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CONTENTS

The effects of furosemide on kidney damage in acute kidney injury rat models

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ABSTRACT

The most frequent cause of acute kidney injury (AKI) is ischemia reperfusion injuries that causes inflammation. Furosemide is still used in AKI's therapy. The advantages and disadvantages of furosemide in AKI remain controversial. The aim of the study was to investigate the effect of furosemide on kidney damage in AKI rat models. Twenty-five male (2-3 months old) Sprague-Dawley rats were divided into 5 groups; sham operation (SO, n=5), ischemic-reperfusion (IR, n=5), IR+furosemide 3.6 mg/kgBW (IR+F1, n=5), IR+furosemide 7.2 mg/kgBW (IR+F2, n=5), and IR+furosemide 14.4 mg/kgBW (IR+F3, n=5). Abdominal surgery was performed under ketamine anesthesia to produce ischemic reperfusion (IR) by mean of renal artery clamping for 45 min. Urine output, serum creatinine level, tubular injury score, and TLR4 gene expression were examined to investigate kidney damage. Periodic acid-schiff (PAS) staining was measured to examine kidney tubular injury. Data were analyzed using One-Way ANOVA and Kruskal-Wallis test with significance level of $p<0.05$. AKI rat models which were given 3.6 and 7.2 mg/kgBW of furosemide (0.014±0.001 mL/min; and 0.012±0.007) showed higher ($p>0.05$) creatinine clearance compared to IR (0.009±0.003) while administration of 14.4 mg/kgBW furosemide (0.009±0.004) denoted equal creatinine clearance to IR ($p>0,05$). Kidney tubular injury score of 3.6 mg/kgBW furosemide (2.89±0.13) was lower ($p>0.05$) than IR (3.26±0.19) whereas 7.2 mg/kgBW and 14.4 mg/kgBW furosemide (3.55±0.26; 3.83±0.19) were higher ($p<0.05$) than IR. Administration of 3.6 mg/kgBW furosemide (0.99±0.08) indicated lower ($p<0.05$) TLR4 gene expression than IR (1.20±0.08) whilst 7.2 mg/kgBW furosemide (1.23±0.13) was not-significantly higher ($p>0.05$) and 14.4 mg/kgBW furosemide (1.63±0.12) was significantly higher ($p<0.05$) than IR. In conclusion, administration of 3.6 mg/kgBW furosemide reduces kidney damage in AKI rat models while higher dosages (7.2 mg/kgBW and 14.4 mg/kgBW) increase kidney damage.

ABSTRAK

Penyebab *acute kidney injury* (AKI) yang paling sering adalah cedera iskemia-reperfusi sehingga menyebabkan timbulnya inflamasi. Pada penatalaksanaan AKI masih banyak digunakan furosemid. Keuntungan serta kerugian penggunaan furosemid pada AKI masih menjadi kontroversi. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian furosemid terhadap kerusakan ginjal pada model tikus AKI. Sebanyak 25 ekor tikus jantan umur 2-3 bulan galur *Sprague Dawley* dikelompokkan menjadi 5 kelompok, yaitu *Sham Operation* (SO, n=5), *Ischemia reperfusion* (IR, n=5), IR+furosemid 3,6 mg/kgBB (IR+F1, n=5), IR+furosemid 7,2 mg/kgBB (IR+F2, n=5), dan IR+furosemid 14,4 mg/kgBB (IR+F3, n=5). Luaran urin, kadar kreatinin serum, skor cedera tubulus, dan ekspresi gen TLR4 diperiksa untuk mengetahui adanya kerusakan ginjal. Dilakukan pewarnaan *Periodic Acid-Schiff* (PAS) pada sediaan histopatologi untuk menilai skor cedera tubulus

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ginjal. Data dianalisis dengan *One Way* ANOVA dan Kruskal Wallis (p<0,05). *Creatinine clearance* pada model tikus AKI yang diberi furosemid dosis 3,6 mg/kgBB (0,014±0,001 mL/min) dan dosis 7,2 mg/kgBB (0,012±0,007) lebih tinggi dari IR (0,009±0,003) (p>0,05), sedangkan dosis 14,4 mg/kgBB (009±0,004) sama dengan IR (p>0,05). Skor cedera tubulus ginjal pada model tikus AKI yang diberi furosemid dosis 3,6 mg/kgBB (2,89±0,13) lebih rendah dari IR (3,26±0,19) (p<0,05), sedangkan dosis 7,2 mg/kgBB (3,55±0,26) dan 14,4 mg/kgBB (3,83±0,19) lebih tinggi dari IR (p<0,05). Ekspresi gen TLR4 pada model tikus AKI yang diberi furosemid dosis 3,6 mg/kgBB (0,99±0,08) lebih rendah dari IR (1,20±0,08) (p<0,05), sedangkan dosis 7,2 mg/kgBB (1,23±0,13) lebih tinggi dari IR (p>0,05), dan dosis 14,4 mg/kgBB (1,63±0,12) mg/kgBB juga lebih tinggi dari IR (p<0,05). Dapat disimpulkan, pemberian furosemid dosis 3,6 mg/kgBB dapat memperbaiki kerusakan ginjal pada model tikus AKI, sedangkan pada dosis lebih besar (7,2 mg/kgBB dan 14,4 mg/kgBB) memperburuk kerusakan ginjal pada model tikus AKI.

Keywords: acute kidney injury - ischemic-reperfusion – furosemide – creatinine – kidney tubular injury

INTRODUCTION

Acute kidney injury (AKI) is a health problem due to increasing the incidence of AKI in both the developed and developing countries that increase mortality rate.[1–5] About 20% of AKI may progress to chronic kidney disease thereby increase the maintenance costs.[6,7] The use of furosemide in the treatment of AKI remains controversies. Furosemide improves GFR in patients with portal hypertension and ascites, as well as lower hyperkalemia, hyperchloremia, acidosis, and fluid overload on patien who are at risk of AKI.[8] Nonetheless, furosemide increase serum creatinine in cardiac surgery.[9] Furosemide is more effective than mannitol when given along with hydration fluids to prevent nephrotoxicity due cisplatin[10,11] but furosemide increase mortality in AKI patients with critical illness.[12] Furosemide in preclinical studies was known to decrease apoptosis and related gene expression in the IRI mouse model.[13] Inflammation is the main mechanism of AKI due to ischemia.[14] *Toll-like receptor4* (TLR4) activation is the major pathway of the innate imune response that started the kidney injury.[15] The study was conducted to investigate the effects of furosemide on kidney damage in AKI rat models.

MATERIALS AND METHODS

Animal model

Twenty-five Sprague-Dawley male 2-3 months old rats were used in the quasi-experimental study with post test only controlled group design. The rats were divided into 5 groups; sham operation (SO, n=5), ischemic-reperfusion (IR, n=5), IR+furosemide 3.6 mg/kgBW (IR+F1, n=5), IR+furosemide 7.2 mg/kgBW (body weight) (IR+F2, n=5), IR+furosemid 14.4 mg/kgBW (IR+F3, n=5). The sham operation used as control, and ischemic-reperfusion as AKI model.[16] Wheres in the group IR+F1, IR+F2, IR+F3 given furosemide 3.6, 7.2, 14.4 mg/BW, respectively, once giving after surgery. Determination of the dosage of furosemide according to Sinto and Nainggolan[17] which says that administration of furosemide can be useful in the first 12 h, and initially can be given intravenous furosemide bolus 40 mg. If the benefit is not visible the dosage can be doubled to 100-250 mg/times in 1-6 h rapidly or 10-20 mg/kgBW/d slowly with a maximum dose of 1 g/d. All animals terminated on day 3. All experimental procedures were conducted according to the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing,

Universitas Gadjah Mada, Yogyakarta. The ischemia-reperfusion injury model was performed under ketamine anesthesia 100 mg/kgBW. The AKI was induced by mean of clamping both renal pedicle using non-traumatic vascular clamp (Hammacher®) for 45 min. Then, both clamps were released and followed by reperfusion. The incision site closed using silk surgical thread 3/0 (One Med®). Blood serum was obtained from the retroorbital vein for creatinine measurement.

Measurement of tubular injury

Tubular injury score measurement was done by mean of Periodic Acid-Schiff (PAS) staining, examined with a light microscope (Olympus CX22®) and portraited with Optilab software with 400x magnification at the corticomedullary junction area as many as 15 fields per kidney. Scoring divided into 4 category, they were: 0-4 (0=normal; 1=tubular injury <25%; 2=tubular injury involve 25-50%; 3=tubular injury involve 50-75%; 4=tubular injury involve >75%). The assessment included renal tubular dilatation, loss of brush border of proximal tubules, depletion of the tubular epithelial cell and the accumulation of intraluminal cast.

Gen expression examination

Examination of TLR4 gene expression used RT-PCR. Total RNA was extracted using RNA Isoplus, followed by quantification of RNA concentration using sphectrophotometry. cDNA was made using Rever TraAce® (Toyobo, Japan, Cat.No.TRT-101) and random primer (Toyobo, Japan, Cat.No 3801), with PCR condition: 30°C for 10 min (denaturation), 42°C for 60 min (annealing), 99°C for 5 min (extension). Reverse transcriptase PCR was carried out to amplify the following specific cDNAs: forward rTLR4: 5' CAGGGAGCACGAGGCTTCTA-ACC-3', and reverse: 5'-CTTGTGCCCT-GTGAGGTCGTTGA-3'). PCR conditions: 94°C for 2 s (initial denaturation), 94°C for 10 s (denaturation), 60°C for 30 s (annealing) and 72°C for 1 min (extension) and 72°C for 10 min (last extension). The gene expression was quantified using ImageJ software. GAPDH was used as housekeeping gene.

Statistical analysis

Data were analyzed using Shapiro-Wilk test for normality and Levene test for homogeneity. Urine output, creatinine urine level, creatinine clearance, tubular injury score were analized by one-way ANOVA and followed by pos hoc LSD test. Creatinine serum level was analized by Kruskal Wallis test and followed by pos hoc Mann-Whitney test. A $p<0.05$ was used to determined the level of significance.

RESULTS

Significantly difference in urine output between the SO group (7.8±1.79 mL) and IR group (15.00±3.08) on day 3 after surgery but non-significantly difference between IR group (15.00±3.08) and the treatment groups IR+F1 (10.30±4.66), IR+F2 (14.2±4.92), IR+F3 (14.40±2.41) were observed. Serum creatinine level of IR group [1.23 mg/dL (1.10-1.66)] was significantly lower than IR+F3 group [3.53 (3.01-4.71)] ($p<0,05$) and non-significantly higher than IR+F1 [0.76 (0.68-1.81)] ($p=0.151$) and IR+F2 [1.19 (1.09-3.01)] groups ($p=0.841$). The urinary creatinine level of IR group (0.98±0.15) was significantly lower than IR+F2 (2.16±1.31) ($p=0.049$) and IR+F3 (3.18±0.61) ($p=0.001$) groups but non-significantly higher than IR+F1 group (1.76±1.18) ($p=0.179$). There was non-significantly higher in creatinine clearance of IR+F1 (0.014±0.001) and IR+F2 (0.012±0.007) compare to IR group (0.009±0.003) and equal to IR+F3 group (0.009±0.004) ($p>0.005$).

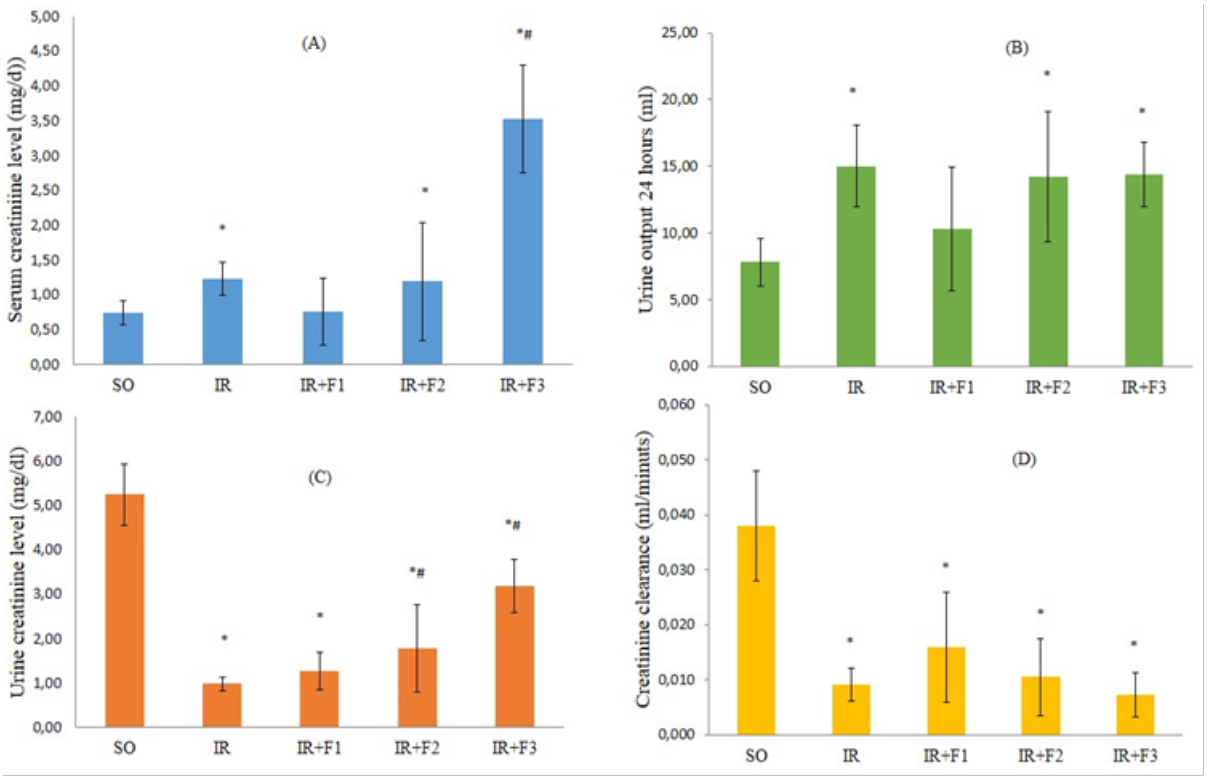

FIGURE 1. The effect of furosemide on serum creatinine level (A), urine output 24 hours (B), urine creatinine level (C), and creatinine clearance (D). * = p<0,05 vs SO, # =p<0,05 vs IR. SO (Sham Operation), IR (Ischemia-reperfusion), IR+F1 (Ischemia-reperfusion+furosemide 3.6 mg/kgBW, IR+F2 (Ischemia-reperfusion+furosemide 7.2 mg/kgBW), IR+F3 (Ischemia-reperfusion+furosemide 14.4 mg/kgBW).

The kidney tubular injury score of IR group (3.26±0.19) was significantly higher than IR+F1 group (2.89±0.13) (p=0.005) but significantly lower than IR+F2 (3.55±0.26) (p=0.024) and IR+F3 (3.83±0.19) (p=0.000) groups. Significantly lower in TLR4 gene expression of IR group (1.20±0.08) than IR+F1 group (0.99±0.08) (p=0.002) but non-significantly higher than IR+F2 (1.23±0.13) (p=0.680) and significantly higher than IR+F3 (1.63±0.12) (p=0.000) groups were reported.

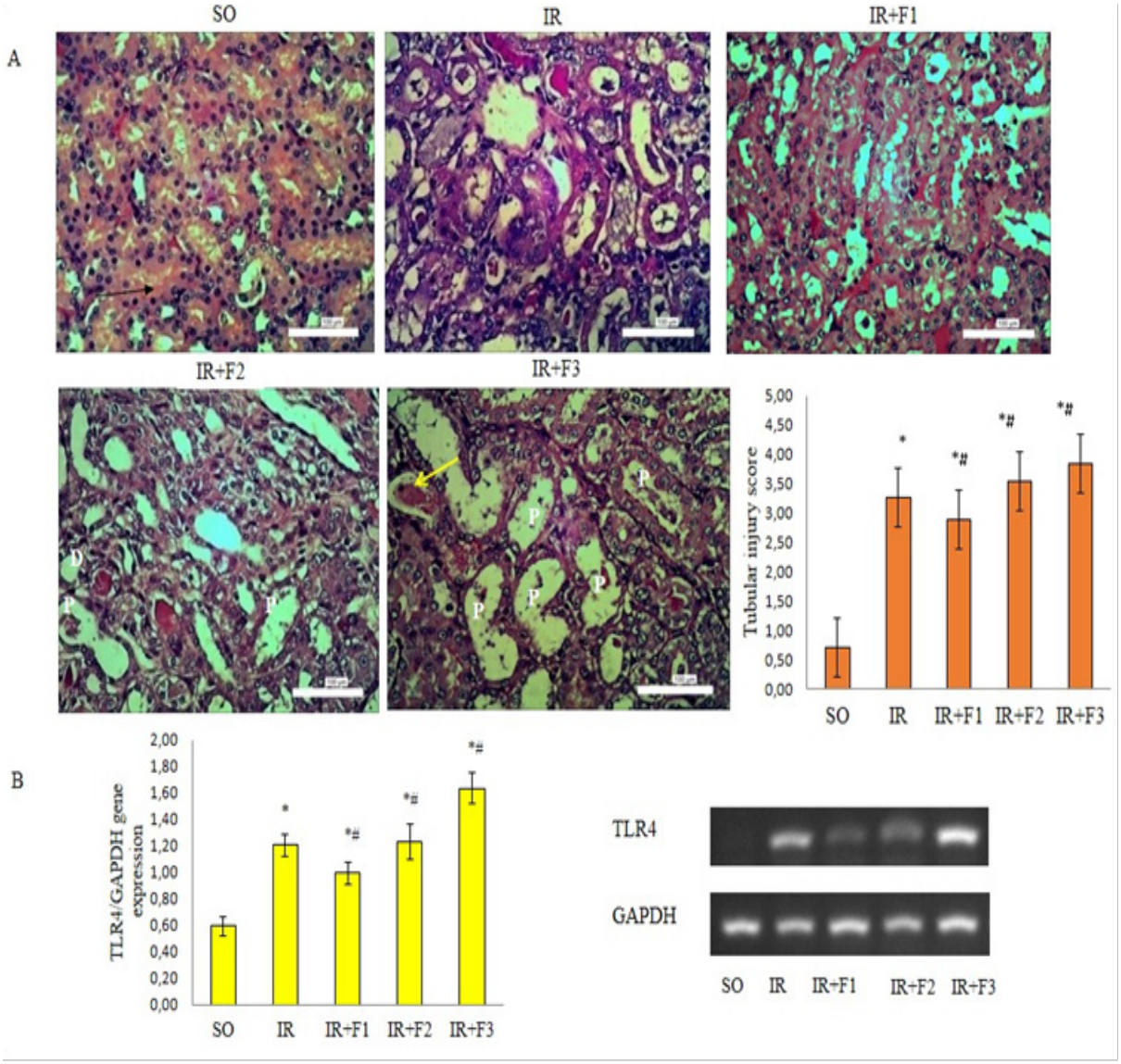

FIGURE 2. Renal histological picture on day 3 with PAS staining and tubular injury score (A). TLR4/GAPDH gene expression (B). * = p<0,05 vs SO, # =p<0,05 vs IR. SO (Sham Operation), IR (Ischemia-reperfusion), IR+F1 (Ischemia-reperfusion+furosemide 3.6 mg/kgBW, IR+F2 (Ischemia-reperfusion+furosemide 7.2 mg/kgBW), IR+F3 (Ischemia-reperfusion+furosemide 14.4 mg/kgBW). Black arrows showed brush border. Yellow arrows showed intraluminal cast. Yellow circle showed tubular dilatation.

DISCUSSION

The volume of urine output in the IR group is higher than the SO group. This result similar with Younan *et al*.[18] study, that in mice IR group had higher urine volume at 24 and 48 h after reperfusion compared with SO. Ischemia-reperfusion causes damage primarily in the proximal tubule S3 segment and the thick ascending loop of Henle. Ischemia causes the cord region becomes increasingly diminished oxygenation, causing severe damage. Due to damage involving the proximal tubules, the renal function related to the formation of urine is impaired, especially reabsorption. If this reabsorption function is impaired by tubular cells damage, then only a little water can be reabsorbed, and most will be excreted. In the IR group that were given furosemide had urine volume higher than the

SO group but lower than the IR group. The volume of urine was higher with the higher dose of furosemide. The timing of giving furosemide may affect the process of kidney damage both the functional and structural. Furosemide guard against partial damage shown by the improvement of the medullary hypoxia during the AKI, the impact is in the early phase after the occurrence of AKI than ongoing AKI. However the greater the dose of furosemide given, the more the urine volume.[19]

Creatinine serum level in IR group was 1.23 (1.10-1.66) higher compared to that of SO group 0.74 (0.48-0.91). As explained by Wu *et al*.[20] and Younan *et al*.[18], there were creatinine level increase in IRI group. In normal condition, creatinine is filtered by glomerulus but not absorbed. About 10-20% creatinine is excreted to proximal tubules. Thus, any damage in tubules will affect the process. Consequently, the creatinine serum level becomes higher. Creatinine serum level in IR group receiving furosemide increased as furosemide dose increase. This is possibly because at the time of ischemia occurs ATP depletion, and when there is furosemide as a ligand which binds to the transporter as a site of action, the ATP should be used for repairs but used to work, and the greater number of ligand binding, ATP getting a much needed so that the kidney getting damaged because the heavier work. In contrast, Lassnigg *et al*.[9] study demonstrated creatinine serum level increase in cardiac surgery. The negative effect of furosemide probably due to neurohormonal activation dan temporary blood pressure increase as the result of sympathetic neural activation dan renin angiotensin system. Those mechanisms might increase peripheral vascular resistance, left ventricle afterload, heart workload, and cardiac output reduction. Thus, they might worsen myocardial ischemia. Moreover, renal blood flow maldistribution induction through medular perfusion diversion due to cortex vascular resistance decline might promote tubular disfunction.[9]

Urine creatinine level was elevated in SO group (5.25±0.69) and depleted in IR group (0.98±0.15). In physiological condition, creatinine was filtrated by the glomerulus and excreted through urine hence, the creatinine level was elevated in urine dan depleted in serum and if there is any damage causing low creatinine level in urine. Urine creatinine level in IR+F1 was higher compared to that of IR group (IR+F1 vs IR (1.76±1.18 vs 0.98±0.15). The greater furosemide dose given the higher urine creatinine level (IR+F1(1.76±1.18), IR+F2 (2.16±1.31), dan IR+F3 (3.18±0.61)). This condition contradicted with serum creatinine level in this study that demonstrated serum creatinine raise as furosemide dose increase.

