>D (Cast-Mest) (n = 6)</td>
<td>p (Manova)</td>
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<td>BW (gram ± SD)</td>
<td>268.64 ± 28.03</td>
<td>267.78 ± 25.20</td>
<td>268.65 ± 28.53</td>
<td>266.42 ± 19.55</td>
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<td>( \text{Bcl}_2 ) penile (%) ± SD</td>
<td>82.64 ± 3.70</td>
<td>73.82 ± 8.40</td>
<td>53.03 ± 21.3</td>
<td>77.12 ± 6.20</td>
<td>0.000</td>
</tr>
<tr>
<td>( \text{Bcl}_2 ) prostate (%) ± SD</td>
<td>84.15 ± 4.07</td>
<td>78.18 ± 7.83</td>
<td>50.90 ± 21.84</td>
<td>78.14 ± 5.40</td>
<td>0.000</td>
</tr>
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http://mji.ui.ac.id
expression was negative (blue) from each five fields observation were counted and results are shown in table 1. This table indicates that the highest Bcl₂ positive expression was in normal group, followed by Cast-Bcg group, Cast-Mest group, and the lowest was in Cast-Plac group (figure 3).

DISCUSSION

The results of the present study indicate that buceng is capable to increase Bcl₂ expression of penile and prostate cells comparable to that of mesterolone and endogenous testosterone. Thus, the effect of buceng is attributable to up-regulation of Te level. This statement is in accordance with previous studies showing that buceng has been proven capable to up-regulate testosterone level in Sprague Dawley male rats as well as to decrease caspase₃ expression and in turn apoptosis of penile and prostate cells. Caspase₃ is an IL-1 beta converting enzyme (ICE) like protease that is able to break peptide chains at the point following aspartic acid residues. At least, there are 10 types of caspases that have been identified and engaged in apoptosis process. Many of those caspases were arranged in a hierarchy, beginning from caspase₁, caspase₄, caspase₅, and caspase₁₀ as initiator caspase, then activates caspase₁₂ which serves as an amplifier,

Of those various caspases are inhibited by Bcl₂, a protein of cytoplasm that extremely testosterone dependent. In conjunction with Bcl₂, they constitute antiapoptotic protein. An important result of this present study is that the increase in Bcl₂ expression of penile and prostate cells in response to the treatment with buceng extract which commensurates with normal and mesterolone groups.

Mesterolone (17-betahydroxy-1-alpha-methyl-5alpha-androstan-3-one, C₁₇H₂₀O₃), is a derivative of dehydrotestosterone (DHT) that is frequently used as replacement therapy in male hypogonadism. It can be administered orally and rapidly absorbed without metabolism or inactivation in the liver. Thus, testosterone serum level will increase in accordance with the dose given. In this study, the dose of mesterolone was 6.75 mg/day or equivalent to 0.02 - 0.06 mg/kg BW/day, based on the physiological calculation for 70 kg man. Higher or supraphysiologic dose of mesterolone will result in intoxication indicated by weak, sluggish, even unmoved rats.

Compared to Protodiocin (Tribulus terrestris L), a natural androgen that has long been launched in the market, buceng that also has long been utilized to increase male vitality and sexual function since about 3 centuries ago possess a little bit superiority in improving sexual and reproductive potencies in male hypogonadism. Various evidences indicated that protodiocin was capable to improve sexual behavior parameters such as intra-cavernous pressure, testosterone level, DHT, DHEA, and NO level in animal models. Likewise, buceng was capable to increase testosterone level, endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), and cyclic guanosin monophosphate (cGMP). Moreover, buceng was able to stimulate both LH and FSH secretions, whereas protodioscin just stimulates LH secretion without affecting increased FSH secretion. Increase in LH secretion results in increasing testosterone level secreted by Leydig cells in testis, while increased FSH secretion induces LH receptor expression on Leydig cells, and in turn up-regulates testosterone level, subsequently increases sexual function and spermatogenesis. As such, an illustration may be presented a report from Phillip and colleagues in 1998 that the patient who had the defect in the beta subunit of FSH and FSH receptor will show delayed puberty and had low serum testosterone and FSH but high LH concentrations. Albeit that report was not exactly appropriate, nonetheless it indicates that the existence of both LH and FSH are essential for maintenance of testicular function like buceng is. The probable similarity in effect of protodiocin and buceng is attributable to the similarity in chemical structure of furostanol contained in protodiocin and sitosterol in buceng. The similarity of chemical structure lies on OH group bound to carbon atom 3 in the nucleus of cyclopentanoprehydrofenantren. The OH group leads to formation of glycoside binding between sitosterol (aglicon) and olygosacharide (hexose or mannose). Consequently, sterol physical properties might be transformed from nonpolar to polar ones. Based on the effect and structure similarities of furostanol and buceng, deduction can be proposed that sitosterol of buceng is included in the family of furostanol compound which is capable to increase DHEA level as well as testosterone. Accordingly, both buceng and furostanol can be
used as hormone replacement therapy in male hypogonadism.

Male hypogonadism is associated with significant increase of morbidity and decline in quality of life, especially related to skeletal muscles frailty caused by sarcopenia, and sarcopenia due to apoptosis.16,27 Various evidences indicated that high level of testosterone capable to decrease in apoptosis on any cells that possess testosterone receptors.31,28 On the other hand, down-regulation of testosterone level gives rise various organs, in particular penile and prostate which is regulated by testosterone experiencing reduction of size and function mediated by increasing apoptosis.11 The previous study showed that buceng was capable to increase testosterone level,2 and in the present study indicates that buceng is capable to increase Bcl-2 expression in penile and prostate tissues, hence decrease their apoptosis.4 Taken together, the results of the previous and the present studies and in line with biological plausibility, could explain the pathway of buceng effect on apoptosis in penile and prostate tissues. The explanation of that pathway is that buceng could inhibit apoptosis through increasing testosterone level and Bcl-2 expression, followed by decreasing caspase expression, and then apoptosis. It can be expected that buceng can be used as a hormone replacement therapy related to male hypogonadism or infertility. In addition, buceng is relatively safe, since the majority of males who experienced decrease in vitality have used it since 3 centuries ago, without adverse effect that has been reported.

In conclusion, the treatment of buceng with dose of 100 mg/day for consecutive 30 days in castrated Sprague Dawley male rats is capable to increase in Bcl-2 of penile and prostate cells, comparable to that in normal rats and mesterolone treated castrated rats.

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Conflict of interest
The author affirm no conflict of interest in this study.

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