GLOBAL JOURNAL OF BIOLOGY, AGRICULTURE & HEALTH SCIENCES
(**Published By**: Global Institute for Research & Education)
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BETA AMYLOID CELLULAR EXPRESSION IN GUINEA PIG (*Cavia cobaya*) BRAIN WITH STEROID HORMONE DEPLETION IN THE ALZHEIMER'S DISEASE STUDY

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Abstract

The study aimed on characterisizing guinea pig as model to AD. It was done by observation in the celuller expression of guine pig brains with an association between depletion testosterone hormone and amyloid β (Aβ) level. Two groups of male guinea pigs (*Cavia cobaya*), six adult old (16-32 months) and six age old (32-48 months) with testosterone depletion as a result castration. Observations of the brain were conducted by histopathology analysis with hematoxylin and eosin staining, and immunohistochemistry with beta amyloid (Aβ) at first, third and fifth month after castration. The result showed that castration of adult and old guinea pigs after three months castration was associated with the formation of Aβ plaques in the brain. Common features which appeared include Aβ deposition in the brain parencym and blood vessels. Therefore, guinea pigs are potentialas animal model to study Aβ formation in relation to the pathogenecity of AD.

Key words : Alzheimer's disease, testosterone, guinea pigs, Aβ, castration.

Introduction

Alzheimer's disease (AD) is an *irreversible* neurodegeneratif disease which characterized by the loss of memory and impairment of multiple cognitive functions (Hirtz *et al*. 2007). The mayor pathological features in the brain of AD patients are presence of intraneufibrillary tangles and extracellular beta amyloid (Aβ) deposits, particularly in the regions related to memory function dan cognition (Reddy and Weeney 2006). The Aβ is a peptide of 40 to 42 amino acids which accumulate as extrasellular amyloid plaques in the brain and cause nerve fiber damage leading to neurodegeneration. The formation of Aβ is predicted to play a major rule in the neuropathology of Alzheimer's disease (Checler 1995; Yanker 1996). Aβ is generated by proteolysis of amyloid precursor protein by the enzymatic actions of *beta-site amyloid precursor protein-cleaving enzim* 1 (BACE-1), β-secretase and ϒ-secretase (Haass and Selkoe 2007). Decreased clearence or the overproduction of Aβ may lead to accumulations of Aβ.

Testosteron is an important hormone that regulate reproduction organ and behaviour in male (Campbell and Reece 2002). With advancing age, men experience a significant decrease in circulating levels of testosterone (Morley *et al*.1997). The decline in total testosterone levels begins in thirthies and progresses at annual rate between 0.2-1%, decreases in other androgens including DHEA and androstenedione are observed with increasing age in men (Feldman *et al*. 2002). Low level of testosteron will increase risk factor for the development of AD (Hogervorst et al. 2003; Moffat et al. 2004; Rosario et al. 2004). Testosterone and its metabolite, dihydrotestosterone (DHT), have several important actions in the brain. Androgen actions are mediated in part via activation if androgen receptors (AR), which localized in many brain areas including regions important for learning and memory, function such as hippocampus and amygdala (Kerr et al. 1995; Simerly et al. 1990; Tohgi et al. 1995).

Studies on the formation of the Aβ plaques and the neuropathology AD in humans are difficult since the diagnosis of AD requires postmortem examination as the final confirmatory diagnosis. Therefore, many animal models have been developed to mimic the biological conditions in humans. Guinea pigs are non-transgenic animals which produce Aβ peptide which is identical to human Aβ in the amino acid sequences. Additionally, Amyloid Precursor Protein (APP) processing in guinea pig, primary neuronal cultures, is demonstrably similar to analogous cultures of human origin (Beck et al. 2003). Therefore, the animal might be an ideal model for the assessment of changes of physiological levels of Aβ as a result of hormone depletion (Beck et al. 1997).

This study aimed on caracterizing the guinea pigs as animal model for AD with observing celullar activity and the metabolisme of Aβ in the brain as a result testosterone depletion. This objective was done by using castrated guinea pigs to mimic the decrease in the testosterone level in older men (Kaufman and Vermeulen 2005).