Creatinine is catabolism yield of muscle creatinine and distributed to entire body fluid. Mostly, creatinine is excreted by the kidney. Creatinine has low molecular weight (113D) that facilitate its simple movement through glomerular filtration barrier into tubular filtrate. Creatinine is not reabsorbed nor affected by urine flow.[21] About 10-20% creatinine is secreted into proximal tubules. Active secretion done by tubular cells is facilitated by that are OAT1 (organic anion transporter), OAT2, OAT3, OAT4, OCT1 (organic cation transporter), OCT2, OCT3, OCTN1 (organic cation transporter novel), OCTN2, MATE1 and MATE2-K.[22–24] Correspondingly, furosemide requires the transporter to reach its target that are OAT1, OAT2, OAT3, and OAT4.[25–27] Kim *et al*.[26] demonstrated OAT1 elevation in rat renal after 7 days furosemide administration. and the rise of OAT1 and OAT3 expression in IR model.[28] There is some similar transporter that involved in creatinine and furosemide secretion. The possible explanation of higher urine creatinine level in higher furosemide dose is due to higher serum creatinine level in IR group receiving furosemide hence, the excreted creatinine in urine is greater.

Creatinine clearance in the SO group was higher than the IR group and differently significant ($p<0.05$), it shows that the function of renal excretion in the SO group is better

than the IR group and vice versa. Creatinine clearance in the IR+F1 and IR+F2 groups were higher than IR group, and IR+F3 equal to IR group ($p>0.05$). In accordance with Heyman *et al*.[29] that creatinine clearance in the group of acute renal failure (ARF) that were given furosemide were higher than the ARF group on the first day ($p<0.05$), and had a tendency to rise on the 3rd day but was not significant ($p>0.05$). Creatinine clearance describes the renal excretory function and can be used to predict GFR. The greater the creatinine clearance value showed the better kidney functions, and vice versa. In this study of kidney function in the group which were given the common dose of furosemide was better than that the group which did not receive furosemide, and the group which was given higher doses of furosemide had worse kidney function than the group that did not receive furosemide. In contrast to Lassnigg *et al*.[9] that administration of furosemide in cardiac surgery showed creatinine clearance lower than for the placebo group. It is associated with the activation of neurohumoral, increased blood pressure as a result of the activation of the sympathetic and the renin-angiotensin system.

The tubular injury is characterized by tubular dilatation, brush border loss, depletion of epithelial cell and intraluminal cast,[30] so that it causing kidney morphology changes.[31] Epithelial cell injury due to ischemic-reperfusion especially in the S3 segment of proximal tubules.[20] Our study showed that increasing of tubular injury score was in-line with increasing of furosemide dose. Heyman *et al*.[32] show that furosemide decreased structural and functional of tubular injury in S3, especially in the middle and the outer zone of the inner stripe of outer medulla kidney that has isolated and perfused. The decline in structural damage assessed from the decrease fragmentation in S3 tubules. The protective effect of furosemide in the kidney was correlated with of active reabsorption and reduced of oxygen required by the mTAL cells with limited oxygen supply. Loop diuretics increase the oxidation potential of cytochrome oxidase in whole perfused kidney and increases oxygen pressure in the outer medulla of kidney. It is showed protective effects toward the proximal tubule in-line with research Heyman *et al*.[32] Several clinical studies in-line with the results of this study, Cantarovich *et al*.[19] showed that high doses of furosemide can maintain urine output but has no effect on median survival and kidney repair in patients with AKI. It was showed by the improvement of medulla hypoxia during AKI. The impact may be more significant in the initial phase of the AKI than after AKI.[19] Furosemide has a weak inhibitor carbonic anhydrase enzyme impact.[33] Carbonic anhydrase enzymes play a role in the reaction between CO_2 and H_2O into H^+ and HCO_3^-. Hydrogen ions that are secreted into the lumen of the kidney tubules to replace reabsorbed Na^+. The hydrogen ions in the luminal kidney tubules react with HCO_3^- to form H_2CO_3. Hydrogen ions also react with the NH_3^- to form NH_4. If the carbonic anhydrase enzyme is inhibited, H^+ will not be secreted into the tubular lumen. Therefore NH_3^- will not be neutralized to NH_4, so NH_3^- will damage kidney.[34]

TLR4 is a pattern recognition molecule doe to ischemic injury. Ischemic caused tubular and microvascular injuries, thus the integrity of cytoskeleton and cell polarity will be lost, brush border of proximal tubules loss, loss of polarity followed by the change of the adhesion molecules location and other membrane proteins such as Na^+K^+-ATPase and β-integrin.[35] The microvascular injury causes the disruption of blood flow, increased of leukocyte adhesion and increased of blood vessels permeability that causes a response inflammation.[36] Activation and epithelial damage led to the formation of inflammatory and vasoactive mediators, which provide feedback on the vasoconstriction and inflammation blood vessel. Furthermore, activation of innate immune system plays an important role in the initiation of acute injuries and acute on chronic.[14] Ischemic-reperfusion injury causes the activation of the innate immune system.

Activation of the innate immune system begins to bond TLR by endogenous ligands. The TLR4 has expressed by the kidney is a potential mediator of innate immune system activation and inflammation. Appropriate research Wu *et al*.[15] TLR4 gene expression in the kidney increased after ischemic, mainly expressed by cells tubulus. In this study, TLR4 gene expression was higher in IR group than SO group. TLR4 mediates the expression of pro-inflammatory cytokines and chemokines in the kidney during an IRI. There are two mechanisms that signaling pathway in TLR4: MyD88-dependent and MyD88-independent, until the process of transcription. In this study, IR+F3 has the highest TLR4/GAPDH among others. Group IR+F3 has the highest score of ischemic injury score in-line with the most severe inflammation. To determine the role of renal tubular epithelial cells in the inflammatory process may need to do further research on markers of epithelial cell damage in the renal tubules.

The results of this study were AKI rat models which were given 3.6 mg/kgBW and 7.2 mg/kgBW of furosemide (0.014±0.001 and 0.012±0.007 mL/min) showed higher creatinine clearance compared to IR (0.009±0.003) ($p>0.05$) while administration of 14.4 mg/kgBW furosemide (0.009±0.004) indicated equal creatinine clearance to IR ($p>0.05$). Kidney tubular injury score of 3.6 mg/kgBW furosemide (2.89±0.13) was lower than IR (3.26±0.19) ($p<0.05$) while 7.2 mg/kgBW and 14.4 mg/kgBW furosemide (3.55±0.26; 3.83±0.19) were higher than IR ($p<0.05$). Giving of 3.6 mg/kgBW furosemide (0.99±0.08) showed lower TLR4 gene expression than IR (1.20±0.08) ($p<0.05$) whereas 7.2 mg/kgBW furosemide (1.23±0.13) was non-significantly higher ($p>0.05$) and 14.4 mg/kgBW furosemide (1.63±0.12) was significantly higher than IR ($p<0.05$).

CONCLUSION

Administration of 3.6 mg/kgBW furosemide reduces kidney damage in AKI rat models while higher dosages (7.2 mg/kgBW and 14.4 mg/kgBW) increase kidney damage in the used models. It shows that administration of 40 mg furosemide in the early phase of human AKI reduces kidney damage, but not to be increased of the dosage.

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Peppermint oil prevented oxidative stress in experimental animal – induced acute single bout of eccentric exercise (ASBEE): study on blood catalase and hydrogen peroxide ($H_2O_{2)}$ and glucose transporter-4 (GLUT-4) expression on the muscle cells

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ABSTRACT

Peppermint oil is one of the essential oils with antioxidant activity that can reduce levels of reactive oxygen species (ROS). An acute single bout of eccentric exercise (ASBEE) is an acute exercise activity that can lead to increased ROS and cause skeletal muscle injury. This study aimed to assess the effect of peppermint oil in experimental animals induced with ASBEE with the purpose to measure catalase, hydrogen peroxide (H_2O_2) blood and glucose transporter-4 (GLUT-4) expression of skeletal muscle cells. A total of 30 Wistar rats *(Rattus norvegicus)* aged 20-24 weeks, weighing 160-350 g were divided into six groups i.e. T_1 (n =5), T_2 (n =4) and T_3 (n =5) given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C_0 (n=5) not given peppermint oil and not induced with ASBEE; C_A (n=5) not given peppermint oil and induced with ASBEE and C_E (n=5) given vitamin E 400 mg/kg one h before induced with ASBEE. ASBEE induction was done by downhill running on a rat treadmill -5^0 with a load index of 70% VO_2 max for 30 min. Twenty four h after induction of ASBEE, blood samples and muscle tissue were taken for examination of catalase, H_2O_2 and GLUT-4 expression. The results showed increased levels of blood catalase and decreased blood H_2O_2 levels in groups T_1, T_2, T_3, and CE. The opposite occurred in the group CA. The GLUT-4 expression did not show any significant difference between groups. It was concluded that peppermint oil can improve the condition of oxidative stress caused by ASBEE.

INTISARI

Minyak pepermin merupakan salah satu minyak esensial yang memiliki aktivitas antioksidan yang dapat menurunkan kadar *reactive oxygen species* (ROS). *Acute Single bout eccentric exercise* (ASBEE) merupakan olahraga akut yang dapat menyebabkan peningkatan ROS dan berakibat cedera otot rangka. Penelitian ini bertujuan untuk mengkaji efek pemberian minyak pepermin terhadap hewan coba yang diinduksi ASBEE terutama mengkaji katalase, hidrogen peroksida (H_2O_2) darah dan ekspresi *glucose transporter* (GLUT-4) sel otot rangka. Sebanyak 30 ekor tikus wistar (*Rattus norvegicus*) usia 20-24 minggu dengan berat 160-350 g dibagi menjadi enam kelompok perlakuan. Kelompok T_1 (n=5), T_2 (n=4) dan T_3 (n=5) diberi minyak pepermin per oral dosis bertingkat 0,25; 0,5 dan 1,0 g/kg BB satu jam sebelum diinduksi ASBEE; kelompok C_A (n=5) tidak diberi apapun dan diinduksi ASBEE; kelompok C_0 (n=5) tidak diberi apapun dan tidak diinduksi ASBEE; dan kelompok

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C_E (n=6) diberi vitamin E dengan dosis 400 mg/kgBB satu jam sebelum induksi ASBEE. Induksi ASBEE dilakukan dengan *downhill running* -5^0 pada treadmill tikus (Gamatread) dengan beban 70% indeks VO2 *max* selama 30 menit. 24 jam setelah induksi ASBEE, sampel darah dan jaringan otot diambil untuk pemeriksaan katalase, H_2O_2 dan GLUT-4. Hasil penelitian menunjukkan terjadi peningkatan kadar katalase darah serta penurunan kadar H_2O_2 darah pada kelompok T_1, T_2, T_3, dan C_E. Terjadi sebaliknya pada kelompok C_A. Ekspresi GLUT-4 tidak menunjukkan adanya pengaruh bermakna pada setiap kelompok. Minyak pepermin dapat memperbaiki kondisi stress oksidatif yang diakibatkan oleh ASBEE akan tetapi tidak terbukti mencegah resistensi insulin sementara setelah induksi ASBEE.

Keywords: peppermint oil - acute single bout of eccentric exercise - glucose transporter - 4 - catalase - hydrogen peroxide

INTRODUCTION

Acute single bout of eccentric exercise (ASBEE) is an exercise activity that is done with a duration of 30 minutes or less and maximum intensity greater than or equal to 80% ± 6 metabolic equivalents (METs). The ASBEE is included in the category of very hard that can cause physical stress due to the increase of free radicals especially reactive oxygen species (ROS).[1,2] Imbalance production of ROS and mitochondrial antioxidant can lead to oxidative stress.[3,4] In addition, the eccentric contractions can induce muscle fibers damage lead to inflammatory process, oxidative stress and delayed onset muscle soreness (DOMS).[5]

The DOMS are the effects of inflammation and oxidative stress in muscles that were damaged during ASBEE. The inflammation further increases ROS production through the activity of neutrophils, lymphocytes and proinflammatory cytokine activity. One of the increased ROS molecules and primary cause of DOMS is hydrogen peroxide (H_2O_2).[6] This molecule can stimulate a number of other ROS molecules, such as the highly reactive radicals hydroxyl.[6] Increasing H_2O_2 molecules that are not the same as antioxidants that reduce hydrogen peroxidase, there will be an increase in oxidative stress that can damage other molecules in the body such as proteins, lipids and DNA.[7]

The increase of H_2O_2 in skeletal muscle tissue causes insulin resistance by disrupting glucose uptake into muscle cells through translocation of glucose transport-4 (GLUT-4) inhibition to the sarcoplasm.[8] The degradation of signal proteins GLUT-4 by ROS during ASBEE increases oxidative stress, insulin resistance, and DOMS.[9,10] These problems can reduce exercise performance and slow the recovery process. To prevent such problems many athletes use exogenous antioxidant supplements, such as vitamin C and E. However, Paulsen *et al.*[11] reported that the vitamins supplementation in athletes for 11 weeks before exercise can reduce proteins that are essential for the adaptation of aerobic exercise. In addition, Steinbachr and Eckl[12] reported that vitamin C and E can inhibit transcription of endogenous antioxidants.

Essential oils from medicinal plants are potential exogenous antioxidants sources. Peppermint oil is one of the essential oils that is widely available in the market and inexpensive. It is widely used for the pharmaceutical, cosmetic, and food industries. Peppermint oil contains high monoterpenes, poliphenol and flavonoids. These molecules were proven to have high antioxidant activity.[13,14] This study aimed to investigate the effects of antioxidant activity of peppermint oil in animal model induced with ASBEE. It's effect on catalase, H_2O_2 blood and GLUT-4 of skeletal muscle cells were reported.

MATERIAL AND METHODS

Animals model and testing materials

Thirty males Wistar rats (*Rattus*

norvegicus) aged 20-24 weeks were used in the study. Rats were divided into 6 groups i.e. T_1 (n =5), T_2 (n =4) and T_3 (n =5) given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C_0 (n=5) not given peppermint oil and not induced with ASBEE; C_A (n=5) not given peppermint oil and induced with ASBEE and C_E (n=5) given vitamin E 400 mg/kg one h before induced with ASBEE. The rats were acclimatized in a 12 : 12-h light-dark cycle in individual cage for 7 days and allowed to food as well as to water *ad libitum*. The protocol of the study was approved by the Ethics Committee of the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta (number: 00 098/04/LPPT/XI/2016).

Peppermint oil was purchased from PT. Bratachem, Indonesia and prepared as solution in 10% Tween 20 at 10% concentration. Vitamin E (E-300 Nature) was purchased from PT Darya-Varia, Indonesia and prepared as solution in 10% Tween 20 at dose of 400 mg/kg.

ASBEE induction

On day 7 of acclimatization, VO_2max in rats were measured. Furthermore, on day 8 the rats were placed on a rats treadmill with the lowest rate (10 m/min) with tilt -5^0, followed by increasing running speed of 5 m/min every 3 min. The treadmill was discontinued when rats were experiencing fatigue as indicated by exposed to foot shock > 3 times or the rats reversed direction and not move. The maximum speed and duration of the running rats were recorded. VO_2max index was calculated by multiplying the maximum achievable speed (m/min) with body weight (kg).[15] The rats were then rested without exercise for 2 days for recovery. Induction of ASBEE was performed on the 11th day by downhill running on a rats treadmill tilt -5^0 with a load index of 70% VO_2max for 30 min. Twenty four hours after ASBEE induction, blood samples and muscle tissue were taken for catalase, hydrogen peroxide and GLUT-4 examinations. During the induction, the other groups of rats were placed in another room to minimize additional stress.

H_2O_2 and catalase measurements

Twenty four hours after ASBEE induction (on day 12), rats were anesthetized using ketamine at a dose of 60 mg/kg. Blood samples were taken and centrifuged at 10,000 g for 15 min at a temperature of 4^0 C. The blood plasma was taken for H_2O_2 and catalase examinations. The H_2O_2 scavenging activity was measured according to the method of Gulcin *et al.*[16] A solution of 40 mM H_2O_2 as control solution (A_0) was prepared in phosphate buffer at pH 7.4. The blood plasma sample as test solution (A_1) was added to 0.6 mL of 40 mM H_2O_2 solution and leaved at room temperature for 10 min. The absorbance of H_2O_2 in control and test solution were measured spectrophotometrically at 230 nm against a blank solution containing phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of the solutions were calculated using the following equation : % scavenged $[H_2O_2] : [(A_0 - A_1)/A_0 \times 100\%]$, where A_0 was the absorbance of the control solution, and A_1 was the absorbance of the test solution.

The catalase activity was measured colorimetrically using colorimetric assay kit (Biovision-US). As much as 2-78 µL of blood samples or 1-5 µL of positive control solution was added and adjusted volume to total 78 µL with Assay Buffer. Sample High Control (HC) was prepared in separate well with the same amount of sample then bring total volume to 78 µL with Assay Buffer. Stop Solution (10 µL) was added into the sample HC into the sample HC, mix and incubated at 25 ^{0}C for 5 min to completely inhibit the catalase activity in samples as High control. Fresh 1 mM H_2O_2 (12 µL) was added into each well of samples, positive control, and sample HC to start the reaction, incubated at 25 ^{0}C for 30 min and then 10 µL Stop Solution into each sample well, except the sample HC, to stop the reaction. The absorbance was then measured colorimetrically at 570 nm. Signal change by catalase in sample was $\Delta A = A_{HC} - A_{sample}$. A_{HC} was the reading of sample High Control, A_{sample} was the reading

of sample in 30 min. ΔA was then applied to the H_2O_2 standard curve to get B nmol H_2O_2 decomposed by catalase in 30 min reaction. Catalase activity was calculated using the following equation : catalase activity = [(B/30 x V) x sample dilution factor mU/mL, where B was the decomposed H_2O_2 amount from H_2O_2 Standard Curve (in nmol), V was the pretreated sample volume added into the reaction well (in mL) and 30 was the reaction time 30 min. One unit of catalse was amount of catalase that decomposed 1.0 µmol of H_2O_2 per min at pH 4.5 at 25 ^{0}C.

GLUT-4 expression examination

The GLUT-4 expression was immunohistochemically analysed using anti GLUT-4 antibody (IF8): sc 53566 (Santa Cruzz Biotechnology, Inc., USA). At the end of the experiment, rats were euthanized through intramuscular injection of ketamin 80 mg kg^{-1}. The muscle tissue was taken from the triceps brachii of the right front foot. The tissue was fixed in formalin solution for 24 h and then processed for immunohistochemical examination by a standard method using paraffin. The tissue was incubated with H_2O_2 in methanol for 15 min and then washed with phosphate bufferred saline (PBS) pH 7.4. The tissue was incubated with background sniper solution at room temperature for 15 min and washed with PBS. The tissue was then spilled with anti-GLUT-4 primary monoclonal antibody and left at room temperature for 2 h. After washing with PBS, the tissue was spilled with biotinylated IgG and left at room temperature for 20 min. Furthermore, the tissue was spilled with avidin biotin HRP and left at room temperature for 30 min and then it was visualized by using diamino benzidine (DAB). The tissue was counterstained with HE and washed with PBS. The observations were performed using a photomicroscopic camera and a light microscope of 10 time-magnifications in 10 visual fields per sample preparation. The results were considered as GLUT-4 positive expression if the cell indicated brown color. The color intensity was calculated by using a computer programs (Adobe Photoshop CS6 and Macbiophotonic Image J) and scored as (negative); 1 (low positive); 2 (positive); and 3 (high positive).

Statistical analysis

Data were presented as mean ± standard error of mean (SEM) and analysed using one-way ANOVA or Kruskal Wallis test depend on normality of the data. A p value < 0.05 was considered to be significant.

RESULTS

The maximum running speed and VO_2 max index

The mean maximum running speed and VO_2 *max index* of each group are presented in TABLE 1. No significantly difference in maximum speed running and VO_2 max index were observed in this study.

