Basic Medical Research

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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ABSTRAK

Latar belakang: *Pemberian buceng kombinasi pasak bumi (Eurycoma longifolia* Jack*) dan purwoceng (Pimpinella alpine* Molk*) telah terbukti mampu meningkatkan kadar testosteron, menurunkan apoptosis, dan ekspresi caspase$_3$. Bcl_2 adalah protein anti apoptosis yang ditemukan dalam sitoplasma. Tujuan penelitian ini adalah untuk mengungkap efek buceng terhadap ekspresi Bcl_2 pada jaringan penis dan prostat tikus.*

Metode: *Studi experimental dengan 24 tikus jantan Sprague Dawley umur 90 hari, berat badan ± 300 gram, dibagi menjadi 4 kelompok secara acak. Kelompok A tikus normal, kelompok B tikus dikastrasi dan diberi buceng 100 mg/hari, per oral (Cast-Bcg). Kelompok C tikus dikastrasi dan diberi air 2 ml sebagai plasebo dari buceng (Cast-Plac). Kelompok D tikus dikastrasi, diberi mesterolon 6,75 mg/hari, per oral sebagai testosteron eksogen (Cast-Mest). Pengamatan dilakukan selama 30 hari. Uji Mannova digunakan untuk menguji perbedaan ekspresi Bcl_2 di antara kelompok dengan tingkat signifikansi $p \leq 0.05$.*

Hasil: *Proses kastrasi menurunkan ekspresi Bcl_2 pada penis dan prostat (53,0 dan 50,9%) secara signifikan dibanding tikus normal (82,6 dan 84,2%, $p < 0.001$). Pemberian mesterolon menyebabkan peningkatan ekspresi Bcl_2 (77,1 dan 78,1%) mendekati normal. Normalisasi ekspresi Bcl_2 juga diperoleh dengan pemberian buceng (73,8 dan 782%).*

Kesimpulan: *Pemberian buceng dapat meningkatkan ekspresi Bcl_2 pada jaringan penis dan prostat, setara dengan normal pada tikus kastrasi yang mendapat mesterolon.*

ABSTRACT

Background: Treatment with *buceng* combination of *Eurycoma longifolia* Jack and *Pimpinella alpine* Molk has been proven to increase testosterone level, decrease apoptosis and caspase$_3$ 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 $p \leq 0.05$.

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%, respectively, $p < 0.001$). 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

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Pasak bumi (*Eurycoma longifolia* Jack) and purwoceng (*Pimpunella alpina* Molk) both are Indonesian indigenous plants[1,2] combination of them so called buceng. Treatment of buceng extract has been proven to increase serum testosterone level, suppress apoptosis, and decrease $caspase_3$ expression.[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.[6] $Caspase_3$ is a proapoptotic protein that serves as an executor of apoptosis,[7] whereas Bcl_2 is an antiapoptotic protein preventing apoptosis.[8] Based on these facts, the decrease in both apoptosis and $caspase_3$ in buceng treatment, physiobiologically will be followed by increase in Bcl_2 expression. However, the effect of buceng extract treatment on Bcl_2 expression is still unknown.

The influence of buceng on Bcl_2 expression is important to investigate, since many antiapoptotic proteins regulate the apoptosis process. When buceng is proven capable to increase in Bcl_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 $caspase_3$,[3-5] whereas the effect of buceng on Bcl_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 $caspase_3$ or Bcl_2 and by both $caspase_3$ and Bcl_2.[7,8] In line with those arguments, study on buceng effect on Bcl_2 expression and its role in the down-regulation of apoptosis in order to enhance male vitality is necessary.

Bcl_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.[9,10] When released into cytoplasm, diablo/Smac will bind IAP in order to activate apoptosome and caspase and then induce cell to undergo apoptosis.[9,10] In addition, Bcl_2 is an antiapoptototic protein whose activity is dependent on testosterone level.[11] Buceng, has previously been proven to up-regulate Te, LH, FSH level, down-regulate apoptosis process and $caspase_3$ expression in Sprague Dawley male rats.[4,5] However, its effect on Bcl_2 expression has not yet been explored. Thus, the aim of the present study was to elucidate the effect of buceng on Bcl_2 expression in penile and prostate tissues of Sprague Dawley male rats.

METHODS

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.[4,5] 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 and taken their tissues of penis and prostate for Bcl_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_2 expression. Measurements of Bcl_2 expression were performed by immunohistochemical method (kit: Bcl-2/100/D5 from novocastra) and then observed by light microscope in 400x magnification in each 5 fields. The Bcl_2 expression was calculated as percentage of total Bcl_2 expressing cells to total cells (total Bcl_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.[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 Bcl_2 expression both in penile and prostate, shown in table 1.

As shown in table 1 both Bcl_2 expression in penis and prostate were significantly different among 4 groups, $p < 0.001$.

Difference of Bcl_2 expression of penile

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

The numbers of cells (cytoplasm), in which Bcl_2 positive expression (brown) and 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 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).