Materials and Methods

The animal husbandry was carried out in the mall animal facility of PT. INDOANILAB, Taman Kencana, Bogor, Indonesia including the daily observation and sampling. Animals were necropsied at the Laboratory of Pathology, Study

Primate Centre, IPB, and the histopathology analysis was conducted at the Laboratory of Pathology, Faculty of Veterinary Medicine, IPB. Ethical approval for the experiment was given by the local ethical committees for animal research.

Two groups of male guinea pigs (*Cavia cobaya*) consisted of six adult old (16-32 months) and six age-old (32-48 months), were castrated to obtain the testosterone depletion. The animals were caged as individual, and fed by commercial pellet and drink with supplementation of vitamin C *ad libitum*. The procedure of castration and sampling of blood and CSF, were carried out under anaesthesia with combination of ketamin 50 mg/kg BW, xylazine 20 mg/kg BW intraperitonel, followed by ketoprofen 20 mg/kg BW intramuscular to provide analgesia after the procedure. The anaesthetic dose above provide 30 minutes of deep anaesthesia. Necropsies were scheduled at the first, thirth and fifth month after castrated with euthanasi. Two animals were sampled for blood twice in the early and the late of the study, precisely before euthanasia. CSF was collected from the cisterna magna by spine needle. Plasma testosterone was measured by ELISA (DRG EIA 1559). Concentration of $A\beta_{42}$ in CSF samples were measured using Invitrogen™ human beta amyloid peptide 42 (catalog number KHB3441). The minimum detectable level of human $A\beta_{42}$ is 10 pg/ml and had no cross-reactivity with other Aβ species ($A\beta_{12}$, $A\beta_{20}$, $A\beta_{28}$, $A\beta_{35}$, $A\beta_{40}$, etc) and other neurodegenerative markers, such as α-synuclein and APP.

Observations of the brain were conducted on macroscopic and histophatological feature in the part of parietal, temporal lobe, hippocampus dan cortical area. Expression of Aβ was tested using Hematoxilin & Eosin (HE) staining and immunohistochemistry. The brain from guinea pigs were fixed in the buffered neutral formalin 10% for 24 hours, and embedded in paraffin wax prior to cut into 5µm sections with a microtome. In order to be able to detect β amyloid in the brain sections, immunohistochemistry technique was conducted by using monoclonal antibody of rabbit anti human Amyloid A4 (United States Biological, Massachusetts). The sections were treated with sodium citrate buffer 95^0 C for 30 minutes for antigen retrieval and blocking of endogenous peroxidase was done by treating the sections with 3% H_2O_2 in methanol for 30 menit in the room temperature. Non spesific binding was blocked by 1% normal serum in 10% skim milk in PBS for 30 minutes at the room temperature. The sections were incubated with a rabbit monoclonal antibody anti human Amyloid A4 (United States Biological, Massachusetts) as the primary antibody (1:500 dilution in PBS) overnight at 4^0C. After washing in PBS, the sections were incubated with a biotinylated secondary antibody for 30 minutes (DAKO-LSAB+ system HRP kit, Dako Corporation, Denmark), and then Streptavidin-Horse Radish Peroxidase (HRP) for 30 minutes at room temperature. This was followed by applying diaminobenzidine (DAB) substrate at room temperature until the intensity of staining was obtained (2-5 minutes). Sections were rinsed in distilled water and then followed by counterstained with hematoxylin to visualize tissue morphology and then mounted with *entellan*. A section was included to serve as negative control. The tissues were observed by light microscope with objective magnification of 20 and 40 times. Positive reaction to Aβ gave a brown colour of the tissue.

Results and Discussion

Castration produced significant decreased of plasma testosterone level from 70% until 80% (Table 1) at the first, third and fifth month after castration from two group. The decline was associated with low Aβ level of plasma and CSF at the adult old group, but inconsistent decrease at the age old group (Table 2). The findings emphasized that testosterone in adult group was related with the amyloid clearance, meaning that the hormonal depletion was effectively reduce the amyloid circulation and a possible amyloid retention in the brain. In contrary with the old group, there was no significant difference occurred among the hormonal depletion with castration. The findings showed that the hormonal depletion at the old stage may not be relevant to stimulate amyloid retention in the brain indicated by a low level of amyloid circulation in CSF or plasma level.