Overview of inflammation of the muscles in general

The HE staining results showed some mononuclear cells slightly more dominant in

TABLE 1. The maximum speed running and VO_2 max index (mean ± SEM) of each group

Group	n	Maximal speed running (m/min)	VO2 max index (kg m/min)
T1	5	41.60±3.867	11.97±1.428
T2	4	48.50±4.907	11.93±1.232
T3	5	42.00±5.366	9.36±1.265
C0	5	-	-
CA	5	42.40±3.600	11.64±1.389
CE	6	46.83±2.508	12.29±0.985

Note: T1, T2, and T3 were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C0 was normal rat which not given peppermint oil and not induced with ASBEE; CA was negative control which not given peppermint oil and induced with ASBEE and CE was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE

group C_A. In this group mononulear collection of cells were found in muscle interstitial tissue. In the other groups (except group C_0) mononuclear cells were also present in the interstitial area. Group C_0 did not appear to have mononuclear cells inmuscle tissue and interstitial area (FIGURE 1).

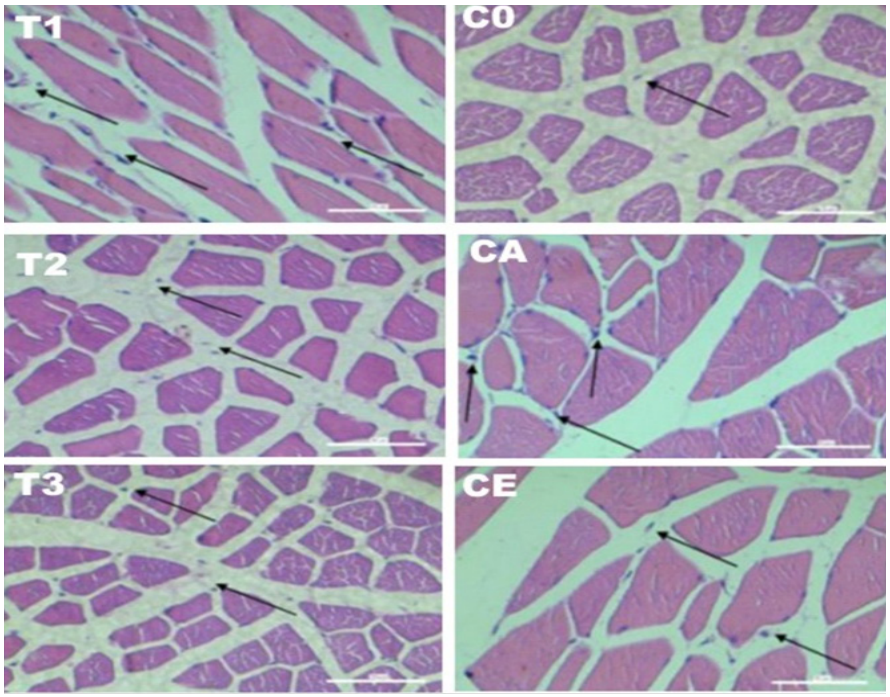

FIGURE 1. Hematoxylin-eosin staining results of skeletal muscle tissues. T1, T2, and T3 were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C0 was normal rat which not given peppermint oil and not induced with ASBEE; CA was negative control which not given peppermint oil and induced with ASBEE and CE was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE. The arrow (→) indicates the collection of mononuclear cells (10x magnification light microscopy) and scale 50 μm.

Levels of catalase and H_2O_2 in Blood

The blood catalase level of rats without peppermint oil administration and induced with ASBEE (C_A group) was significantly lower and the blood H_2O_2 level was significantly higher than that without induced with ASBEE (C_0 group) ($p<0.05$). In addition, the blood catalase level of rats after peppermint oil administration and induced with ASBEE (T_1, T_2 and T_3 groups) was significantly higher than that C_A group and it was significantly lower than that C_0 group. In contrast, the blood H_2O_2 level of T_1, T_2 and T_3 groups were significantly lower than that C_A group and they were significantly higher than that C_0 group ($p<0.05$). No significantly different in blood catalase and H_2O_2 levels between C_0 group and C_E group were observed ($p>0.05$).

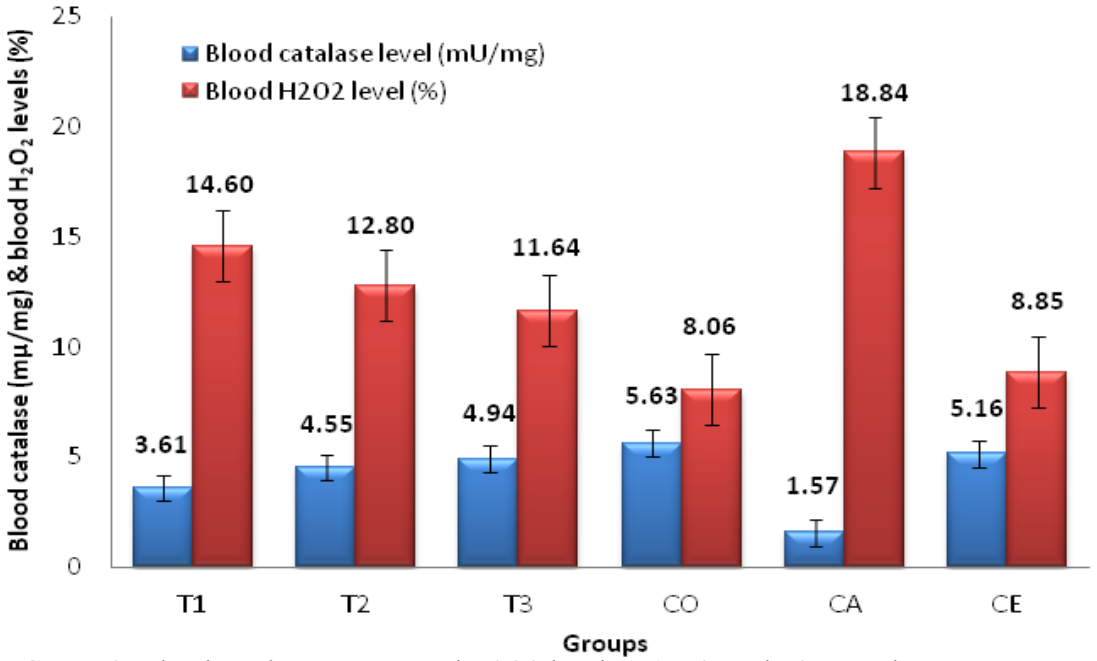

FIGURE 2. Blood catalase enzyme and H2O2 levels. T1, T2, and T3 were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C0 was normal rat which not given peppermint oil and not induced with ASBEE; CA was negative control which not given peppermint oil and induced with ASBEE and CE was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE

GLUT-4 expression

Low positive in the GLUT-4 expression (score <1) in the muscle tissue was observed in all groups (FIGURE 3 and 4). Moreover, no significantly different in the GLUT-4 expression was reported ($p>0.05$). In general, the GLUT-4 expression was observed in closed sarcoplasma. However, in the C_A group the GLUT-4 expression appeared less strong and was observed cytoplasm. The GLUT-4 expression in group C_E, C_0, T_1, T_2 and T_3 was similar in color and location of the expression.

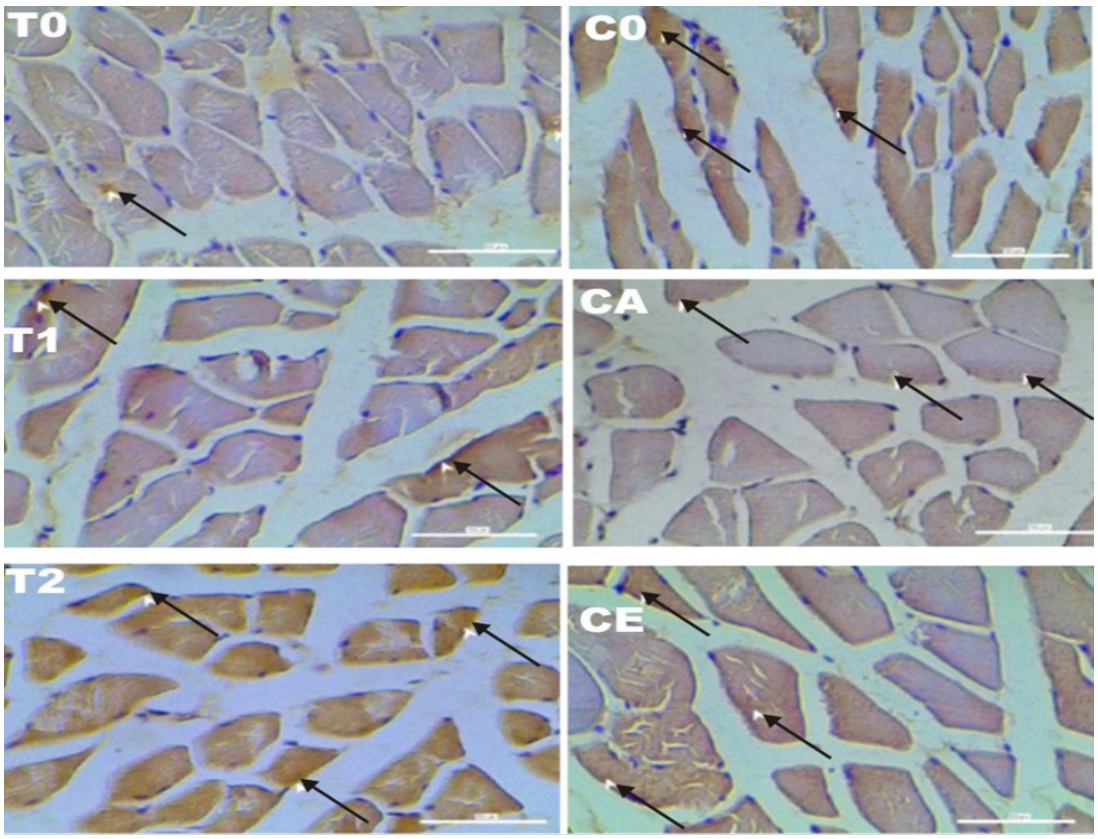

FIGURE 3. The expression of GLUT-4 in skeletal muscle tissue in each group. T_1, T_2, and T_3 were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C_0 was normal rat which not given peppermint oil and not induced with ASBEE; C_A was negative control which not given peppermint oil and induced with ASBEE and C_E was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE.

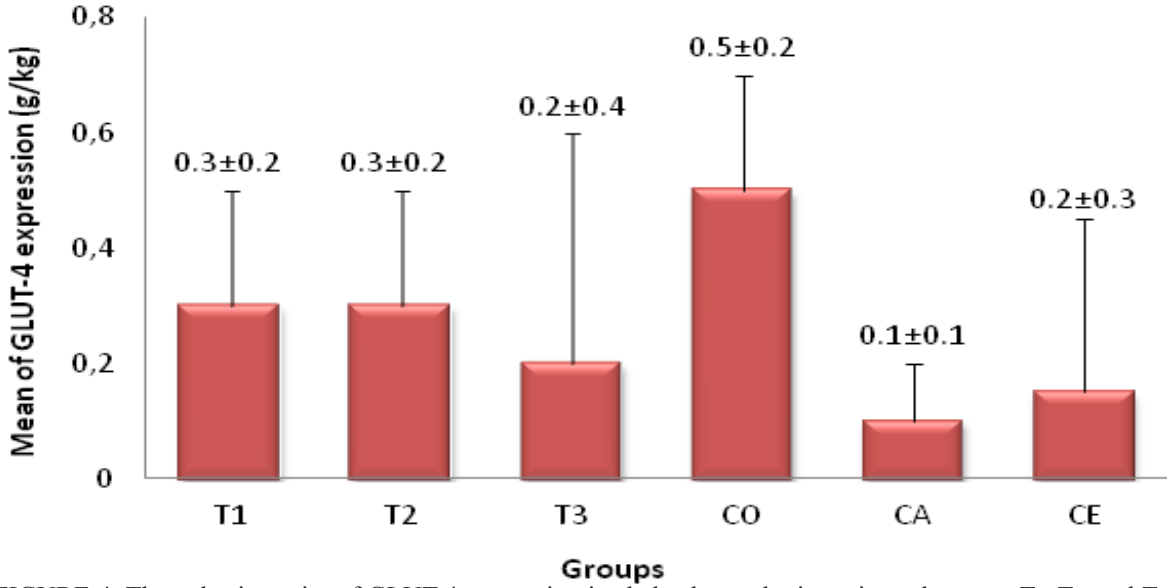

FIGURE 4. The color intensity of GLUT-4 expression in skeletal muscle tissue in each group. T_1, T_2, and T_3 were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C_0 was normal rat which not given peppermint oil and not induced with ASBEE; C_A was negative control which not given peppermint oil and induced with ASBEE and C_E was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE

DISCUSSION

This study showed that the peppermint oil influenced oxidative stress and insulin resistance in dose dependent manner (FIGURE 2). The catalase and H_2O_2 of rats given peppermint oil (T_1, T_2 and T_3) were higher than those of rats not given peppermint oil (C_A). Moreover, the GLUT-4 expression in skeletal muscle tissue of the rats given peppermint oil (T_1, T_2 and T_3) was not significantly different compared to that of the rats not given peppermint (C_A). This effect demonstrates the ability of peppermint oil to donate a proton or hydrogen to a molecular oxidant and turn it into a molecule that reduces oxidative stress.[13,17] These results are consistent with a study reported by Sroka et al.,[14] that peppermint oil contain seriocitrin and rosmarinic acid, which have a considerable effect on the action of H_2O_2 scavenging.

The blood H_2O_2 levels of C_E group was not significantly different with group C_0. It was indicated that vitamin E could decrease the blood H_2O_2 levels similar to the group that not induced with ASBEE. Vitamin E can prevent lipid peroxidation in cell membranes by means of interaction with unsaturated fatty acids and protection of the polypeptide chain protein. In addition, vitamin E acts as a peroxide scvenger.[18] The blood H_2O_2 levels of T_2 and T_3 groups were not significantly different. However, they were significantly lower than that of C_A group, but significantly higher than that C_E groups (FIGURE 2). It was indicated that the peppermint oil at dose of 1.0 g/kg (T_3 group) had an antioxidant activity although its effect was lower than vitamin E at dose of 400 mg/kg (C_E group).

The antioxidant activity of peppermint oil was associated with its ability as a modulator to increase the internal antioxidants, such as catalase. This activity showed in dose dependent manner. In addition, post-hoc statistical analysis showed no significantly different in blood catalase levels between the T_3 and C_E groups. It was indicated that the antioxidant activity of peppermint oil was similar with vitamin E. Song and Park[19] reported that flavonoids (luteolin) in peppermint oil can increase endogenous antioxidant enzyme activities.

Blood catalase enzymes act as a catalyst for changing of H_2O_2 back into water. Catalase can reduce the levels of ROS molecules of H_2O_2 and prevent damage to biological molecules such as lipids, proteins and DNA in the body.[3] ASBEE induced muscle tissue damage can cause additional oxidative stress which influences the metabolism of glucose in damaged muscle tissue.[9] Glucose metabolism in muscle proteins involves GLUT-4 as the transporter of glucose into muscle cells.

The blood H_2O_2 levels of the C_A group was highest but the blood catalase levels was lowest compared to those of other groups. In addition, the blood H_2O_2 and catalase levels were likely associated with the GLUT-4 expression (FIGURE 2 and 4). These findings are consistent with a study reported by Aoi et al.[20] that many oxidative damage in lipids, proteins and DNA found in the skeletal muscle due to high levels of ROS. Wei et al.[21] also reported that the interference in the GLUT-4 translocation due to high levels of ROS found in skeletal muscle oxidative damage. Furthermore, Maddux et al.[22] showed that one of the ROS molecules that interferes with the molecule GLUT-4 translocation to the cell membrane is the H_2O_2.

The score of GLUT-4 expression in all groups was between 0 to 1 (FIGURE 4). It was indicated that the expression of each sample is low positive quality. However, the control group (C_0) had the highest expression. Possible causes include induction of ASBEE that caused increased oxidative stress. Higaki et al.[23] reported that in low concentrations, H_2O_2 will stimulate GLUT-4 translocation and entry of glucose in the skeletal muscles and inhibit the entry of glucose if the concentration of H_2O_2 is high.

No significantly difference in the GLUT-4 expression between groups was observed in this study (FIGURE 4). The possible causes are inflammatory conditions of muscle

tissue mostly happed in muscle interstitial areas. Panza et al.[5] reported that the peak of inflammation in muscle contraction due to improper eccentric activity is 24-48 h. Variations in peak inflammation may occur in the sample population. A study conducted by Armstrong et al.[24] reported the similar results. A total of 140 male rats Sprague-Dewley were subjected to acute exercise running downhill -16 ^{0} for 90 min and the speed of 16 m/min. Twenty four h after exercise they found that the inflammation was in the interstitial area of the muscles. These conditions allow the levels of ROS in muscle tissue derived from the inflammation to remain low. In addition, due to the eccentric contractions, ASBEE causes damage to the collagen matrix of the muscle tissue. Damage to the collagen can invite mononuclear cells to the interstitial area.[24] One h after ASBEE, ROS is generated as a result of the ischemia-reperfusion process of muscle tissue and activates NFkB xanthine oxidase. Active NFkB causes the appearance of the inflammatory cascade to procede slowly (inflammation after 24 h). Inflammation after 24 h of injury may increase the production of ROS. ROS are actively produced from the aging process of cell neutrophils and mononuclear phagocytes. This increase in ROS can enhance the increase damage in muscle tissue and cause widespread inflammation. As a result, DOMS can occur for an extended time.[20]

One limitation of this stuy is in addition to the time variation of inflammation, the blood H_2O_2 levels were measured from venous blood and not derived from muscle. This complicates the direct identification of oxidative stress that occurs in the muscles since H_2O_2 content of the blood is an accumulation of all body tissues involved in the sport. The GLUT-4 expression in cell membranes and oxidative stress can not be separated from the other intracellular signaling proteins. One of the proteins that affect the activities above is peroxisom proliferator activated receptor γ coactivator 1-α (PGC1-α). This protein is a central regulator of energy metabolism and numerous in cells that have a high metabolism.[25] Chun and Arany[26] concluded that PGC1-α may decrease the activation of phosphorilation of Akt protein on insulin-dependent path ways that prevents insulin resistance. Pagel-Langenickel et al.[27] reported that PGC1-α prevents insulin resistance by inducing mitochondrial metabolism so diasilglycerol (DAGs) moleculesare decreased. The DAG molecules inactivate phosphorilation of Akt protein. If the DAG molecules is reduced, then the phospforilation can be increased.

Related to the importance of antioxidants, proteins PGC1-α activity increases cellular defense against oxidative stress. PGC1-α is one of the molecular regulators of the energy metabolism and is associated with the expression of antioxidants.Valle et al.[25] said that down regulation of PGC1-α protein can decrease the production of Mn-SOD (superoxide dismutase mangan-) on endothelial cells. This result may occur in skeletal muscles. Barbosa et al.[7] added explaination that increased expression of the enzyme catalase appears to increase transcription PGC1-α.

This study has some limitations in linking the findings with molecular PGC1-α. In addition, this study is limited to groups of adult male Wistar rats. Further research is recommended to study the the difference in subjects samples if studies are conducted in groups of older male Wistar rats and the results are associated with the process of degeneration (ageing). Furthermore, exploratory study of the relationship between the regulator molecule PGC1-α with the effects of peppermint oil as an exogenous antioxidant can confirm our results as an effective antioxidant. This study contributes a review related to the benefits of peppermint oil in preventing the negative effects of exercise attributed to oxidative stress from ASBEE.

CONCLUSION

Peppermint oil can increase blood catalase levels and decrease blood H_2O_2 levels in adult male Wistar rats with oxidative stress

induced by ASBEE. However, peppermint oil has not been shown to increase the GLUT-4 expression in this study.

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The effect of measurable and regular exercise on ovariectomized Sprague Dawley rats in improving skin quality

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ABSTRACT

The decline of estrogen level causes various skin changes including amount of fibroblast, the thickness of epidermis and dermis. Physical exercise is believed can increase the estrogen level and give benefit impacts on skin. It mechanism is often associated with the increase of extragonadal aromatization and estrogen serum, the activation of insulin-like growth factor (IGF), and the expression of estrogen receptor (ER). This study was conducted to investigate the effect of physical exercise in ovariectomized rat on the amount of fibroblast, the thickness of epidermis and dermis, the levels of ER β in skin and serum estrogen. Eight female Sprague Dawley rats aged 3 months were used in this study. Rats were divided into two groups i.e. Group 1 consisted of ovariectomized rats without physical exercise and Group 2 consisted of ovariectomized rats and given measurable and regular physical exercise. Rats ran on treadmill with the speed 18 m/min, the tilt of 5%, duration for 60 min/experiment/day, 5 times a week for 12 weeks. The amount of fibroblast, the thickness of epidermis and dermis, the levels of ERβ in skin and serum estrogen were measured after physical exercise. The result showed that there was a significant difference amount of fibroblast between group 1 and group 2 ($p<0.05$). However, no significant difference the levels of serum estrogen, ERβ, and the thickness of epidermis and dermis between Group 1 and Group 2 ($p>0.05$). There was a significant positive correlation between the level of serum estrogen and the thickness of epidermis ($p<0.05$), and negative correlation between the serum estrogen level and the level of ERβ ($p<0.05$), and negative significant correlation between the level of ERβ and the thickness of epidermis ($p<0.05$). In conclusion, the amount of dermal fibroblast of ovariectomized rats increase after underwent measurable and regular exercise. There is correlation between the serum estrogen level and the thickness of epidermis as well as ERβ.