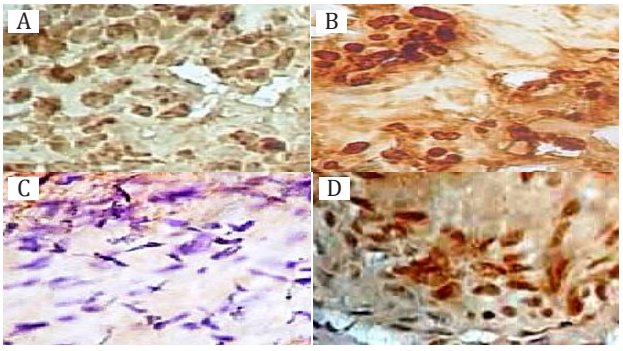

Figure 1. Bcl_2 expression in penile tissue. a) normal, b) castrated-buceng group, c) castrated-placebo group, d) castrated-mesterolone group.

Difference of 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 Bcl_2 expression was positive (brown) and Bcl_2

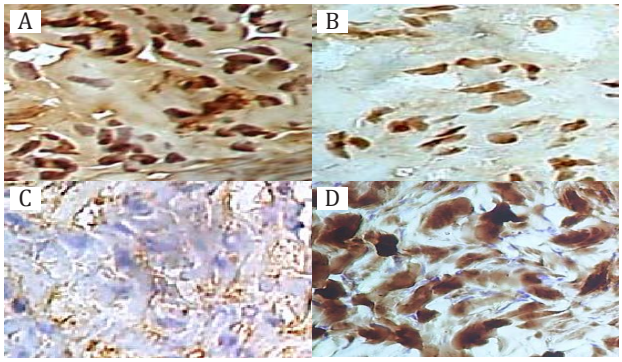

Figure 2. Bcl_2 expression in prostat tissue. a) normal, b) castrated-buceng group, c) castrated-placebo group, d) castrated-mesterolone group

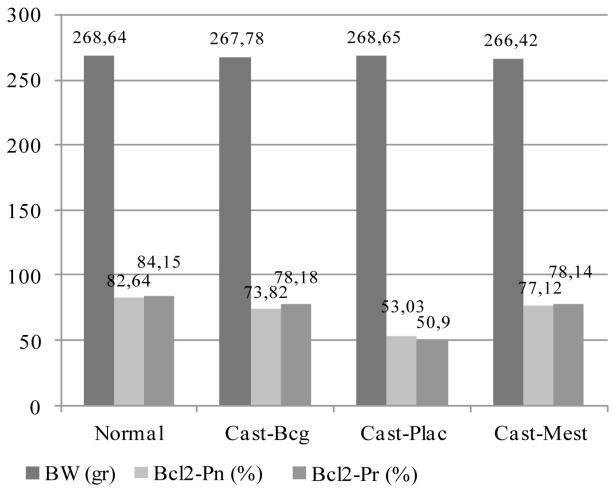

Figure 3. Body weight and Bcl_2 expression in penile and prostate cells

Table 1. Body weight and Bcl_2 expression in penile and prostate cells

Variables	Groups				
	A (Normal) (n = 6)	B (Cast-Bcg) (n = 6)	C (Cast-Plac) (n = 6)	D (Cast-Mest) (n = 6)	p (Manova)
BW (gram ± SD)	268.64 ± 28.03	267.78 ± 25.20	268.65 ± 28.53	266.42 ± 19.55	0.994
Bcl_2 penile (% ± SD)	82.64 ± 3.70	73.82 ± 8.40	53.03 ± 21.3	77.12 ± 6. 20	0.000
Bcl_2 prostate (% ± SD)	84.15 ± 4.07	78.18 ± 7.83	50.90 ± 21.84	78.14 ± 5.40	0.000

expression was negative (blue) from each five fields observation were counted and results are shown in table 1. This table indicates that the highest 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 3).

DISCUSSION

The results of the present study indicate that buceng is capable to increase Bcl_2 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 able to decrease $caspase_3$ expression and in turn apoptosis of penile and prostate cells.[4,5] $Caspase_3$ is an IL-1 beta converting enzyme (ICE) like protease that is able to break peptide chains at the point following aspartic acid residues.[13] 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 $caspase_2$, $caspase_8$, $caspase_9$, and $caspase_{10}$ as initiator caspase, then activates $caspase_1$ which serves as an amplifier,[14,15] and finally activates $caspase_3$, $caspase_6$, and $caspase_7$ as an execution machinery. Of those various caspases are inhibited by Bcl_2, a protein of cytoplasm that extremely testosterone dependent.[11] In conjunction with Bcl_{xL}, they constitute antiapoptotic protein.[14] An important result of this present study is that the increase in Bcl_2 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-5-alpha-androstan-3-one*, $C_20H_32O_2$), is a derivative of dehidrotestosteron (DHT) that is frequently used as replacement therapy in male hypogonadism.[16] 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.[17] 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.[18] Higher or supraphysiologic dose of mesterolone will result in intoxication indicated by weak, sluggish, even unmoved rats.

Compared to Protodiocin (*Tribullus terestris* L), a natural androgen that has long been launched in the market,[19] buceng that also has long been utilized to increase male vitality and sexual function since about 3 centuries ago posseses 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.[20] Likewise, buceng was capable to increase testosterone level, endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), and cyclic guanosin monophosphate (cGMP).[21] Moreover, buceng was able to stimulate both LH and FSH secretions,[3] whereas protodioscin just stimulates LH secretion without affecting increased FSH secretion.[22,23] 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.[24] 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.[25] 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 cyclopentanoprehidrofenantren. 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.[26] 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.[20] 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.[11,28] 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,[3] 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 $caspase_3$ 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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