Table 1 Mean differences of plasma testosteron level from treatment group (ng/ml)

Group	Period					
	1 month		3 month		5 month	
	Before castrated	After castrated	Before castrated	After castrated	Before castrated	After castrated
I	1.95±0.91	0.4±0.28	1.96±0.91	0.31±0.08	2.95±0.14	0.35±0.23
II	2.83±0.38	0.68±0.17	2.24±0.76	0.74±0.09	2.42±1.62	0.3±0.21

Table 2 Mean differences of amyloid-β in the blood plasma and CSF levels from treatment group (pg/ml)

Parameter	Group	Period		
		1 month	3 month	5 month
Mean plasma Aβ level	I	65.75±37.12	61.5±15.90	37.625±6.18
	II	28.25±15.90	40±13.08	33.875±7.95
Mean CSF Aβ level	I	69.625±41.54	39.5±7.42	32.375±5.83
	II	49±31.81	53±1.59	37.375±1.23

Pathological changes could be observed in the guinea pig brain with immunohistochemistry staining. There was clear positive staining of Aβ which began third month after castrated of adult and age old guinea pigs. Accumulations of Aβ were observed in the vessel walls of the cortex, frontal lob, temporal lob, hippocampus and brain parencym. Expression of Aβ was also detected in first month after castrated but not clear (Figure 1). Positive immunostaining of Aβ

was clear at thirth month after castrated in the adult group and old group. The findings showed that the formation of Aβ associated with depletion hormone and long of castration.

The problem of this study, the risk of mortality of the guinea pigs during and or after castration, become the major constraint in this study. Therefore, high numbers of animals were included at the beginning of study to avoid loss of animal at the end of the study. We must avoid to contamination during the study and stress of guinea pigs.

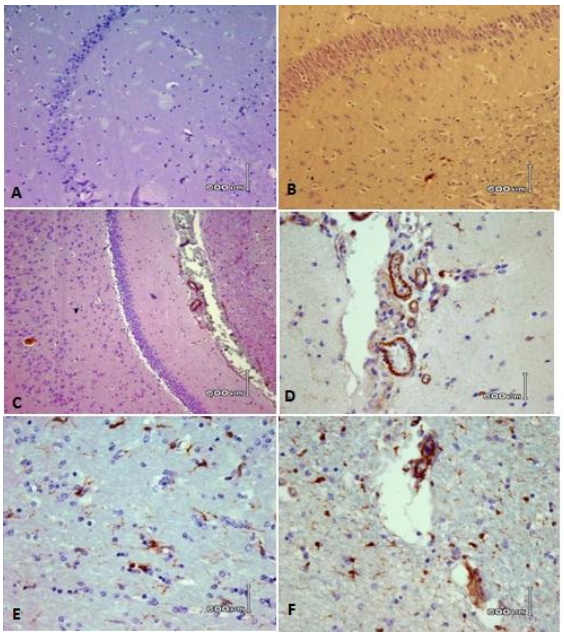

Figure 1: Histopathological features of the brain of the guinea pigs. A. Negative control staining, B. Unclear expression of Aβ in the first month necropsied of castrated guinea pigs (Aβ, 20x). C. Positive Aβ immunostaining in the hippocampus (Aβ, 20x). D. Aβ accumulation in the walls of cerebral arteries and capillaries (Aβ, 40x). E. Aβ accumulation in the brain parencym of cortex (Aβ, 40x). F. Aβ accumulation in the brain parencym and vessel walls of temporal lobe (Aβ, 40x)

This study is conducted to observe the correlation between testosterone, CSF and plasma Aβ levels as the role of Alzheimer's disease pathogenesis. Plasma testosterone level significantly declined after castrated, and this is associated with age-related risk factor for development of AD. Testosterone, the primary male sex steroid hormone, is depleted gradually as a normal consequence of aging (Morley 2001). Testosterone is an androgen steroid hormone that binds and activates androgen reseptors (AR) and influence of the neuron functions (McPhaul and Young 2001) and the level is modulated by androgen (Lu *et al*.1998). Neuron morphology in the area of hippocampus is very sensitive to androgen level change. High level of AR is expressed in the brain area which important to cognition function, such as hippocampus, amygdala and cerebral cortex in the rodentia (Simerly *et al*. 1990). Testosterone has been shown to regulate levels of Aβ in the cell culture, rodent's model and human brain (Pike et al. 2006). The mechanism by which androgens regulates Aβ is not clearly understood, but presumably involves one or more between three general pathways, which are direct action through AR-dependent pathways, indirect actions through estrogen pathways via testosterone aromatization to estradiol, and indirect actions through gonadotropin pathways via testosterone modulation of the hypothalamic-pituitary-gonadal axis (Rosario and Pike 2008). Additionally, the low testosterone level resulting from castration cause a loss negative feedback of testosterone on the gonadotropin luteinizing hormone (LH), leading LH levels, and in vitro to favor the amyloidogenic pathway, increasing Aβ production (Bowen et al.2004).