ABSTRAK

Penurunan kadar estrogen dapat menyebabkan perubahan kulit termasuk jumlah fibroblast, ketebalan epidermis dan dermis. Latihan fisik dapat meningkatkan kadar estrogen dan berdampak positif pada kulit. Mekanismenya berhubungan dengan peningkatan aromatisasi ekstragonadal, peningkatan estrogen serum, aktivasi *insulin-like growth factor* (IGF), serta ekspresi reseptor estrogen (RE). Penelitian ini bertujuan mengkaji pengaruh latihan fisik pada tikus yang dilakukan ovariektomi terhadap jumlah fibroblast, ketebalan epidermis dan dermis, kadar REβ kulit, dan kadar estrogen serum.

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Delapan ekor tikus Sprague Dawley betina umur 3 bulan digunakan dalam penelitian ini. Tikus dibagi menjadi dua kelompok yaitu Kelompok 1 tikus dilakukan ovariektomi dan tidak diberi latihan fisik dan Kelompok 2 tikus dilakukan ovariektomi dan diberi latihan fisik teratur dan terukur. Tikus berlari di atas *treadmill* dengan kecepatan 18 m/menit, kemiringan 5%, durasi 60 menit/kali/hari, dan frekuensi 5 kali per minggu, selama 12 minggu. Setelah latihan fisik teratur dan terukut, jumlah fibroblast, ketebalan epidermis dan dermis, kadar REβ kulit dan estrogen serum diukur. Hasil penelitian menunjukkan bahwa terdapat perbedaan bermakna rerata jumlah fibroblast antara Kelompok 1 dan Kelompok 2 ($p<0,05$). Namun, tidak terdapat perbedaan bermakna untuk rerata kadar estrogen serum, kadar REβ, ketebalan epidermis dan dermis antara Kelompok 1 dan Kelompok 2 ($p>0,05$). Terdapat hubungan positif yang bermakna antara kadar estrogen serum dengan ketebalan epidermis ($p<0,05$) dan hubungan negatif antara kadar estrogen serum dengan kadar REβ kulit serta hubungan negatif yang bermakna antara kadar REβ kulit dengan ketebalan epidermis ($p<0,05$). Dapat disimpulkan, jumlah fibroblas dermis tikus yang menjalani latihan fisik teratur dan terukur lebih besar dibandingkan tanpa latihan fisik teratur dan terukur pada tikus yang dilakukan ovariektomi. Terdapat hubungan antara kadar estrogen serum dan ketebalan epidermis dan juga REβ.

Keywords: physical exercise - serum estrogen - estrogen β receptor - fibroblast - dermis - epidermis.

INTRODUCTION

The decline of estrogen level in postmenopausal woman shows various impacts such as low content of collagen in skin, increase of the numbers and the depth of wrinkles, skin depletion, dryness in skin and low elasticity.[1,2] In addition, low estrogen level may cause long wound healing and atrophy.[3] In young rats after ovariectomy (OVX), visible skin deteriorations are found such as low number of collagen fibers in skin,[4] the decrease of subcutaneous tissue's thickness, the increase of breaking strength, tensile strength and Young's skin modulus.[5] The decrease in the thickness of epidermis, dermis and the percentage of dermis collagen are also found in the young rabbits after OVX.[6]

One of most convenient methods to elevate estrogen level is physical exercise. Regular physical exercise with medium intensity is proven can increase the serum estrogen level on rats after OVX[7] and on post-menopausal women.[8] Regular physical exercise can also increase the CYP19 expression in adrenal cortex and fat tissue.[9,10] Furthermore, estrogen level in cerebellum[11] and hippocampus[12] of rats after OVX increases as the result of measurable and regular physical exercise for 12 weeks

Physical exercise gives also significant impact on the expression of estrogen receptor (ER) in liver, heart and muscles.[7,13] The expression of particular hormone's receptor can change along with the physiologic need of one tissue on that hormone.[14] Physical exercise also elevates the level of insulin-like growth factor-1 (IGF-1).[15] The IGF-1 is promoting cell growth involved in keratinocytes proliferation[16] and fibroblast so that the decreasing number of IGF-1 contributes to the decrease of skin thickness.[17] It is also able to form cross talk (cross interaction which influences each other) with ER. The IGF-1 activates mitogen activated protein kinase (MAPK) cascade which phosphorylates transcription factor leading to increasing the transcription activity of ER.[18] The ER **β** plays dominant role in regulating the action of estrogen in skin.[19]

This study aimed to investigate the effect of measurable and regular physical

exercise on serum estrogen level and ER β level in skin on ovariectomized Sprague Dawley rats. Their effect on the amount of fibroblast and the thickness of epidermis and dermis of the rats was also evaluated.

MATERIALS AND METHODS

Animal model procedure

This was an experimental study using post test only control group design involving eight female Sprague Dawley rats aged about 3 months. Before experimental, rats were adapted in individual cage for 7 days before OVX. Following after OVX, the rats were adapted again for 7 days for OVX wound healing and hormonal adaptation. On the 15th day, these rats were randomly divided into two groups with four rats for each group. Group 1 consisted of rats underwent OVX without physical exercise, whereas Group 2 consisted of rats underwent OVX with regular and measurable physical exercise.

Gradual adaptation of the physical exercise on the rats of Group 2 was conducted on the 15th to 21st day. Furthermore, the physical exercise was initiated for 5 days a week (Monday, Tuesday, Wednesday, Friday, and Saturday) for 12 weeks with medium intensity and the tilt of 5%. The duration of the physical exercise was 60 min consisting 10 min of warming up with the speed of 12 m/min, 40 min for main exercise with the speed of 18 m/min, and 10 min for cooling down with the speed of 12 m/min.[7] On the 106th day, the rats were anesthetized using HCl ketamine. Following after the rats lose their consciousness, blood and skin samples were taken. The blood sample was taken through orbitalis sinus for the estrogen serum level measurement. Two sample of skin tissues were taken by using 5 mm-punch biopsy on left leg. One fresh the skin tissue sample was used for the skin ERβ level measurement and another one was dipped into formalin 10% to produce paraffin block. Furthermore, HE staining was conducted to examine the amount of fibroblast and the thickness of dermis and epidermis.

Examination of serum estrogen level, ERβ, dermis fibroblast and epidermis thickness

The serum estrogen and skin ERβ levels were measured by using ELISA technique. The amount of dermis fibroblast was measured in 10 microscope fields of view at a magnification of 200 x after HE staining. The thickness of epidermis and dermis were measured by using Optilab®Image Raster version 2.1on 10 different places.

Statistical analysis

Data were presented as mean ± SEM. The different of serum estrogen level, skin ERβ level, dermis fibroblast and epidermis thickness of the both groups were analyzed using paired t test. Moreover, the relationship between the variables were analyzed using Pearson correlation. A p value <0.05 was considered to be significant.

RESULTS

Serum estrogen and ER β levels, amount of fibroblast, thickness of epidermis and dermis

No significantly different in the serum estrogen level, skin ERβ level and thickness of epidermis as well as thickness of dermis between the rats underwent OVX with physical exercise (Group 2) and those without physical exercise (Group 1) were observed. However, the amount of fibroblast on Group 2 (15.75±1.70) was significantly higher than that on Group 1 (8.75±0.48) ($p<0.05$) as presented in TABLE 1. It was indicated that the regular and measurable physical exercise on the rats underwent OVX increased the amount of fibroblast, however, did not influence the serum estrogen level, skin ERβ level, thickness of epidermis and thickness of dermis.

TABLE 1. Serum estrogen and ER β levels, amount of fibroblast, thickness of epidermis, and thickness of dermis (mean±SEM) in both groups of rats

Variable	Group	n	Mean ± SEM	p
Serum estrogen level (ng/mL)	1	4	24.23±10.99	0.761
	2	4	28.03±4.59	
ERβ level (pg/mL/50 mg tissue)	1	3	0.028±0.067	0.260
	2	4	0.019±0.004	
Amount of fibroblast	1	4	8.75±0.48	0.007*
	2	4	15.75±1.70	
Thickness of epidermis (μm)	1	4	2.93±0.24	0.199
	2	4	3.35±0.17	
Thickness of dermis (μm)	1	4	63.13±2.52	0.112
	2	4	72.43±4.31	

Note: Group 1 : rats underwent OVX without physical exercise; Group 2: rats underwent OVX with regular and measurable physical exercise; *significant ($p<0.05$)

Correlation between the serum estrogen, ERβ levels and amount of fibroblast, the thickness of epidermis as well as dermis

Negative correlation between the serum estrogen level and the thickness of epidermis ($r = -0.832$; $p = 0.029$), positive correlation between the skin ERβ level and the thickness of epidermis ($r = 0.759$; $p = 0.040$) and negative correlation between the serum estrogen level and skin ERβ level ($r = -0.911$; $p = 0.011$) were reported in this study (TABLE 2). It was indicated that the serum estrogen level increases the thickness of epidermis. In contrast, the skin ERβ decreases the thickness of epidermis.

TABLE 2. The Pearson Correlation analysis

Correlation		r	p
Serum estrogen level	Amount of fibroblast	-0.244	0.861
	Thickness of dermis	0.564	0.582
	Thickness of epidermis	-0.832	0.029*
Skin ERβ level	Amount of fibroblast	-0.074	0.641
	Thickness of dermis	-0.231	0.244
	Thickness of epidermis	0.759	0.040*
Serum estrogen level	Skin ER β level	-0.911	0.011*

* significant ($p<0.05$)

DISCUSSION

The serum estrogen level of the rats underwent OVX with physical exercise (G2) was higher than those without physical exercise (G1), although it was not significantly different (TABLE 1). The mechanism underlying the effect of physical exercise on serum estrogen level is not quite understood. After menopause or OVX, the serum estrogen is produced and secreted by peripheral steroidogenic active glands through extragonadal aromatization process.[20,21] Due to the following explanation, physical exercise is assumed to increase extragonadal aromatization process. Its mechanism has correlation with the production of IL-6 during performing endurance exercise.[22,23]

IL-6 is involved in immune response and acute phase reaction. Cytokine that is produced during inflammation can influence the communication between the hypothalamus-pituitary-adrenocortex (HPA) axis and immune system.[24] Inflammatory stimuli activates anti-inflammation signals from the central nervous system, whereas inflammation in peripheral tissues triggers neural signals in hypothalamus.[25] Acute phase response stimulates the expression of mRNA IL-6R in hypothalamic nucleus causing IL-6 activates HPA axis.[26] IL-6 is presumably produced as acute phase response after physical exercise.[27] These signal induces hypothalamic releasing factors which eventually enhance the secretion of hormone pituitary including adrenocorticotropic hormone (ACTH).[28]

ACTH which is released will result in rapid increase in the formation of pregnenolon and its derivatives, including cortisol and androgen adrenal. In longer period, ACTH elevates the synthesis of P450 which influences the formation of adrenocortex hormones.[29] P450 aromatase is an enzyme responsible for converting testosterone into estradiol and androstenedion into estron.[30] P450 aromatase can be activated by inflammatory mediator,[31] one of them is IL-6,[24] so that it increases the capability of extragonadal tissue in synthesizing estrogen from androgen.[31] Estrogen tissues will join the circulation if they do not take part in local metabolism.[32]

The test result on mean of ER β level of group given OVX without physical exercise (G1) and group given OVX and physical exercise (G2) shows that mean of those groups have no significant difference $p > 0.05$ (TABLE 2). However, mean of ER β level of G1 is higher than mean of ER β of G2 (TABLE 2). It means that physical exercise decreases ER β level of skin in rats after OVX. Factors that influence this to happen are not quite understood, but it is assumed that physical exercise influences steroidogenesis process in skin. DHEA is proven to be converted into estradiol in skin.[33] Physical exercise is proven to influence steroidogenesis process in several tissues which eventually elevates local estrogen level in tissues.[11,12] The increase of estrogen level in local tissues is assumed to contribute to ER β transcription process leading to decreased level of ER β level in skin. Mechanism refers to adaptation of receptor's need on its physiologist need because the adaptation of the number of receptor is very specific in each network.[14]

Besides, it is possible that estrogen mechanism in skin after OVX and physical exercise does not frequently involve genomic response. This assumption is who found that estradiol stimulates the production of skin procollagen in rats and human, although it does not activate ER. Thus, it needs further research focusing on estrogen action mediator.[34] Mean of the number of fibroblast on group given OVX without physical exercise (K1) compared to group given OVX and physical exercise is significantly different ($p<0.05$) (TABLE1). Mean of the number of dermal fibroblast of Group 2 is significantly higher than Group 1 (TABLE1). It shows that physical exercise increases the amount of fibroblast in skin. However, the result is still lower than mean of group which gets neither OVX nor

physical exercise.

No research has ever been conducted on examining the influence of physical exercise on the amount of skin fibroblast. However, the mechanism that might happen correlates with the effect of physical exercise on local steroidogenesis process in skin tissue. Local estrogen plays important role on skin physiology especially fibroblast.[35] Fibroblast migration and proliferation increase along with the existence of DHEA; however, the effect is hampered by aromatase inhibitor. It shows that fibroblast migration and proliferation depend on the conversion of 17β- estradiol.Mechanism of intracrine estrogen action in skin fibroblast indicates that skin fibroblast contains an enzyme converting DHEA into estradiol.[33]

Fibroblasts and dermal papilla cells express 5α-reductase tipe 2.[36] Skin fibroblast express P450 (aromatase).[37,38] However, this research does not measure the level of estrogen in tissues, enzymes and genes involving in steroidogenesis process in skin; therefore, its underlying mechanism can be clearly explained. Research show that measurable and regular physical exercise increase antioxidant enzyme which serves as the protector against skin deterioration due to free radicals.[39] The increased expression of catalase enzyme and glutathione peroxidaseon rats after 16 week-physical exercise using freewheel running. Glutathione peroxidase and superoxide dismutaseon rats increase after swimming.[40] Therefore, measurable and regular physical exercise elevates the ability to prevent any destruction resulted from free radicals.[41] Physical exercise is also proven to increase the level Insulin-like growth factor-1(IGF-1).[15] IGF-1 is a growth factor involved in fibroblast proliferation.[42] Thus, the increased level of IGF-1 elevates fibroblast proliferation.

Physical exercise endurance also significantly increases blood flow.[43] The increased microcirculation causing increases tissue oxygenation and nutrient delivery.[44] This mechanism is assumed to increase fibroblast proliferation. The result test on mean of the thickness of epidermis and dermis for group given OVX without physical exercise (G1) compared to group given OVX and physical exercise (G2) is not significantly different $p>0.05$ (TABLE 1 and TABLE 2). It means that measurable and regular physical exercise increases the thickness of epidermis and dermis.

Previous research examining the effect of physical exercise on the thickness of epidermis and dermis has never been conducted before. Mechanism that might happen has correlation with the effect of physical exercise on the local steroidogenesis process in skin tissues. Therefore, local estrogen level will improve the physiology of skin tissue. Moreover, physical exercise elevates antioxidant enzyme will prevent the damage on the component of connective tissue in dermis due to the formation of ROS on skin of OVX-induced rats. [39,45,46]

The changes of skin mechanical structure is associated with age and period after menopause, as it has been stated.[5] Therefore, age possibility and the length of time after OVX influence the result of research in skin thickness. The age of rats which get OVX also influences the result.[46] the uses of Wistar rats aged 5 months for research on the biomechanical decrease on rats skin after OVX.[5] Thus, the age of rats undergoing OVX also has possibility to affect end result of physical exercise on skin thickness.

Besides, there is a final phase in tissue rejuvenation process, namely the remodeling process. This phase has long duration in which incessant synthesis of collagen happens. Collagen damages are renovated and stabilized in a stable condition for 21 days or even weeks after. Remodeling phase occurs during the first week until the 12th week or more.[47] Hence, before reaching the last period of remodeling, skin thickening will not stop. When reaching stable extracellular matrix, skin thickness will not lengthen as the time passes by. Based on period after OVX, treatment in this research will stop

in the 12th week because tissue rejuvenation has reached the final phase of remodeling. However, it need time-series based research to ensure this assumption.

A study should be conducted to investigate the relation between ER β level and the thickness of epidermis and dermis as well as serum estrogen level with the thickness of epidermis and dermis. From the correlation test on the relation between ER β level and the thickness of epidermis, the result shows significant correlation. A significant correlation is visible in serum estrogen level with the thickness of epidermis (TABLE 2). The function of epidermis is proven to be regulated by the circulation of estrogen level, even though estrogen metabolism process and estrogen sensitivity in peripheral tissue also greatly contribute to epidermis homeostasis. In epidermis, estrogen plays important role in keratinocyte proliferation.[48]

Receptor upregulation is one of method to scrutinize the response of intracellular receptor on the increased hormone level.[49] However, this research finds no significant difference on ER β level between G1 and G2. It is possible that estrogen action in skin in order to maintain the thickness of epidermis and dermis genomically occurs through classic pathway depend on ligan. It does not only involve ER β but also ER α; however, this research will not further discuss this area. Besides, it can also occur through gnomic pathway which does not depend on ligan; however, the capability of GF to activate ER β is lower than ER α.[50]

CONCLUSION

Amount of dermal fibroblast on rats increases after measurable and regular exercise. The increase on the level of serum estrogen elevates the thickness of epidermis and decrease the level of ERβ. The increase on the level of ERβ decrease the thickness of epidermis. It is suggested to conduct further study about the influence of OVX and physical exercise on the expression of skin steroidogenesis enzymes, skin estrogen level and IGF-1 level. The study can be conducted on older rats using much more samples. It can also use time series research, in order to examine the changes on skin structure due to longer period of physical exercise and after OVX.

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Moderate-intensity interval exercise but not high-intensity interval exercise improves the spatial memory of ovariectomized rats

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ABSTRACT

Physical exercise exerts beneficial effects on the spatial learning and memory. High-intensity interval exercise (HIIE) has been proposed as a time-efficient physical exercise regimen. On the other hand, there were evidences that HIIE increased oxidative stress biomarkers and reduced antioxidant capacity, which resulted in oxidative damage. The present study aimed to investigate the effects of high-intensity interval exercise and moderate-intensity interval exercise on oxidative stress biomarkers and oxidative enzymes activity in the hippocampus and the spatial memory of ovariectomized rats. A total of 16 female Sprague Dawley rats aged 12 weeks were randomly assigned into 4 groups, i.e. the sham-operated (SO), ovariectomized without exercise (O), ovariectomized with high-intensity interval exercise (HIIE), and ovariectomized with moderate-intensity interval exercise (MIIE) groups. Rats of the exercise groups (HIIE & MIIE groups) performed 6 sessions of interval exercise per week for 6 weeks. The spatial memory of rats was measured using the Morris water maze procedure. The malondialdehyde (MDA) levels and activity of catalase (Cat) as well as glutathione peroxidase (GPx) in hippocampus were determined using spectrophotometry method. The spatial learning and memory retention of the moderate-intensity interval exercise group was significantly better than that of the high-intensity interval exercise group. The GPx activity of MIIE group was higher than any other groups. The SO group had the lowest hippocampal MDA level and highest Cat activity among groups. Moderate-intensity interval exercise reduces the ovariectomy induced-oxidative stress in the hippocampus and improves spatial learning and memory retention of ovariectomized rats.

ABSTRAK

Latihan fisik memberikan efek menguntungkan pada pembelajaran spasial dan memori. Latihan fisik interval intensitas tinggi (HIIE) telah diusulkan sebagai rejimen latihan fisik yang efisien waktu. Di sisi lain, ditemukan bukti bahwa HIIE meningkatkan *biomarker* stres oksidatif dan mengurangi kapasitas antioksidan, yang mengakibatkan kerusakan oksidatif. Penelitian ini bertujuan untuk menguji efek latihan fisik interval intensitas tinggi dan latihan fisik interval intensitas sedang pada *biomarker* stres oksidatif dan aktivitas enzim oksidatif di hippocampus serta memori spasial tikus yang diovariektomi. Sebanyak 16 tikus Sprague Dawley betina berusia 12 minggu secara acak dibagi menjadi 4 kelompok, yaitu operasi palsu (SO), ovariektomi tanpa latihan fisik (O), ovariektomi dengan latihan fisik interval intensitas tinggi (HIIE), dan kelompok ovariektomi dengan latihan fisik interval intensitas sedang (MIIE). Tikus dari kelompok latihan fisik (kelompok HIIE & MIIE) melakukan 6 sesi latihan interval per minggu selama 6 minggu. Memori

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spasial tikus diukur menggunakan prosedur *Morris water maze*. Kadar malondialdehide (MDA) dan aktivitas katalase (Cat) serta glutation peroksidase (GPx) di hippocampus diukur menggunakan metode spektrofotometri. Pembelajaran dan retensi memori spasial dari kelompok latihan fisik interval intensitas sedang secara signifikan lebih baik daripada kelompok latihan fisik interval intensitas tinggi. Aktivitas GPx kelompok MIIE lebih tinggi daripada kelompok lain. Kelompok SO memiliki tingkat MDA hippocampus terendah dan aktivitas Cat tertinggi di antara kelompok-kelompok. Latihan fisik interval intensitas sedang mengurangi stres oksidatif yang diinduksi ovariektomi dan meningkatkan pembelajaran dan retensi memori spasial pada tikus yang diovariektomi.

Keywords: ovariectomy - spatial memory - interval exercise – malondialdehyde - catalase

INTRODUCTION

The effects of exercise on health have been extensively investigated, including the prevention of cognitive impairment.[1] Although a substantial amount of evidence of the beneficial effects of exercise on health has been presented, many people are still reluctant to participate in exercise for various reasons, such as "lack of time"[2] or "lack of enjoyment".[3] Recently, high-intensity interval exercise (HIIE) has been proposed as a time-efficient physical exercise regimen that could generate comparable benefits to moderate-intensity continuous physical exercise (MICE).[4,5] HIIE has also been found to increase enjoyment[6] and improve patient adherence to physical activity.[5]

Studies evaluating HIIE and MICE have revealed some advantages of the HIIE over MICE, including the improvement of VO_{2max}[7] the increase of the maximal activities of mitochondrial enzymes,[8] the reduction of lactate accumulation during exercise,[9] and the improvement of metabolic adaptation.[4] Compared to moderate-intensity interval exercise (MIIE), HIIE is better in improving insulin sensitivity of obese adolescent girls,[10] and decreasing body mass, body fat, and waist circumference of healthy obese female adolescents.[11] Despite these many advantages of the HIIE, there were evidences that HIIE increased oxidative stress biomarkers and reduced antioxidant capacity,[12] induced brain mitochondrial dysfunction and decreased BDNF levels in the frontal cortex of mice.[13]

Previous studies have shown that MIIE increased the serum BDNF levels of Parkinson disease patients,[14] and increased the expression of hippocampal BDNF gene of juvenile rats greater than HIIE.[15] The present study intended to compare the effects of HIIE and MIIE on the spatial memory of ovariectomized rats and the association between the spatial memory and oxidative stress biomarkers (MDA) as well as the antioxidant enzymes activity i.e. Cat and GPx.

MATERIALS AND METHODS

Animals and reagents

A total of 16 female Sprague Dawley rats aged 12 weeks, which were initially weighing 150 - 200 g, were used in this study. The rats were obtained from the Animal House of Universitas Gadjah Mada. They were housed in cages under 12-h of natural light-dark cycle. Food and water were given *ad libitum* throughout the experiment. The experimental protocol and animal handling was approved by the Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (ethical number KE/FK/217/EC/2016). After one week of familiarization to the experimental room, the rats were randomly assigned into two main groups, i.e. ovariectomy (12 rats) and sham-operated without exercise (SO; n = 4) groups. Both ovaries of the 12 rats of the first group were removed via a 2–3 cm ventral midline incision on the abdomen

under anesthesia (ketamine HCl 60 mg/ kg body weight; PT Guardian Pharmatama, Jakarta, Indonesia). The remaining 4 rats underwent sham surgery. Seven days after ovariectomy, the 12 rats of the first group were divided further into three groups, i.e. ovariectomy without exercise (O; n = 4), ovariectomy with high intensity interval exercise (HIIE; n = 4), and ovariectomy with moderate intensity interval exercise (MIIE; n = 4) groups.

Exercise training protocol

The protocol of exercise was conducted according to the protocol developed by Afzalpour *et al.*[16] with slight modifications. Briefly, the protocol consisted of two periods, i.e. adaptation period and exercise period. The rats of the exercise group were adapted to the exercise protocol and treadmill apparatus (Gama Tread version 2010, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada) for one week. During the adaptation period, the rats had to run on the treadmill with the running speed of 10 m/min, the treadmill slope of 0°, and the duration of exercise of 10 min/day for 6 days. During the exercise period, the rats had to perform 6 sessions of running per week for 6 weeks. The number of intervals and running speeds of training were distinguished between odd and even days. In the odd days, the rats had to run at a high intensity running speed (36 m/min) in the HIIE group and a moderate intensity running speed (18 m/min) in the MIIE group for 30 sec, interspersed with one-minute intervals of running at low intensity running speeds (10 m/min) for recovery phase. The number of intervals started with 2 intervals in the first week up to 6 intervals in the fourth week. In the even days, the rats had to run at the running speeds of 40 m/min for HIIE and 20 m/min for MIIE for 30 sec and interspersed with recovery phase at the same speed (10 m/min) and duration as odd days. The interval increased daily starting at 3 intervals in the first week and settled after reaching 20 intervals by the end of the fourth week. At the beginning and the end of high intensity and moderate intensity interval exercise training procedures, warming-up and cooling-down were performed at 10 m/ min for 5 min. The O and SO groups were only moved to the training room at the same time when the exercise groups performed exercise.

Morris water Maze task

The Morris water maze (MWM) test was conducted based on the protocol described elsewhere.[17] The test consisted of two phases, i.e. escape acquisition and memory persistence phases, and an additional visible platform test. The test apparatus consisted of a white-painted circular pool with a diameter of 150 cm and a height of 40 cm. The pool was filled with fresh cow milk to hide the platform, up to the depth of 18 cm. A circular white platform was placed 2 cm below the surface of the water. The temperature of the water was around 25°C. To record the movement of animals in the pool, a video camera was installed above the center of the pool, and linked to a personal computer. Several geometric pictures with different colors were attached to the white curtain wall around the pool. The pool was divided into four equally imaginary quadrants. The circumference wall of the pool was marked with 8 equally spaced starting points.

Six days before the exercise training finished, the MWM test began. In order to familiarize with the test room, the rats were moved to the room twenty-four hours before the trials. On the day of testing, the platform was positioned in the center of a randomly chosen quadrant for each rat. One starting point was randomly chosen for each trial. The test began when any randomly selected rat was placed at this starting point with its head facing toward the inner side of the circumference wall of the pool, and then allowed to swim and find the hidden platform as a way to escape.

Escape acquisition test. Each rat was given four trials each day for 4 consecutive days with 60 sec inter-trial interval. The rat was allowed to swim for a maximum of

60 sec to find the hidden platform at each trial. The time ('escape latency') for the rat to find the platform was recorded. Once the rat reached the platform, it was left there for 20 sec. If the rat failed to find the platform within 1 min, the rat was given a latency score of 60 sec and placed on top of the platform for 20 sec.

Memory persistence test. To examine the animal ability in retaining the spatial memory about the location of the platform, the rats underwent memory persistence test, 24 h after the escape acquisition test. Each rat was allowed to swim in the pool without a platform for 60 sec. The latency of each rat to swim in the quadrant where the platform was previously placed during the escape acquisition test was recorded. The percentage of time expended in the correct quadrant to a total of 60 sec was calculated.

Visible platform test. In the same day with and after the memory persistence test, the rats were given a visual test to examine their sensory and motor functions. Before the test began, a starting point was randomly selected for each rat. The platform was located in a different place from where the platform was previously positioned during the escape acquisition and memory persistence tests. The platform was made visible with a flag was attached on the platform up to 2 cm high from the surface of the water. The test consisted of three trials which lasted for a maximum of 60 sec per trial. The latency was recorded for each test. If a rat failed to find the platform within 60 sec, the latency was recorded as 60 sec. The data on latency were then used for further statistical analysis.

Hippocampal tissue collection

The rats were euthanized under anesthesia (ketamine HCl 100 mg/ kg body weight; PT Guardian Pharmatama, Jakarta, Indonesia) approximately 24 h after the last exercise training. The hippocampi of the rats were removed from their skulls and subsequently were extracted from the forebrains of the rats in iced phosphate buffered saline (PBS). The extracted hippocampus was homogenized in 10% PBS. The homogenates were then centrifuged at 14.000 rpm for 5 min at 4°C, and the supernatants of the homogenates were used for the examination of the activity of Cat and GPx, as well as the level of MDA using spectrophotometry method.

Statistical analysis

The data from the acquisition phase of the spatial memory test were analyzed using two-way repeated measures analysis of variance (Anova). The memory persistence tests data, level of MDA and the activity of antioxidant enzymes (Cat and GPx) were analyzed using one-way Anova. The post hoc least significant difference (LSD) test was performed when appropriate. The Pearson correlation tests were conducted to assess the correlation between the spatial memory persistence test data with the hippocampal MDA level as well as the activity of Cat and GPx enzymes. The statistical analyses were performed using either SPSS (version 19) or Sigmastat (version 4.0) software. All data were presented as the means ± standard error of the mean (SEM) and the significance levels were set at $p < 0.05$.

RESULTS

The sensory-motor functions of rats

Visible platform test. All groups performed equally well on the three trials of the visible platform test (FIGURE 1). The escape latencies data of the first trial in visible platform test were not homogenous. The Kruskal-Wallis analysis of these data did not show any significant difference between groups ($p = 0.414$). One-way Anova of the escape latencies of the second and third trials of the visible platform test did not show any significant difference between groups ($p = 0.297$ and $p = 0.269$, respectively). Overall, there was no significant difference in the sensory-motor functions between all groups of rats.

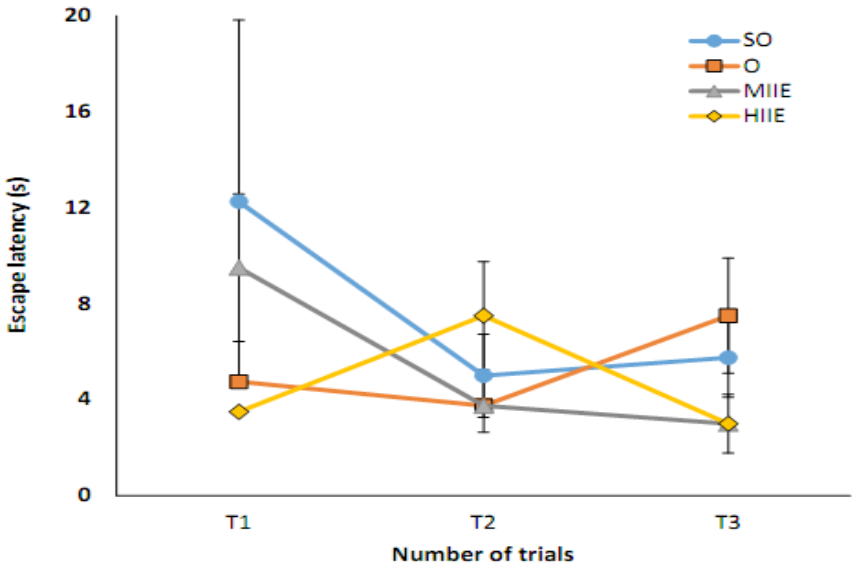

FIGURE 1. Means ± SEM of escape latency (s) of sham-operated (SO) group, ovariectomized (O) group, moderate-intensity interval exercise (MIIE) group and high-intensity interval exercise (HIIE) group rats during sensory-motor test of the Morris water maze procedure; T, trial. Result of Kruskal-Wallis of T1 wasp > 0.05, Results of one-way Anova of T2 and T3 were p > 0.05

The effects of exercise on the spatial memory

Escape acquisition test. The data of the escape acquisition test are shown in FIGURE 2. The two-way repeated measures Anova of these data showed significant main effects of groups (df = 3, 180; F = 3.996; p = 0.035) and day/trial (df =15, 180; F = 10.773; p = <0.001), but not groups x day/trial interaction. The post-hoc LSD test (complete data not presented) showed that the escape latency of the MIIE group was significantly shorter than the other three groups.

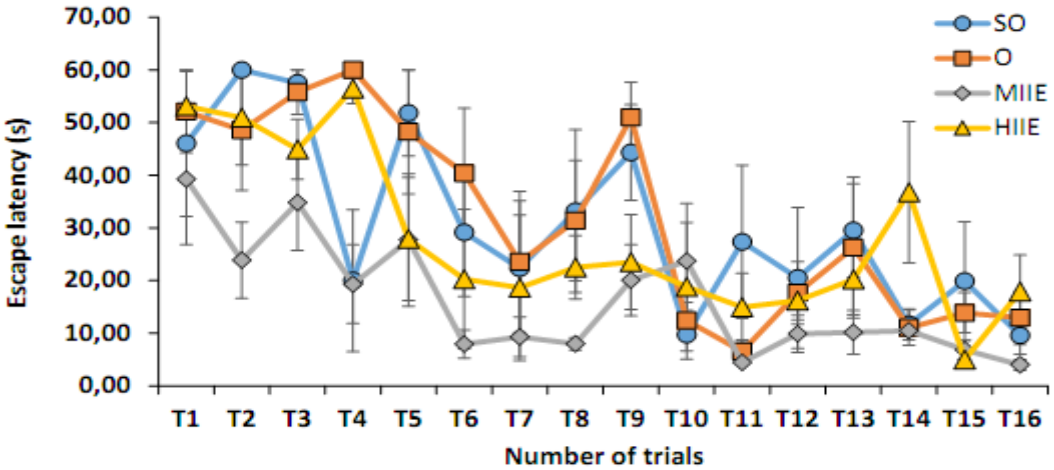

FIGURE 2. Means ± SEM of the $\log_{10}$ of the escape latency (s) of the sham-operated (SO) group, moderate-intensity interval exercise (MIIE) group and high-intensity interval exercise (HIIE) group rats during 4 consecutive escape acquisition test days of the Morris water maze procedure; T, trial. Results of two-way Anova repeated measures. Groups; df = 3, 180; F = 3.996; p = 0.035. Day/trial; df= 15, 180; F = 10.773; p = <0.001. Groups x day/trial interaction; df = 45, 180; F = 1.301; p = 0.117. Anova, analysis of variance; df, degree of freedom; F, F value; p, p value

Memory persistence test. The memory persistence ability was analyzed from the data of percentage **of** time spent in the target quadrant of the probe test (FIGURE 3). One-way Anova of these data showed that there was a significant main effect of groups ($p = 0.005$). The post-hoc LSD test of these data (complete data not presented) revealed that the percentage of time expended in the correct quadrant of the MIIE group was significantly shorter than that of the O ($p = 0.023$) and HIIE ($p = 0.027$) groups but not significantly different from the SO group ($p= 0.232$).

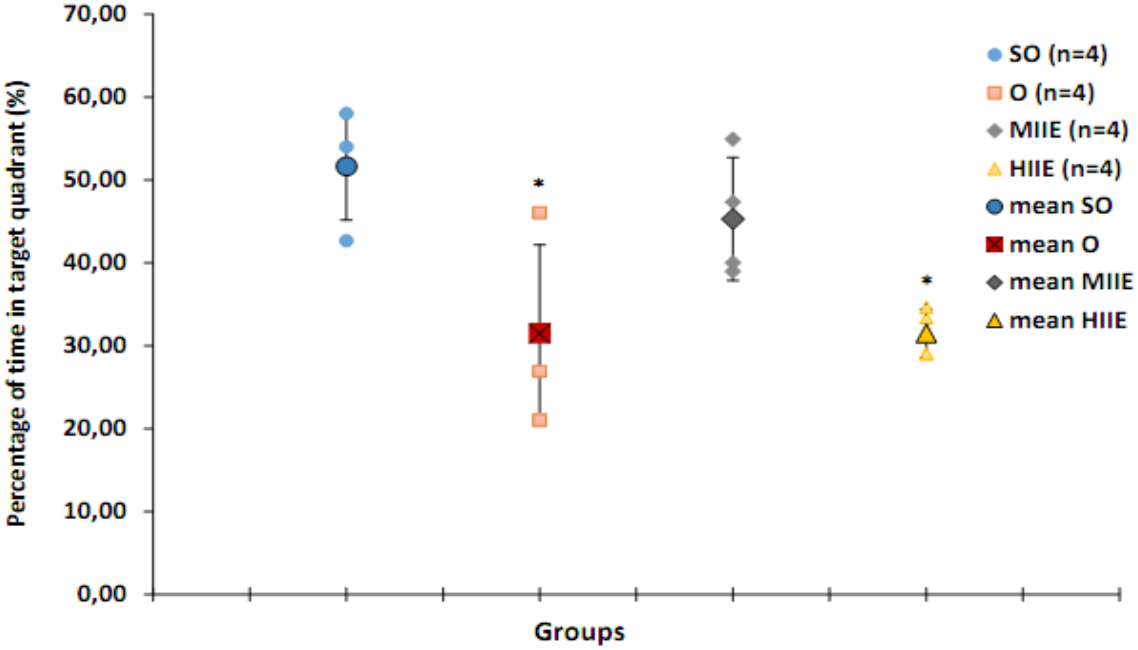

FIGURE 3. Means ± SEM of the escape latency (s) in memory persistence test of the Morris water maze test; SO, sham-operated group, O, ovariectomized group, MIIE, moderate-intensity interval exercise group, HIIE, high-intensity interval exercise group. *, $p < 0.05$ compared to SO group

The effects of exercise on the hippocampal MDA level

FIGURE 4 presents the data of hippocampal MDA concentration of all groups of rats. One way Anova of these data showed a significant main effect of groups ($p < 0.001$). The post-hoc LSD test of the data revealed that the mean level of hippocampal MDA of the SO group (1.49 ± 0.065 nmol/g tissue weight) was significantly lower than that of the O (6.16 ± 0.241 nmol/g tissue weight), MIIE (3.90 ± 0.229 nmol/g tissue weight), and HIIE (2.46 ± 0.144 nmol/g tissue weight) groups ($p<0.05$). The mean of hippocampal MDA level of the HIIE group was significantly lower than that of the O and MIIE groups ($p<0.05$).

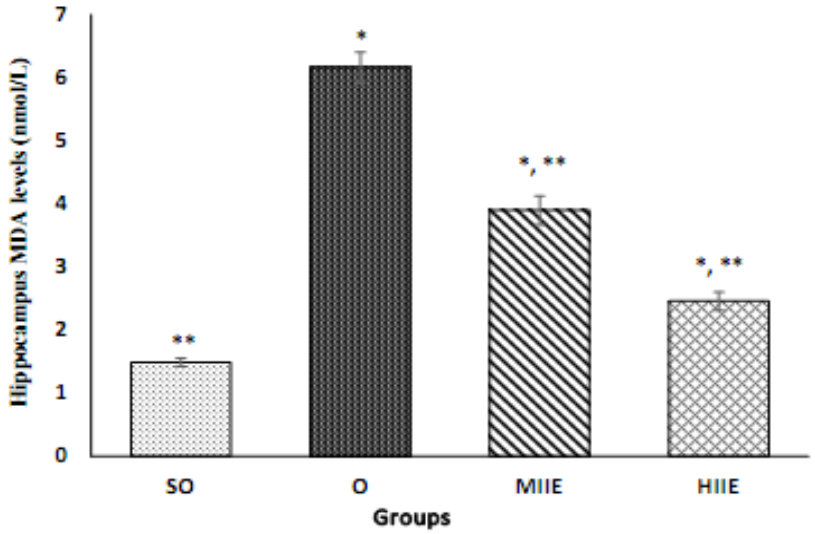

FIGURE 4. Means ± SEM of the levels of malondialdehyde (MDA) in the hippocampus of the sham-operated (SO) group, ovariectomized (O) group, moderate-intensity interval exercise (MIIE) group and high-intensity interval exercise (HIIE) group rats. *, $p < 0.05$ compared to the SO group.

The effects of exercise on activity of antioxidant enzymes in hippocampus

FIGURE 5 presents the data of the activity of GPx in hippocampus of all rats. One way Anova of these data revealed a significant main effect of groups ($p < 0.001$). The post-hoc LSD test of the data demonstrated that the GPx activity in the hippocampus of the O group (33.38 ± 1.456 IU/mL) was significantly lower than that of the SO (56.72 ± 2.388 IU/mL), MIIE (61.54 ± 2.978 IU/mL), and HIIE (54.98 ± 2.051 IU/mL) groups.

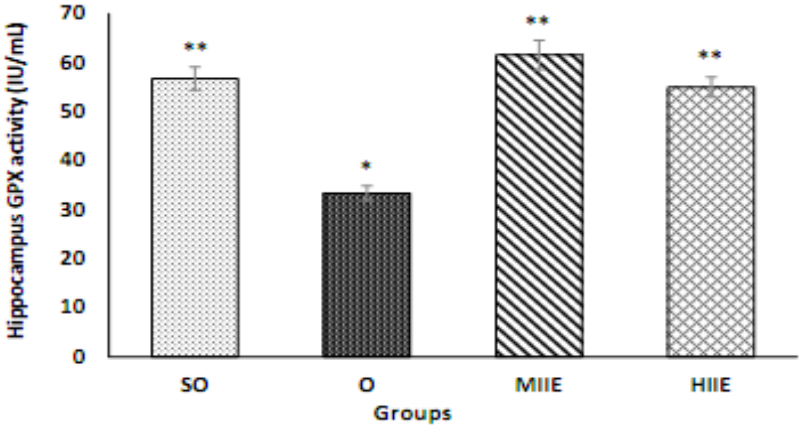

FIGURE 5. Means ± SEM of the activity of glutathione peroxidase (GPx) in the hippocampus of the sham-operated (SO) group, ovariectomized (O) group, moderate-intensity interval exercise (MIIE) group and high-intensity interval exercise (HIIE) group rats. *, $p < 0.05$ compared to the SO group

FIGURE 6 shows the data of the activity of Cat in hippocampus of all groups of rats. One way Anova procedure of these data showed a significant main effect of groups ($p < 0.001$). The post-hoc LSD test of the data showed that the Cat activity in the hippocampus of the SO group (6.40 ± 0.034 IU/mL) was significantly higher than that of the O (1.46 ± 0.037 IU/mL), MIIE (2.13 ± 0.035 IU/mL), and HIIE (5.60 ± 0.016 IU/mL) groups.