Castrated animals had significantly decreased Aβ level in cerebrospinal fluid (CSF) and plasma in the adult and age old group for along time (5 months). In elder human with lower testosterone level, the amyloid aggregation tend to increase in the brain parenchyme and resulting to low circulated level, both in CSF and plasma (Gillet et al. 2003). Therefore, our castrated guinea pigs agree with above human's phenomenon which indicate the experimentally castrated guinea pigs might be an ideal animal model for amyloid related disease such as Alzheimer's disease. The lower Aβ level in the plasma and CSF showed that an age-related failure of clearances Aβ from the brain. They are associated with the accumulation of Aβ in the vessel walls and brain parencyma. The histopathologic changes are consistent with an accumulation of Aβ in the walls of cerebral arteries and capillaries, in the cortex, temporal lobe in the thirth necropsied of castrated guinea pigs. However, our result was contrasted with the research conducted by Wahyoepramono et al. 2008 that explained castrated guinea pigs had significantly increased Aβ level in CSF on day-18 and day-36. The concentration of Aβ in plasma tended to increase only after day-36 following castration, suggesting it cleared from the body.

The changes were observed in the brains of these adult and age old guinea pigs after three months castration, which emphasize their value as animal model for studies of human aging and AD. Common features which appeared include Aβ deposition in the brain parencym and blood vessels. We found typical changes of AD including neuritic plaques, in addition cerebral amyloid angiopathy (CAA) in the brain parencym. These findings indicate that pathological protein deposits may be detectable in the brain tissue at three months after castrated guinea pigs. In an aggressive model of transgenic use expressing double mutation (APP and PS1), Aβ deposits appear as early as 2.5 months (Blanchard et al 2003). Deposition of Aβ in basement membrane in artery walls appear to impair the perivasculer damage of interstitial

fluid (ISF) and solutes from the brain. Perivascular lymphatic drainage pathway by which ISF and solutes drain from the brain have been defined in experimental animals and Aβ is deposited in those pathway in CAA as its drainage fails with age (Carare et al. 2008). Guinea pigs are non-transgenis animal which produce Aβ peptide which explain that it requires more time to be developed in the brain. There was no positive staining of Aβ in the brain of guinea pigs after day-36 following castration (Wahjoepramono et al. 2009).

In humans, various propotions of neuritic and diffuse plaques are found in aging and AD, and neocortical neuritic plaques are considered as a key feature in the histopathological diagnosis in AD (Mirra et a. 1991). Amyloid is deposited in the walls of arteries and capillaries as cerebral amyloid angiopathy (CAA) in the brains of older individuals and AD. CAA reflects age-related failure elimination of amyloid-beta from the brain along perivasculer lymphatic drainage pathways. Failure of elimination of Aβ along perivasculer pathways coincides with a reduction in enzymatic degradation of Aβ, reduced absorption of Aβ into the blood and related stiffening of artery walls that appears to reduce the force for lymphatic drainage (Weller et al. 2009). Deposition of Aβ in the walls of cerebral arteries and capillaries has a prevalence of 90% to 96% in patients with AD (Love et al. 2009) and is present in 30% of non-demented individuals over the age of 60 years (Esiri and Wilcock 1986).

Conclusion

Castration of adult guinea pigs after three months was associated with the formation of $A\beta_{42}$ plaques in the brain and lower circulating CSF level of $A\beta_{42}$ indicate an acceleration of $A\beta_{42}$ formation was able to modulate by testosterone depletion. Therefore adult guinea pigs are potential as animal model to study Aβ formation in the pathogenecity of Alzheimer's disease.

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