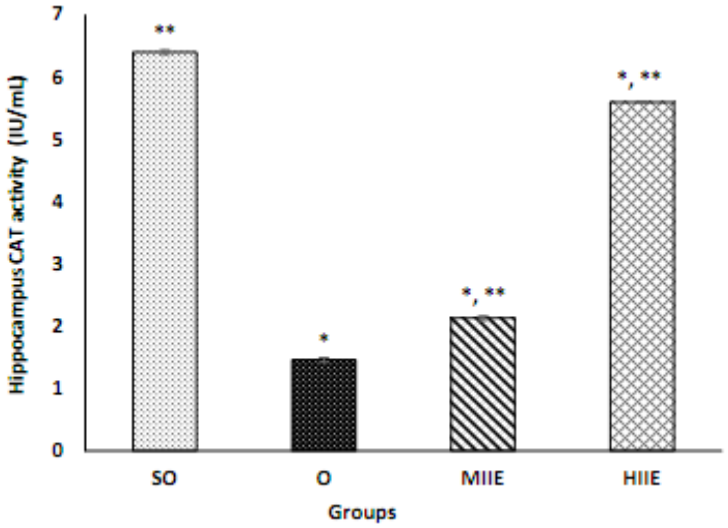

FIGURE 6. Means ± SEM of the activity of catalase (Cat) in the hippocampus of the sham-operated (SO) group, ovariectomized (O) group, moderate-intensity interval exercise (MIIE) group and high-intensity interval exercise (HIIE) group rats. *, $p < 0.05$ compared to the SO group. **, $p < 0.05$ compared to the O group.

Correlation

The Pearson correlation test revealed a significantly ($p = 0.019$) positive correlation ($r = 0.576$) of the percentages of time spent in the target quadrant during the memory persistence test with the activity of GPx enzyme in hippocampus. On the other hand, the hippocampal MDA levels had a significantly ($p = 0.047$) negative correlation ($r = -0.502$) with the percentages of time expended in the correct quadrant of the memory persistence test. There was no significant correlation ($p > 0.05$) between the percentages of time expended in the correct quadrant with the activity of catalase. The regression analysis could not be conducted because the data do not fulfill the requirements for the analysis.

DISCUSSION

The present study found that the spatial learning of the MIIE group was significantly better than the O, SO, and HIIE groups. The escape latency of trials 1- 4 of the fourth day of all groups was significantly shorter than that of the first day. Although all groups of rats demonstrated a similar trend of final escape latencies (trials 13 - 16 in the fourth day), the latency curve of the MIIE group already declined since trial 6. This may indicate that the MIIE group learned the spatial information faster than any other groups. The spatial memory retention of the MIIE group was not different from the SO group. However, it was significantly better than the O and HIIE groups. Therefore, moderate-intensity interval exercise may inhibit the spatial memory decline induced by ovariectomy.

There was a significantly positive correlation between the hippocampal GPx activity and the percentage of time in the target quadrant in the memory persistence

test. In contrast, there was a significantly negative correlation between the percentage time in the target quadrant in the memory persistence test with the hippocampal MDA level. This may suggest that the spatial memory retention of ovariectomized rats was affected by the activity of antioxidant enzyme (GPx) and the level of oxidant (MDA) in the hippocampus.

The hippocampal MDA level of the O group was higher than that of the SO group. This suggests that ovariectomy induces oxidative stress. The hippocampal MDA level of the MIIE group was lower than the O group but still higher than that of the SO and HIIE groups. In addition, the MDA level of the HIIE group was lower than that of the O and MIIE groups, but still higher than that of the SO group.

The hippocampal GPx activity of the O group was lower than that of the SO group ($p < 0.01$). This indicates that ovariectomy suppresses the GPx activity. The hippocampal GPx activity of the MIIE and HIIE groups was not significantly different from the SO group. The GPx/MDA ratio of the HIIE group was higher (1: 26) than that of the MIIE group (1:15). It appears that HIIE-induced oxidative stress triggered the increase of GPx response that rapidly counteracted the increase of the MDA level of the ovariectomized rats.

Despite the fact that the hippocampal MDA level of the HIIE group was the lowest among all groups and the GPx/MDA ratio of the HIIE group was higher than the MIIE group, the spatial learning and memory persistence of the HIIE group was not as good as the MIIE group. It seems that a sufficient hippocampal MDA level, such as that in the MIIE group, is required to maintain an adequate response of the antioxidant and oxidative damage repair systems against oxidative stress to yield an optimum improvement of the spatial learning and memory functions of the hippocampus.

It has been shown that ovariectomy triggers oxidative stress, including lipid peroxidation.[18] MDA was an aldehyde compound produced by lipid peroxidation.[19] It has been found in the current study that the SO group had the lowest hippocampal MDA level, which was followed by the HIIE group. Probably the capability of the HIIE to decrease the MDA level in the hippocampus of ovariectomized rats was better than the MIIE group, although the decline of the MDA level did not reach the level of the SO group. The present study also revealed an inverse correlation between the MDA level in the hippocampus and the spatial memory persistency test performance. This result corroborate other studies showing that the parenteral administration of MDA decreased the ability of learning and spatial memory of rats,[20] and the decrease of spatial memory function was parallel with the hippocampal MDA level in the STZ-induced diabetes mellitus type 2 in mice.[21]

Exercise-induced mild oxidative stress seems to be able to reduce oxidative damage by upregulating antioxidant enzymes e.g. GPx and Cat.[22] GPx is known as an enzyme that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or alcohols, respectively, using reduced glutathione (GSH) as the reductant.[23] Decreased GPx activity was found in tissues suffering from oxidative stress.[24] In this study, the lowest and highest GPx activities were observed in the O and SO groups, respectively. The GPx activity of both HIIE and MIIE groups was not significantly different from the SO group. This indicates that both HIIE and MIIE regimens have a similar ability to increase the GPx activity of ovariectomized rats.

Another antioxidant enzyme that was affected by oxidative stress was catalase. Cat is an antioxidant enzyme that catalyzes the conversion of H_2O_2 into water and oxygen.[25] Thus catalase is effective in reducing H_2O_2 levels.[26] In this study, the lowest and highest Cat activities were observed in the O and SO groups, respectively. The catalase activity of the HIIE group was higher than the MIIE group, but still lower than that of the SO group. This suggests that HIIE has a

better ability to increase the Cat activity of ovariectomized rats than MIIE. The increase of the Cat activity of the HIIE group was possibly induced by the increase of H_2O_2 level after exercise, which was higher in the HIIE group than the MIIE group.[27]

The correlation analysis revealed that the spatial memory persistence of the rats was positively correlated with the GPx but not with the Cat activity in the hippocampus. The reasons of this pattern of correlation are probably that GPx is more potent than catalase[28] in that GPx affinity against H_2O_2 is higher than catalase[26] and that GPx not only breaks down H_2O_2 but also breaks down lipid peroxide (LOOH).[26]

Physical exercise modulates oxidative stress which in turn stimulates an adaptation as homeostatic responses. An increase in the free radical level initiates an adaptive response of the antioxidant and oxidative damage repair systems which may lead to the increase in the activity of suitable antioxidant enzymes.[29] The beneficial effects of repeated physical exercise obtained from the adaptation process serve as an answer to the oxidative stress. The adaptive effects of regular exercise are systemic and specific, depending on the characteristics of exercise and target organ.[30] The adaptation process may also result in a failure which is primarily due to inappropriate/very light stimuli, or incomplete recovery.[29,31] In the present study, the MIIE and HIIE was comparable in terms of their capability to increase the GPx activity of the ovariectomized rats. However, the MIIE seems to be greater in improving learning and spatial memory function of ovariectomized rats than the HIIE.

The beneficial effects of physical exercise depend on the adaptive responses of genes expression.[30] Physical exercise gives rise to the increase in the hippocampal growth factors, for instance brain-derived neurotropic factor (BDNF),[32] insulin-like growth factor-1 (IGF-1)[33] and vascular endothelial growth factor (VEGF).[34] The positive effects of exercise on the hippocampal function is thought to occur through hippocampal neurogenesis stimulation by BDNF,[33] IGF-1[33] and VEGF.[35] The underlying mechanisms of hippocampal-dependent spatial learning and memory improvement in the present study, however, are beyond the scope of the present study since it is primarily designed only to compare the effects of MIIE and HIIE on the spatial memory of ovariectomized rats

CONCLUSION

In conclusion, our study found that moderate-intensity physical exercise prevented the ovariectomy-induced spatial memory retention deficits of the Sprague Dawley rats. Physical exercise may exert these beneficial effects on the hippocampus via its modulation on the hippocampal GPx activity. The detailed mechanism of these effects, however, remains unclear at present, and therefore requires further investigations

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The effect of tonsillectomy on formant sound frequency

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ABSTRACT

Vocal tract resonance characteristics are reflected by formant frequencies which are determined by the size and shape of the vocal tract. Tonsillectomy may cause changes in the structure of the oral cavity as a resonator in the speech process. The aim of this study was to evaluate the effect of tonsillectomy on formant sound frequency. This was a pre-test and post-test only design study conducted in the Department of Otorhinolaryngology, Dr. Sardjito General Hospital, Yogyakarta between August and November 2012. The inclusion criteria were chronic tonsillitis; tonsil size was T3-T4, and age up to 45 years, whereas the exclusion criteria were craniofacial abnormality and neurological disorders. All patients performed a sound analysis by recording formant frequencies 1 (F1), F2 and F3 vowels /a/, /e/, /i/, /o/, and /u/ at 1 day before and 4 weeks after surgery. Twenty (60%) female patients and 16 (40%) male patients were involved in this study. The T3-T3 tonsil size were 25 (62.5%) samples and the lowest in T3-T4/T4-T3 were 6 (15%) samples. Significantly increase of all F1 vowels (paired t-test $p<0.05$) except for vowel /i/ ($p>0.05$) was observed. However, no significantly change of F2 except for vowel /e/ and F3 except for vowel /u/ were observed ($p>0.05$). In conclusion, tonsillectomy generally affects F1 but not affect F2 and F3.

ABSTRAK

Karakteristik resonansi traktus vokalis ditunjukkan oleh frekuensi *formant* yang ditentukan oleh ukuran dan bentuk traktus vokalis. Tonsilektomi dapat menyebabkan perubahan pada struktur rongga mulut yang berfungsi sebagai resonator proses bicara. Tujuan penelitian ini untuk menentukan pengaruh tonsilektomi terhadap frekuensi *formant* suara. Penelitian dengan rancangan *pre-test and post-test only experimental* ini dilakukan di Departemen Telinga Hidung Tenggorok-Kepala Leher, RSUP Dr. Sardjito, Yogyakarta periode bulan Agustus sampai November 2012. Kriteria inklusi adalah tonsilitis kronis; ukuran tonsil T3-T4 dan usia hingga 45 tahun. Kriteria eksklusi adalah kelainan kraniofasial dan gangguan neurologis. Seluruh pasien dianalisis suaranya dengan merekam frekuensi *fromant* 1 (F1), F2 dan F3 vokal /a/, /e/, /i/, /o/, dan /u/ pada 1 hari sebelum dan 4 minggu setelah tonsilektomi. Sebanyak 24 (60%) subjek perempuan dan 16 (40%) subjek laki-laki terlibat dalam penelitian. Ukuran tonsil T3-T3 terbanyak adalah 25 (62,5%) subjek dan T3-T4/T4-T3 sebanyak 6 (15%) subjek. Terjadi kenaikan bermakna semua vokal pada F1 (uji t pasangan $p<0.05$) kecuali vocal /i/ ($p>0.05$). Akan tetapi, tidak terjadi perubahan F2 kecuali vokal /e/ dan F3 kecuali vokal /u/ ($p>0.05$). Dapat disimpulkan, tonsilektomi secara umum mempengaruhi F1 tetapi tidak terhadap F2 dan F3.

Keywords **:** tonsillectomy – formant frequency – sound analysis – adenoidectomy – resonator structure

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INTRODUCTION

Tonsillectomy with or without adenoidectomy is a common surgical procedure on children in Western countries. A study showed that on average adeno-tonsillectomy varies widely between countries. Adeno-tonsillectomy in Canada in 1998 reported 19 cases per 10,000 children and 19 cases per 10,000 adults, in Northern Ireland there were 118 cases per 10,000 children, in UK 65 cases per 10,000 children, in the Netherlands 115 cases per 10,000 children and in Finland 76 per 10,000 adults.[1] Data from the National Center for Health Statistics show about 418,000 tonsillectomy with or without adenoidectomy were done in the United States in 1996.[2]

Although there are many advantages of tonsillectomy, but treating physicians also need to consider the complications from surgery such as sore throat, nausea and vomiting after surgery, delayed intake of food, bleeding and changes in voice and death although rare.[3] Tonsillectomy can cause changes in the structure in the mouth cavity that play a role as a resonator in the speech formation process.[4] Changes in the resonator structure after tonsillectomy can cause changes in individual speech characteristics which can be viewed from the sound parameter analysis that include frequency formant, jitter, shimmer, harmonic noise ratio (HNR).[5-7] voice turbulence index (VTI), soft phonation index (SPI), degree of voiceless (DUV), and degree of voice breaks (DVB). Sound analysis has begun to be used for assessing characteristic the changes of speech in patients who undergo surgery in the airway area especially tonsillectomy with varying results.[6,7] Study to evaluate the change in the sound color (timbre) after surgery has not yet been extensively conducted in Indonesia.

Human voice characteristics is divided into two types: 1) non-acoustic characteristics, examples are pulses and time; and 2) acoustic characteristics, which are composed from pitch, formant and formant bandwidth. Pitch is formant to zero (F0). Formant is defined as the spectrum of wave crest to wave crest in the human voice. Formant bandwidth is the width of a formant. When humans are speaking and pronouncing vowels they can produce more than four formants. To distinguish a sound vowel requires two formants, namely the first formant (F1), which corresponds to the position of the tongue against soft palate while talking and the second formant (F2), which is associated with position the tongue in front or behind while speaking. The third formant (F3), the fourth formant (F4) and so on, affect the sound (timbre) color.[8]

Based on the above reasoning it is necessary to do speech characteristic analysis before and after tonsillectomy with or without adenoidectomy objectively so that it can be used as a basis to explain about the change of speech characteristics based on the parameters of sound analysis after tonsillectomy. This study determined the effect of tonsillectomy to the vocal tract using sound analysis parameters that include the formant frequency (F1, F2 and F3).

MATERIALS AND METHODS

Study design

This research used a pretest and posttest only experimental design (before and after). The data were collected from the measurements of the sound analysis using PRAAT 5.1.05 software on 1 day before and 4 weeks after tonsillectomy with or without adenoidectomy. PRAAT is a computer program for analyzing, synthesizing, and manipulating speech. PRAAT 5.1.05 software is currently the most accepted, standardized and popular for speech analysis. It is particularly helpful in phonetics classes and academic sessions for making spectrogram, pitch tracks and similar functions. Whatever be the level of knowledge about speech analysis and phonetics, this tool can be easily used to achieve desired results. Analyzing speech with PRAAT allows us to record a sound with a microphone or any other audio input device, or to read a sound from a sound file on disk. It will then be able

to have a look `inside' this sound. The upper half of the sound window will show a visible representation of the sound (the wave form). The lower half will show several acoustic analyses: the spectrogram (a representation of the amount of high and low frequencies available in the signal) is painted in shades of grey; the pitch contour (the frequency of periodicity) is drawn as a cyan curve; and formant contours (the main constituents of the spectrogram) are plotted as red dots. PRAAT is most often used with speech sounds, in which case the pitch contour is associated with the vibration of the vocal folds and the formant contours are associated with resonances in the vocal tract.[9]

Time and location

This research was conducted in the Ear Nose and Throat Department of Dr. Sardjito General Hospital Yogyakarta, Dr. Soeradji Tirtonegoro Hospital Klaten-Central Java and Saras Husada Hospital Purworejo-Central Java between August and November 2012.

Population and samples

The study population was patients who underwent tonsillectomy with or without adenoidectomy. The inclusion criteria were: 1) chronic tonsillitis patients with or without adenoid hypertrophy (Mallampati class II-III), 2) tonsil size greater than or equal to T3, 3) in the past 1 week did not experience cough and cold, 4) normal mandibular and neck anatomy structures 5) method of tonsillectomy with Guillotine or dissection, 6) age equal to or more than 5 years, and 7) willing to follow the research and sign the informed consent or proxy consent form. The exclusion criteria in this study were craniofacial abnormalities and neurological disorders.

This study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta (Ref. KE/FK/775/EC). All patients performed a standard sound analysis by recording vowel /a/, /e/, /i/, /o/, and /u/ (Boersma, and van Heuven) in 1 day before and 4 weeks after surgery using PRAAT 1.5.05 software.[9] Based on the sample size calculation for different mean of variables: F1, F2, F3 before and after surgery, the number of sample size used in this study was 36 plus estimated drop out 10%, which were 40 samples.

Statistical analysis

Data were presented as mean ± standard deviation (SD) or percentage. The difference of the formant frequency (F1, F2, and F3) before and after tonsillectomy was analyzed using paired t-test with p value < 0.05 as considered significant.

RESULTS

The characteristics of subjects by group age, sex, BMI (body mass index), tonsil size, type of operation, and method of operation are presented in TABLE 1. From the all subjects, 24 (60%) subjects were female and 16 (40%) samples were male. Based on the age distribution, it was divided into two groups, age 5- <18 years old were 32 (80%) samples and ≥18 years old were 8 (20%) samples. The youngest age was 5 years old and the oldest was 42 years old. Based on the distribution of BMI, obtained the largest frequency on underweight group of 19 (47.5%) sujects and fewest frequencies in the overweight group of 6 (6%) samples. Most tonsil size is T3-T3 which is 25 (62,5%) samples and lowest is T3-T4 / T4-T3 as many as 6 (15%) samples. Based on the type of operation, total tonsillectomy in all samples were 40 (100%) samples. There were 18 (45%) samples with Guillotine method and 22 (55%) samples with dissection method.

TABLE 1. Characteristics of research subjects

Variable	n (%)
Gender	
• Female	24 (60)
• Male	16 (40)
Age (years)	
• 5-18	32 (80)
• ≥ 18	8 (20)
BMI (body mass index)	
• Underweight	19 (47.5)
• Normal	15 (37.5)
• Overweight	6 (15.0)
Palatine tonsil size	
• T3-T3	25 (62.5)
• T3-T4/T4-T3	6 (15.0)
• T4-T4	9 (22.5)
Surgery type	
• Tonsillectomy	40 (100)
• Adeno-tonsillectomy	0 (0)
Surgery method	
• Guillotine	18 (45)
• Dissection	22 (55)

An average change of F1, F2, and F3 before and after surgery in all vowels was observed in this study (TABLE 2). An increase in mean F1 for all vowels, F2 /e/ & /i/, and F3 for almost all vowels except /a/. Significant mean increases occurred in almost all F1 vocals ($p < 0.05$) except /i/ ($p > 0.05$). This difference was probably because in making the vowel /i/, the highest position of the tongue was in front and did not require sticking the tongue on the pharynx wall so that on the enlargement of the tonsils and after tonsillectomy did not affect F1.

TABLE 2. Average difference of F1, F2, and F3 before and after tonsillectomy on vowels

Vowel	Operation	n	F1 (Mean ± SD)	p	F2 (Mean ± SD)	p	F3 (Mean ± SD)	p
/a/	Pre-op	40	882±177	0.007	1537±167	0.956	2756±392	0.056*
	Post-op	40	965±142		1535±186		2653±490	
/e/	Pre-op	40	588±114	0.008	2093±299	0.009*	2904±268	0.360
	Post-op	40	629±114		2142±409		2948±244	
/i/	Pre-op	40	502±99	0.382*	2362±285	0.057	3166±218	0.090
	Post-op	40	514±112		2461±327		3232±231	
/o/	Pre-op	40	602±103	0.018	1148±188	0.989*	2896±378	0.192*
	Post-op	40	631±126		1137±249		2971±401	
/u/	Pre-op	40	533±79	0.035	1132±244	0.638*	2940±278	0.035*
	Post-op	40	557±95		1101±284		3043±288	

Note: Pre-op = before operation; Post-op= after operation; n= number of samples, SD=standard deviation; p= p value; * = Wilcoxon test.

DISCUSSION

The significantly increases on F1 for letter /a/, /e/, /o/ and /u/, in F2 for /e/ and F3 for /u/ after tonsillectomy were observed in this study ($p < 0.05$) (TABLE 2). Furthermore, no significantly different on F1, F2 and F3 for all vowels between female and male before and after tonsillectomy was observed ($p > 0.05$).

The studies of the effect of tonsillectomy on formant sound frequency have been reported by some authors with different results. Heffernan & Raffety[10] reported that the increase on F1 for /a/ and the decrease in F2 and F3 after tonsillectomy was observed. Furthermore, changes of F3

and the third formant bandwidth (B3) for vowel /o/ and /a/, a slight decrease in the first formant bandwidth (B1), formant the second bandwidth (B2) for the vowel /a/, along with a mild decrease in the NHR which means diminished nasalized vowels, and the glottal consonants /h/ on patients after tonsillectomy were reporte.[4] Whereas another study reported that F0, F1, F2 and F3 unchanged in females patients after tonsillectomy but F1 and F2 significantly changed in males patients.[5] A preliminary study on sound acoustic changes after upper airway surgery in OSAS patients showed no F0 change, while F1 for vocal /a/ and F2 for vocal /e/ were significantly higher, and F1 for vowel /i/ and F2 for vowels /o/ and /u/ were significantly lower than before surgery.[11]

The effects of adeno-tonsillectomy on the spectrum speech in children aged 4-14 years old has been studied and the results showed that F0, jitter, shimmer, NHR, VTI, SPI, DUV, and DVB 1 month after surgery significantly decreased. In addition, there were significant differences of NHR, VTI, and DVB after surgery, which was close to the value at healthy group.[6] The mirror-fogging test showed a decrease in hyper-nasality from an average of 3.2 before operation to 0 after surgery, while the Gutzman test showed the value of 1 before and 0 after the operation, and the average nasality severity index changed from 3.7 before surgery to 0.6 after surgery.[12]

The impact of tonsillectomy with or without adenoidectomy on speech and voice has been studied on children aged 4-12 years olds. A significant decrease in F0, Jitt, Shim, NHR, VTI, SPI, DUV, DVB, and peak amplitude variation (vAm) in the children underwent adenotonsillectomy was reported one month after surgery indicating the improvements of the speech and voice quality.[6] Furthermore, the effect adenotonsillectomy on the speech spectrum in children aged between 5 and 14 years old, with enlarged palatine tonsils and hypetrophic adenoids has been also reported.[7] An improvement in all the parameters include Fo, Jitt, Shim, NHR, VTI, SPI, DUV and DVB after adenotonsillectomy was observed. In addition a postoperative normalization of NHR, VI and DVB compared with healthy children were also reported.

The mechanism of human speech production (vocal organ) is divided into 3 parts i.e. lung, vocal cord and vocal tract.[13,14] Included in the vocal tract are larynx, pharynx, oral cavity and nasal cavity. The vocal organs include: 1) blowing: lungs, 2) air ducts: trachea, 3) ballot box: larynx 4) resonator: pharynx, mouth and nose.[15,16] The speech formation mechanism consists of 4 processes namely: 1) processing language; the content of the greeting is changed to symbols phonemically in the brain of the language center, 2) the generation of motor commands for vocal organs in the brain of the motor center, 3) articulatory movements by vocal organs under motor command, and 4) air emissions from the lungs in shaping speech.[17]

The speech process is initiated by the stimulating activity of the central nervous system to respiratory tract and vocal tract. Speech is a complex acoustic signal where information is sent to the brain at such high speed with 3-4 syllables per second that are spoken during a conversation. The phoneme is a discrete perception unit composed of complex sound elements that are encoded into neural release patterns in the lower hearing center of the brain for decoding at the higher hearing center.[18]

The sound source comes from the air in the lungs that is the result of inhalation and will be passed on larynx under control of the respiratory muscles, thus causing the vocal cord vibration.[14,18,19] This mechanism produces periodic wave complexes known as glottal flows or voice sources in the form of a hum. The vocal cord vibration in female ranges from 200-300 per second, while in men about 100 per second and in children higher at 400 per second so the sound in male sounds heavier. Good muscular coordination and flow rate according to the subglottal pressure for certain articulation movements is very important to produce a normal amplitude speech pattern.[14]

Frequency of glottal or vocal cord is called frequency fundamental (F0) or otherwise known as pitch.[13,19] The F0 on average at talk time is 120 Hz in males, 250 Hz in females and 400 Hz on children.[14] There is another term used called frequency. The fundamental speech moment is 100 Hz in males and 200 Hz on female, with about 40 harmonization (formant frequency) of fundamental frequency represented in the form of a wave and transmitted to the vocal tract through vibration thus forming speech sounds.[19] The vocal tract (sound resonator) functions as a multi resonant filter (influenced by the articulator movement) for the transmission of the sound wave with its particular shape and spectrum derived from the larynx, so that the hum sounds into a speech or an output sound of a vowel or sound meaningful consonants.[14,18]

In this study almost all F1 average for all vowels except /a/ & /u/ before tonsillectomy had a significant difference in the age group 5 - <18 years and the age group ≥18 years (TABLE 2). The total mean of F1 for all vowels after tonsillectomy was significantly different in the age group 5 - <18 years and the age group ≥18 years. The average F2 and F3 for all vowels before and after the tonsillectomy was not significantly different in the age group of 5 - <18 years and age group ≥18 years except the mean F2 /o/ after tonsillectomy with ($p < 0.05$). In the mean delta of F1, F2, and F3 were not significantly different in the 5- <18 years age group and in the age group ≥18 years.

No significantly difference before and after operation in F1, F2, & F3 mean delta in all vowels on the Guillotine method and dissection was observed except F1 /i/ ($p < 0.05$). There is no prior research comparing Guillotine and dissection methods. The average change before and after the operation and the mean delta of F1, F2, and F3 in each of the vowels /a/, /e/, /i/, /o/, and /u/ based on the operation type as variables are not typically discussed. In this research there were no research samples undergoing adenoidectomy and surgery (TABLE 2)

All subjects of the study were patients undergoing tonsillectomy. There are significant differences in mean scores before and after tonsillectomy based on the tonsil size distribution for F1 /a/, /e/, & /o/ and the mean delta for F1 /a/ and /i/. In F1 /i/ and /u/ and F2, F3 for all vowels there was no difference in total mean and mean delta. TABLE 2 showed that there are significant differences in total mean before and after tonsillectomy based on the BMI distribution for F1 in all vowels except /e/ before tonsillectomy, F2 /a/ before tonsillectomy, and F2 /o/ and /u/ after tonsillectomy.

CONCLUSION

In conclusion, tonsillectomy generally affects F1 but not affect F2 and F3. It is recommended that patients who will undergo tonsillectomy need to be educated regarding the potential change of the F1. Further study is needed with longer sound evaluation time after tonsillectomy to evaluate whether the effect of tonsillectomy on F1 is temporary or permanent.

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Accuracy of albumin creatinine ratio in comparison with albumine excretion rate for diagnosis diabetic nephropathy in type 2 diabetes mellitus

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ABSTRACT

Diabetic nephropathy (DN) is one of complications in diabetic patients manifested by microalbuminuria with minimal level of 30 mg/24 hour which is measured at least 2 times in the period of 3 to 6 months. Microalbuminuria can be measured either albumin excretion rate (AER) or albumin creatinine ratio (ACR). Measurement of ACR is an alternative parameter recommended by WHO in 2011 to diagnose diabetic nephropathy since it is more convenient, fast and not requires special preparation. The purpose of this study was to investigate accuracy of ACR to diagnose DN in type 2 diabetes mellitus (T2DM) patients.This was a diagnostic test study involving 80 T2DM patients. In this study ACR value equal or more than 30 mg/g was independently and blindly compared with AER as the gold standard. The data were analyzed using 2x2 tables in order to calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Other data were analyzed using statistic descriptive. Eighty T2DM patients consisting of 38 males and 42 females participated in this study. They had suffered from T2DM on average 9.5 years, and the average of ACR value was 55.5 mg/g. Total result of true positive and true negative was 77. Three result were false negative but none of false positive result. The ACR value equal or more than 30 mg/g had sensitivity, specificity, PPV, and NPV of 95.9%, 100%, 100%, and 66.7% respectively. In conclusion, the ACR value equal or more than 30 mg/g derived from morning urine sample can be used to diagnose DN in T2DM patients.

ABSTRAK

Nefropati diabetik (ND) merupakan salah satu komplikasi pada pasien diabetes yang dimanifestasikan sebagai mikroalbuminuria dengan nilai minimal 30 mg/24 jam yang diukur setidaknya 2 kali dalam selang waktu 3 sampai 6 bulan. Mikroalbuminuria dapat diukur dengan laju eksresi albumin (*albumin excretion rate*/AER) atau rasio albumin terhadap kreatinin (*albumin creatinine ratio*/ACR). Pengukuran ACR merupakan parameter alternatif yang direkomendasikan Organisasi Kesehatan Dunia pada tahun 2011 untuk mendiagnosis ND karena lebih nyaman, cepat dan tidak memerlukan persiapan khusus. Penelitian ini bertujuan untuk menentukan ketepatan ACR dalam mendiagnosis nefropati diabetik pada pasien diabetes mellitus tipe 2 (DMT2). Dalam penelitian ini nilai ACR sama atau lebih dari 30 mg/g dibandingkan dengan baku emas AER secara bebas dan tersamar. Data dianalisis menggunakan tabel 2 X 2 untuk menetapkan sensitivitas, spesifisitas, nilai ramal positif, dan nilai ramal negatif. Data lain dianalisis menggunakan statistik deskriptif. Sebanyak 80 pasien DMT2 yang terdiri dari 38 laki-laki dan 42 perempuan terlibat dalam penelitian ini. Mereka rata-rata menderita penyakit ini 9,5 tahun dengan rerata nilai ACR 55,5 mg/g. Total hasil positif benar dan negatif benar sebanyak 77. Tiga hasil menunjukkan negatif palsu dan tidak ditemukan hasil positif palsu. Nilai ACR sama atau lebih dari 30 mg/g memiliki sensitivitas, specifisitas, nilai ramal positif, dan nilai ramal negatif secara berurutan sebesar 95,9%, 100%, 100%, and 66,7%. Hal ini dapat disimpulkan bahwa nilai ACR sama atau lebih dari 30 mg/g yang diperoleh dari sampel urin pagi dapat digunakan untuk mendiagnosis ND pada pasien DMT2.

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Keywords: diabetic nephropathy - albumin creatinine ratio (ACR) - albumin excretion rate (AER) – sensitivity - diagnostic test

INTRODUCTION

Diabetic nephropathy (DN) is the most frequent single cause of end-stage renal disease (ESRD) in many countries. Hyperfiltration and microalbuminuria characterize the clinical stages of DN. Increased levels of albumin in the urine have been clearly established as an important determinant for renal complication of diabetes. Screening for increased albumin excretion has therefore been advocated to identify individual at risk for renal disease progression in a timely manner. However, there is still continuing uncertainty as to how urine should be collected and which urinary proteins should be specifically measured for prediction of renal events.[1]

Albumine creatinine ratio (ACR) has been proposed as both a screening and diagnostic test for kidney disease. Screening for microalbuminuria is essential as it allows interventions aimed at preventing diabetic nephropathy and part of the everyday treatment of diabetic patient for detecting kidney disease progression and also evaluation of treatment effect, therefore samples providing immediate and reliable results are highly desirable. Numerous recent studies have been shown that early morning urinary ACR for screening purpose is also a predictor of overt DN and is useful to identify patient at risk as it is less influenced by the factors such as hydration status, physical activity and concentration bias.[2]

Type 2 diabetes mellitus (T2DM) typically asymptomatic in the early stages of development and is generally identified only after 4 to 7 years after onset of the disease.[3] Diabetes mellitus is often not diagnosed until complications appear, and approximately 30% of patients with diabetes may be left undiagnosed. Microvascular and macrovascular complications sometimes already developed, even in pre-diabetic stage. As many as 25% of people with a new diagnosis of diabetes already have diabetic retinopathy (DR) or microalbuminuria.[4]

It is estimated that one-third of patients with type 1 diabetes mellitus (T1DM) and one-sixth of patients with T2DM will develop DN. When DN has developed, the interval to ESRD varies from the first 4 years to more than 10 years. Based on more recent studies, it was similar between T1DM and T2DM. In present study using T2DM patients because its prevalence in Dr. Sardjito General Hospital was higher than that T1DM.[5]

Microalbuminuria is a laboratory parameter that can be used as predictors for the development of nephropathy in diabetic patients. However, it has an ostacle since it requires a 24-hour urine sample which causes a lot of discomfort for patient. Fortunely, nowadays it has been developed other tool to measure microalbuminuria by measuring either albumin excretion rate (AER), or ACR.[2] The lack of information regarding the use of ACR in the determination of microalbuminuria has an impact on delay in diagnosis of DN. Therefore, efforts have been done in order to avoid the development of terminal renal failure that require multidisciplinary management in addition to its poor prognosis and cost.[1]

Currently, the data regarding ACR especially its sensitivity and specificity are still not sufficient, thus its implementation is still limited. In Indonesian population, especially in the Yogyakarta Special Region, ACR test for the diagnosis of DN in T2DM population is rarely used. Therefore, this study tried to measure the sensitivity and specificity of ACR with a cutoff value of 30 mg/g compared with AER value using 24-hour urine sample for diagnosis of DN.

MATERIALS AND METHODS

Reagents and subjects

Sample used in this study were 24-hour urine, urine at random, EDTA blood

sample as well as blood serum. In addition, reagensia kit to measure albumin, creatinine, HbA_{1c}, blood glucose were also needed. This was a diagnostic test study where the ACR was independenly and blindly compared with the AER involving 80 T2DM patients with suspected DN who visited in Internal Medicine Outpatient Clinic of Dr. Sardjito General Hospital and agreed to undergo ACR and AER tests throughout 2015. The inclusion criteria were diabetic patients (a minimum of 4 years) with suspected DN and give written informed consent. Those patients who suffered from either urinary tract infections, or congestive heart failure, or liver dysfunction, or pregnancy, or incomplete or missing medical record data were excluded from this study. Protocol of this study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.

Protocol of the study

Subjects had their urine albumin and urine creatinine levels measured using morning urine sample. The ACR values were calculated manually, dividing urinary albumin by urine creatinine levels. Gold standard in this study was AER that measured from urine albumin level with 24-hour urine. Standard microalbuminuria criteria used in this study were in accordance with 2002 NKF/KDOQ recommendation, ACR between 30-300 mg/g, while AER 30-300 mg/24 hour.[6]

Eligible subjects filled out a questionnaire for baseline characteristics data such as age, sex, education, occupation, history of hypertension, history of heart disease, duration of diabetes and blood pressure (BP). Physical examination was conducted which also include weight, height, BP measurements.

Laboratory tests were carried out for 2 day. In day I, samples were taken for fasting blood glucose, 2 hour post prandial blood glucose, HbA1c, and spot urine. Fasting blood glucose tests, 2 hour post prandial blood glucose and HbA1c were measured from 2 mL of venous blood sample and the tests were condected in the same day of sampling. Subjects on the day I was also asked to collect 5 mL urine sample for urine albumin and creatinine measurements. Urine samples were then transferred into Eppendorf tubes using a standardized micropipette, then labeled and kept at -20°C, pooled them up to sufficient number of samples were acomplished. Subjects were also given written and oral instruction for 24-hour urine collection (when collection began, first urination was discarded then second and subsequent urination collected for 24 hour) into a 2.500 mL threaded cap bottle urine container.

On day 2, 24-hour urine samples were collected. The collected 24-hour urine samples were homogenized by flipping the bottles as much as 5 times, after which the urine volume was recorded. Most homogeneous urine samples were transferred into eppendorf tubes using a standardized micropipette, then labeled and stored at -20°C, pooled until urine albumin tests performed after certain number of samples were collected.

Fasting and 2 hour post prandial blood glucose level were measured according to hexokinase method using COBASS 6000 ANALYZER. The assay is based upon the reduction of NAD^+ through a coupled reaction with glucose-6-phosphate dehydrogenase and is determined spectrophotometrically by measuring the increase in absorbance at 340 nm. Hemoglobin A_1c (HbA_1c) was measured with ion-exchange High-performance liquid chromatography (HPLC) method using Biorad D-10 ANALYZER. The ion-exchange high-performance liquid chromatography (HPLC) is based on chromatographic separation of HbA1c on a cation exchange cartridge in human whole blood. Urinary albumin was measured using a immunoturbidimetric method. The principle of immunoturbidimetric assay is albumin in the urine reacts with specific antibodies, then added polyethylene glycol will precipitate immune complexes that

would cause turbidity. Turbidity is measured by photometric and its value is proportional to the levels of albumin in the urine. Urinary creatinine was measured using Jaffe method using COBASS 6000 ANALYZER. The principle Jaffe method is in alkaline atmosphere with picric acid will form compounds saffron. This color change will be measured by using a photometer.[6]

Albumin excretion rate was urinary albumin excretion within 24 hours. It was measured using immunoturbidimetric method using COBASS 6000 ANALYZER. Albumin excretion rate is the gold standard to assess microalbuminuria. In current study, DN is defined by AER value of ≥30 mg/24 hour. The ACR was calculated manually by dividing the urinary albumin by urinary creatinine values, and the results were expressed in mg/g. In the current study, DN is defined by ACR value of ≥30 mg/g.[2] To assess reliability, before running any tests, analytic tests were first completed using calibration test, accuracy test and precision test.[6]

Data analysis

Data characteristics was presented descriptively in mean and standard deviation (SD) if data had normal distribution or in median and minimum-maximum values if did not have normal distribution. Normality for continuous data was tested using Kolmogorov-Smirnov test. Categorical data were presented in proportion. To test differences in mean age, fasting and 2 hour post prandial blood glucose, independent t-test was used when data were normally distributed and Mann-Whitney test when data were not normally distributed. Meanwhile, to investigate differences in proportion, Chi-Square test was used. Results were considered significant if p values less than 0.05 with 95% confidence intervals.

Diagnostics performance were analyzed by 2 X 2 table and result was presented in sensitivity (Sn), specificity (Sp), accuracy, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio (LR) for test positive and negative results.

RESULTS

Characteristics of subjects

A total of 110 subjects were given a questionnaire, unfortunately 16 subjects did not return their questionnaires, thefore they were considered resigned from study. From the remained subjects (94 subjects), nine subjects did not collect 24-hour urine sample, five subjects without data of volume of 24-hour urine sample so that they were excluded from the study. Finally, 80 subjects were included in analysis. Total subject of the study were 80 subject consisted of females 42 (52.5%) and males 38 (47.5%). Based on age, 40 subjects were more than 60 years (50%) and 40 subjects were less than 60 years (50%). The characteristics of subjects are presented in TABLE 1.

TABLE 1. Characteristics of subjects

Characteristics	n	%
Gender		
• Male	38	47.5
• Female	42	52.5
Age		
• ≤ 60 years	40	50.0
• > 60 years	40	50.0
Education		
• Elementary School	8	10.0
• Junior High School	5	6.3
• Senior High School	37	46.3
• Higher education	30	37.5
Occupation		
• Household	20	25.0
• Professional	2	2.5
• Government employees	6	7.5
• Private	2	2.5
• Entrepreneur	6	7.5
• Other	44	55.0

Total subject of the study were 80 subject consisted 74 subject with DN and 6 subject non DN. No significantly different in body mass index (BMI), history of hypertension, heart disease, dyslipidemia, stroke, duration of DM, fasting and 2 hour post prandial lood glucose level, and HbA_{1C} level between subjects with DN and non DN was observed (TABLE 2).

TABLE 2. Characteristics of subject between ND and Non ND groups

Characteristics	ND (n=74)	Non ND (n=6)	p
BMI (mean±SD kg/m^2)	24.86 ± 3.37	25.24 ± 2.81	0.790
Hipertension [n (%)]			
• Yes	42 (56.8)	3 (50.0)	1,000
• No	32 (43.2)	3 (50.0)	
Heart disease [n (%)]			
• Yes	23 (31.1)	3 (50.0)	0.384
• No	51 (68.9)	3 (50.0)	
Dyslipidemia [n (%)]			
• Yes	47 (63.5)	5 (83.3)	0.659
• No	27 (36.5)	1 (16.7)	
Stroke [n (%)]			
• Yes	7 (9.5)	2 (33.3)	0.133
• No`	67 (90.5)	4 (66.7)	
Duration of DM (mean±SD year)	10.12 ± 7.21	9.33 ± 9.05	0.728
BP (mean ± SD mmHg)			
• Sistolic	129.84±16.17	125.00±10.49	0.521
• Diastolic	79.39±9.43	80.00±5.48	0.854
Fasting glucose (mean±SD mg/dL)	160.38 ± 68.61	139.83 ± 17.95	0.826
2 h PP glucose (mean±SD mg/dL)	227.05 ± 91.58	195.17 ± 52.09	0.517
Creatinine serum (mean±SD mg/dL)	1.10 ± **0**.23	0.87± 0.15	0.630
HbA_1c (mean±SD %)	7.97 ± 2.21	7.67 ± 0.68	0.891

Note: BMI (body mass index); BP (blood pressure); 2 h pp (2 hours post prandial)

FIGURE 1. Scatter plot of AER value based on duration of DM

It was found that AER values tend to be higher in patients with longer duration of DM. Patients with longer duration of DM normally had higher AER (FIGURE 1).

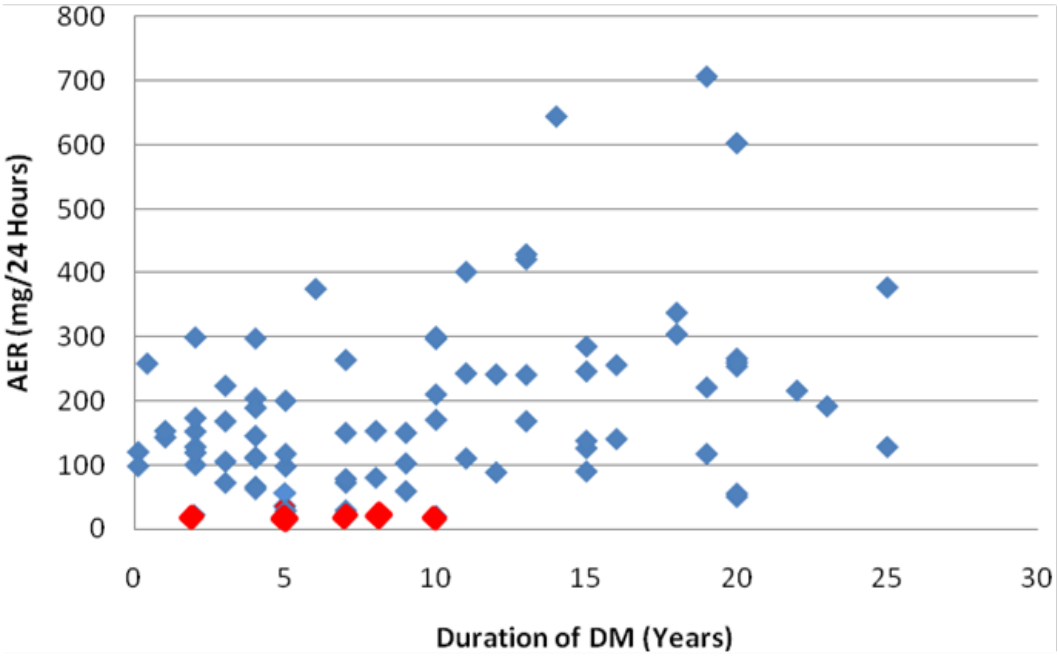

FIGURE 1. Scatter plot of AER value based on duration of DM

ACR diagnostic performance

The ACR diagnostic performance was tested using 2x2 table with AER as the gold standard for DN. It was found that both true positive and true negative rate totally 77. It was also found 3 false negative result but none of result was false positive (TABLE 3). In present study, there were 3 subjects of false negative diagnostic test result means when viewed from the ACR value, was not the subject with DN, but when viewed from the AER value included in subject with DN. The performance of ACR to diagnose DN were as follows sensitivity 95.9%; specificity 100%; positive predictive value 100%; negative predictive value 66.7% (TABLE 4).

TABLE 3. Analysis using 2 x 2 table to define ACR performance to diagnose DN

	ND	Non ND	Total
ACR			
• ≥ 30 mg/g	71	0	71
• < 30 mg/g	3	6	9
Total	74	6	80

TABLE 4. Diagnostic performance of ACR to detect DN

Performance of ACR	Value	95% CI
Sensitivity (%)	95.9	88.6 – 99.2
Specificity (%)	100	54 – 100
Accuracy (%)	96.3	92.0 – 100
Positive predictive value (%)	100	100 – 100
Negative predictive value (%)	66.7	35.9 – 97.5
Positive likelihood ratio (LR+)	~	~
Negative likelihood ratio (LR-)	0.04	0.01 – 0.1

TABLE 5 as well as FIGURE 2 show the diagnostic performance of ACR ranging from 28 to 32 mg/g with 1 mg/g interval. It was shown that ACR value equal or more than 30 mg/g has the best performance to diagnose DN.

TABLE 5. Diagnostic performance of ACR at several cutoff values

ACR level (mg/g)	Sensitivity (%)	1-Specificity (%)
≥ 32 < 32	97.3	16.7
≥ 31 < 31	95.9	0
≥ 30 < 30	95.9	0
≥ 29 < 29	93.2	0
≥ 28 < 28	90.5	0

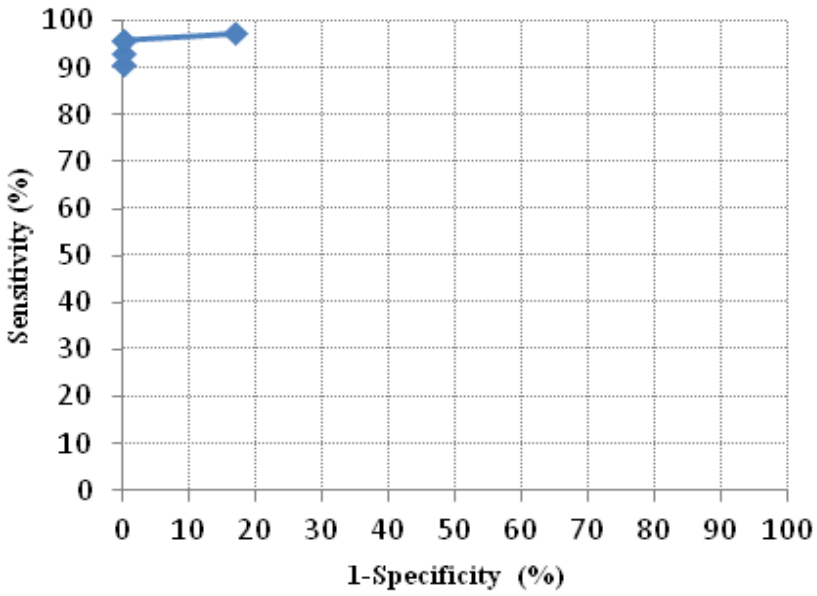

FIGURE 2. Receiver operating characteristic curve (ROC) of ACR

DISCUSSION

The study found higher AER values in subjects with longer duration of DM. It proved that the disease has a progressive characteristic. Generally DN will be diagnosed after 4 years duration of DM and level of AER might be more than 300 mg/24 hours. It indicated that kidney function start to decline due to lack of blood glucose control or the start of chronic hyperglycemia. After the tenth year of disease, AER values tend to return into the range of 200-300 mg/24 hours. This is because of management with anti-diabetic and anti-hypertensive that have also function as renal protector.[7,8]

The study found at the level equal or more than 30 mg/g, ACR has sensitivity and specificity 95.9% and 100% respectively. In present study obtained high sensitivity ACR values. High sensitivity ACR values can be used for screening DN especially for patients with chronic hyperglycemia. Previous studies reported higher specificity ACR values can be used for diagnostic purposes. Valizadeh *et al.*[9] reported a similar study involving 201 T2DM outpatients, aged more than 40 years, and use spot urine sample. They use the same cut off value of ACR report a different result i.e. sensitivity of 100% and specificity of 93.8%. They also found that ACR value was correlated with AER ($r = 0.89$; $p<0.0001$). Another similar study conducted by Jafar *et al.*[10] involved 577 subjects, and used spot urine sample. They reported that ACR value with cut off value 30 mg/g has sensitivity and specificity 86% and 90%, respectively. Another study conducted by Sampaio *et al.*[11] involved T1 and T2DM patient with majority female, and mean of age was 31 years old. Based on cut off value of ACR 27.3 mg/g they reported that ACR has sensitivity and specificity 100% and 73%, respectively. Yamamoto *et al.*[12] conducted a study on 228 T2DM outpatients with female majority with mean of age of 60 years. Twenty four urine samples were tested for albumin, while early morning urine samples were tested for albumin and creatinine. They found that the use of ACR with cutoff value of 28.7 mg/g, it has sensitivity and specificity 88% and 88%, respectively.

Chae *et al.*[13] conducted a study with the aim to investigate the correlation between ACR and AER. Subjects consisted of 113 children and adults with T1- and T2DM patients, with mean age of 15 years and predominantly male subjects. They found that ACR value has a good correlation with AER value.

Wu *et al.*[14] conducted a study which involved 200 T2DM patients with mean age of 40 years to assess the accuracy of ACR compared with the gold standard AER. They reported that ACR with cut off value of 30 mg/g has sensitivity and specificity 87% and 88% respectively. Both sensitivity and specificity reported was lower than that in the our study, because the urine sample used were not in accordance with NKF/KDOQ 2002 recommendations.

Another study was conducted to investigate the accuracy of total protein creatinine ratio in urine to diagnosis DN among T2DM patients. This ratio was independently and blindly compared with AER as the reference standard. The result showed that this ratio had a sensitivity, specificity, PPV, and NPV of 97.4%, 100%, 100%, and 81.8% respectively.[15]

CONCLUSION

Albumin creatinine ratio with cutoff value of 30 mg/g has sensitivity, specificity, PPV, NPV, LR-, and LR+ of 95.9%, 100%, 100%, 66.7%, 0.04, and infinite respectively for the diagnosis of DN. The ACR value of 30 mg/g with a morning urine sample can be used to diagnose DN in T2DM population.

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The expression of COX-2 and iNOS in ethanol and aspirin induced gastric ulcer rat models

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ABSTRACT

Aspirin or ethanol induced gastric ulcer rat models are the most frequently used in studies. Aspirin and ethanol induced gastric ulcers through different pathways involving COX-2 and iNOS. The aim of this study was to examine the expression of COX-2 and iNOS in gastric ulcer rat model induced by ethanol and aspirin. Twenty-one Sprague Dawley rats were divided into 7 groups i.e. control group (CA), ethanol 1st day (ED_1), ethanol 3rd day (ED_3), ethanol 5th day (ED_5), aspirin 4th day (AD_4), aspirin 6th day (AD_6), and aspirin 8th day (AD_8). Oral administration of aspirin was at 200mg/kgBW and the 100% ethanol at 1mL/200gBW. Macroscopic and microscopic observations were done to examine the gastric mucosal damage, COX-2 and iNOS expressions. Severe gastric ulcers were observed in ED_1 and AD_4 groups and mild gastric mucosal damage was observed in ED_3, ED_5, AD_6 and AD_8 groups. Microscopically, light erosion was shown by the CA and AD_8 groups. Erosion was also shown by ED_3, ED_5, and AD_6 groups. The most severe damage with ulcers and heavier bleeding were shown by the ED_1 and AD_4 groups. Weak COX-2 expression was found in the CA, while the highest COX-2 expression was found in the ED_1. The iNOS expression in the ethanol groups was still increasing until the 5th day (ED_5). In the aspirin groups, it reached the peak on the 3rd day (AD_6), and already declined on the 5th day (AD_8). In conclusion, the damage process of ethanol induced gastric ulcer occurred faster than that by aspirin. The highest COX-2 expression in the ethanol and aspirin groups were shown at the onset begin. iNOS expression in ethanol induced ulcer groups still increased until the 5th day, while in the aspirin induced ulcer groups already declined in the 5th day.

ABSTRAK

Tikus model ulkus lambung yang diinduksi oleh aspirin atau etanol adalah model yang paling sering digunakan dalam penelitian. Ulkus lambung yang diinduksi aspirin mempunyai jalur pathogenesis yang berbeda dengan yang diinduksi oleh etanol, namun keduanya melibatkan COX-2 dan iNOS. Tujuan penelitian ini adalah mengkaji ekspresi COX-2 dan iNOS pada tikus model ulkus lambung yang diinduksi oleh ethanol dan aspirin. Dua puluh satu tikus Sprague Dawley dibagi menjadi 7 yaitu kelompok kontrol (CA), etanol hari pertama (ED_1), etanol hari ketiga (ED_3), etanol hari kelima (ED_5), aspirin hari keempat (AD_4), aspirin hari keenam (AD_6), dan aspirin hari kedelapan (AD_8). Perlakuan diberikan secara oral, dengan dosis aspirin sebesar 200 mg/kgBB dan dosis 100% etanol sebesar 1mL/200gBB. Pengamatan makroskopis dan mikroskopis dilakukan untuk menilai kerusakan mukosa lambung, ekspresi COX-2 dan iNOS. Ulkus lambung berat terlihat pada kelompok ED_1 dan AD_4 dan kerusakan mukosa lambung ringan terlihat pada kelompok ED_3, ED_5, AD_6 and AD_8. Secara mikroskopis, erosi ringan terlihat pada kelompok CA dan AD_8. Erosi juga terlihat kelompok ED_3, ED_5, dan AD_6. Kerusakan paling berat dengan ulkus dan pendarahan terlihat pada kelompok ED_1 dan AD_4. Ekspresi COX-2 lemah terlihat pada kelompok CA, sedangkan ekspresi terkuat terlihat pada kelompok ED_1. Ekspresi iNOS pada kelompok etanol masih mengalami peningkatan sampai hari

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kelima (ED_5), sedangkan pada kelompok aspirin ekspresi iNOS mencapai puncak pada hari ketiga (AD_6), dan telah menurun pada hari kelima (AD_8). Dapat disimpulkan proses kerusakan mukosa lambung yang diinduksi oleh etanol terjadi lebih cepat dibandingkan yang diinduksi oleh aspirin. Ekspresi COX-2 tertinggi pada kelompok aspirin dan etanol tampak pada saat mulai terjadi ulkus. Ekspresi iNOS ulkus yang diinduksi etanol masih meningkat sampai hari ke-5, sedangkan pada yang diinduksi aspirin telah menurun pada hari ke-5.

Keywords : gastric mucosal damage - gastric ulcer - aspirin - ethanol - COX-2 - iNOS

INTRODUCTION

Gastric ulcer is the most prevalent digestive problem in clinical examination and affects 5 to 10% of people during their life.[1] Gastric ulcer is a complex, multifactorial disease and its aetiology not fully understood. It appears as a pathologic lesion in the digestive tract exposed to ulcerogenic agents. Gastric ulcer is considered as a result of an imbalance between invasive and defensive factors.[2] Bicarbonate and prostaglandin (PG) are considered as the defensive factors, whereas ethanol and aspirin as the external invasive factors.[2,3]

Ethanol- and aspirin-induced gastric ulcer are the most common experimental models.[4] In daily life, people use aspirin as NSAID drugs, which are usually important for cardiovascular and cerebrovascular therapy.[5] Alcohol consumption is also common for people in several parts of the world. Previous studies proved that ethanol and aspirin can induce the production of inflammatory mediators, such as tumour necrosis factor alpha (TNF-α) and reduce the production of PG. When the condition continuously occurs, it can induce gastric ulcers. Ethanol induced gastric damage develops by different mechanisms compared to the one induced by aspirin.

Gastric lesions caused by aspirin occur due to the cyclooxygenase (COX) inhibition. Cyclooxygenase consists of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2).[6,7] The use of low dosage aspirin inhibits COX-1 production resulting in the reduction of PG production. This condition will disrupt the blood flow in the mucosa of gastric resulting in the production of inflammatory mediators, such as TNF-α and interleukin-1 beta (IL-1β). Moreover, these conditions will induce COX-2 production resulting in the production of more PG for the mucosa healing process.[8]

Ethanol induced gastric mucosa damage is caused by free radicals, such as reactive oxygen species (ROS), the by-products of metabolism.[9] Accumulation of ROS induces oxidative stress. The imbalance of ROS and antioxidants will trigger scavenger enzyme dysfunction.[10] Ethanol has a robust and easy penetration nature to the mucosa gastric layers.[9] Disturbance of the integrity of gastric mucosa will induce the production of gastro-protective mucosa agents, such as nitric oxide (NO). Ethanol can inhibit the production of NO, which results in more severe gastric mucosa damage.[9] The gastric mucosa damage can induce inducible nitric oxide (iNOS) activity to produce NO again for healing mucosa.[11]

In order to confirm and to understand the different mechanisms of gastric ulcers induced by ethanol and aspirin, this study aimed to examine the gastric mucosal damage caused by ethanol and aspirin. Furthermore, this study purposed to examine the COX-2 and iNOS expression on the tissue of gastric ulcer rat models induced by ethanol and aspirin.

MATERIALS AND METHODS

Animals, treatments and termination

This experimental study used post-test only control group. Twenty-one healthy adult male Sprague Dawley rats with 200g weight were used in this study. They were

adapted to the standard laboratory conditions for one week. The rats received pellets, were allowed free access to water during the experiment, and they were kept under controlled environment of 12-hour light-dark cycle. The animals were numbered, weighed and divided into seven groups of 3 (TABLE 1). The dosage and termination time of the rats in ethanol groups were determined according to the study by Al-Qaraghuly.[12]

TABLE 1. The animal groups, treatment and termination time

Groups	Induction (treatment) type	Termination time after induction
CA	Aquadest	1st day
ED_1	Ethanol 100%	1st day
ED_3	Ethanol 100%	3rd day
ED_5	Ethanol 100%	5th day
AD_4	Aspirin	4th day
AD_6	Aspirin	6th day
AD_8	Aspirin	8th day

Note: Aspirin dosage was 200mg/kg BW, ethanol dosage was 1mL/200 g BW

After the adaptation, the rats were fasted for 24 hours and followed by the induction and then were euthanized according to the termination time as seen in TABLE 1.

Gastric organ uptake, macroscopic observation of gastric ulcer and slide preparation

The stomachs of the rats were removed, opened along the greater curvature, rinsed with saline, and examined for the severity and number of mucosal gastric lesions. The lesions were blindly examined according to the appearance and evaluated by a modified scoring system.[11] The score was evaluated by two trained observers. The criteria of the mucosal damage and the scoring can be seen in TABLE 2. The mean scores for each group were calculated.

TABLE 2. Criteria and score of gastric mucosa damage macroscopic

Score	Criteria
0	Normal stomach
1	Red coloration
2	Spot ulcer
3	Bleeding
4	Ulcer with bleeding/deep ulcer
5	Perforation

The tissues with gastric ulcer were sliced at 1 cm thickness, fixed in buffered formalin for maximum 24 hours and processed for paraffin embedding using standard protocol.[28] The formalin fixed paraffin embedded (FFPE) tissues were sliced at 6 μm thick for haematoxylin eosin (HE) staining and at 4 μm thick for evaluating COX-2 and iNOS expression by immune histochemistry (IHC). The COX-2 and iNOS expressions were examined in 3 slides for each subject with 100 μm distance between each slide.

Mucosal damage scoring

The mucosal injury was examined under light microscopy by two blinded examiners. The examination was quantified according to a two-step scoring system (length score and depth score)[12] as seen in TABLE 3 and 4. The total microscopic score resulted from the sum of the two partial scores and ranged from 0-6. Each sample was observed in 5 views foreach group by using microscope at 100x and 400x magnification.

TABLE 3. Criteria and the length of damage area score

Score	Criteria
0	no lesion
1	lesion involving 1%-10%
2	lesion involving 11%-20%
3	lesion involving >20%

TABLE 4. Score and criteria of deep lesion in gastric mucosa

Score	Criteria
0	no change
0.5	Superficial erosion
1	ulcer involving one internal third of the mucosa
2	ulcer involving the two internal thirds of the mucosa
3	ulcer involving almost the entire mucosal thickness

IHC for COX-2 and iNOS expression and the scoring

The IHC was performed according to the manual of the kit (Bio Care Medical STUHRP700 H, L10). The dilution of antibody anti COX-2 (Abcam ab15191) was 1:200 and antibody anti iNOS (Abcam ab15323) was 1:100 with the incubation time for both antibodies was 24 hrs in 4^{o}C. After the IHC procedure, the slides were counterstained with haematoxylin (Meyers, Inc.), and then dehydrated and mounted.

The slides were examined under light microscopy for COX-2 and iNOS; and scored according to the modification of Allred score protocol.[13] Cells with immunoreactivity toward antibody anti-COX-2 appeared as brown stained cells in the cytoplasm and-iNOS appeared as brown stained cells in the cytoplasm and nucleus. The calculation of COX-2 and iNOS expressions were done semi-quantitatively. Calculation of proportion of positive cells was done under light microscope at 400x magnification at 5 different fields of view. The proportion and intensity score can be seen in TABLE 5. The final score was the summing up of the proportion and intensity score. The minimum score is showed in TABLE 6. The observations were done by 2 blinded observers. The results were documented by using Optilab®.

Table 5. Proportion score and intensity score

Proportion score	Intensity score
0 = no positive immunostaining	0 = negative
1 = < 1%	1 = weak
2 = 1-10%	2 = moderate
3 = 10-33%	3 = strong
4 = 33-66%	
5 = >66%	

TABLE 6. Interpretation of final score of COX-2 and iNOS expression

Final score	Interpretation
0-2	Negative
>2-5	Weak positive
>5-8	Strong positive

Statistical analysis

The data were initially analysed by using Shapiro-Wilk test and the results showed that the data was not in normal distribution. Therefore, to analyse the mean difference, it was analysed by using Kruskal-Wallis test, and further by using Mann Whitney test.

RESULTS

The dosage of ethanol used in this study was 1 mL/200g BW of 100% ethanol. Due to the high dosage of ethanol, the ulcer came up on the first day after induction. For the aspirin induced ulcer, the determination of the dosage was according to the preliminary study. The preliminary study was performed based on several previous studies, which mentioned that the dosage for ulcer induction is 200-400mg/kgBW.[6,14,15] Termination time

for aspirin treatment in the previous research was done on the first and third days after treatment.[6,15]

Preliminary study on aspirin induction

Two aspirin dosages were used in the preliminary study, 200mg/kgBW and 400mg/kgBW. The termination time for 200mg/kgBW were days 3, 4, and 5 after treatment; and for 400mg/kgBW were days 1, 2, and 3 after treatment. The results showed that aspirin at 400mg/kgBW did not cause any ulcer at all. For the aspirin at 200mg/kgBW, the ulcer began to come up on the 4th day, therefore, the real experiment for aspirin used dosage at 200mg/kgBW with termination time on the 4th, 6th and 8th days after treatment.

Assessment of gastric mucosal damage

Macroscopically, assessment of gastric mucosal damage was done by observing the characteristics that were categorized and rated into normal (0), red colour (1), spot ulcer (2), bleeding (3), ulcer with bleeding (4), and perforation (5). After the stomach was removed and opened along the greater curvature, the macroscopic conditions of the gastric mucosa of the rats for each group can be seen in FIGURE 1.

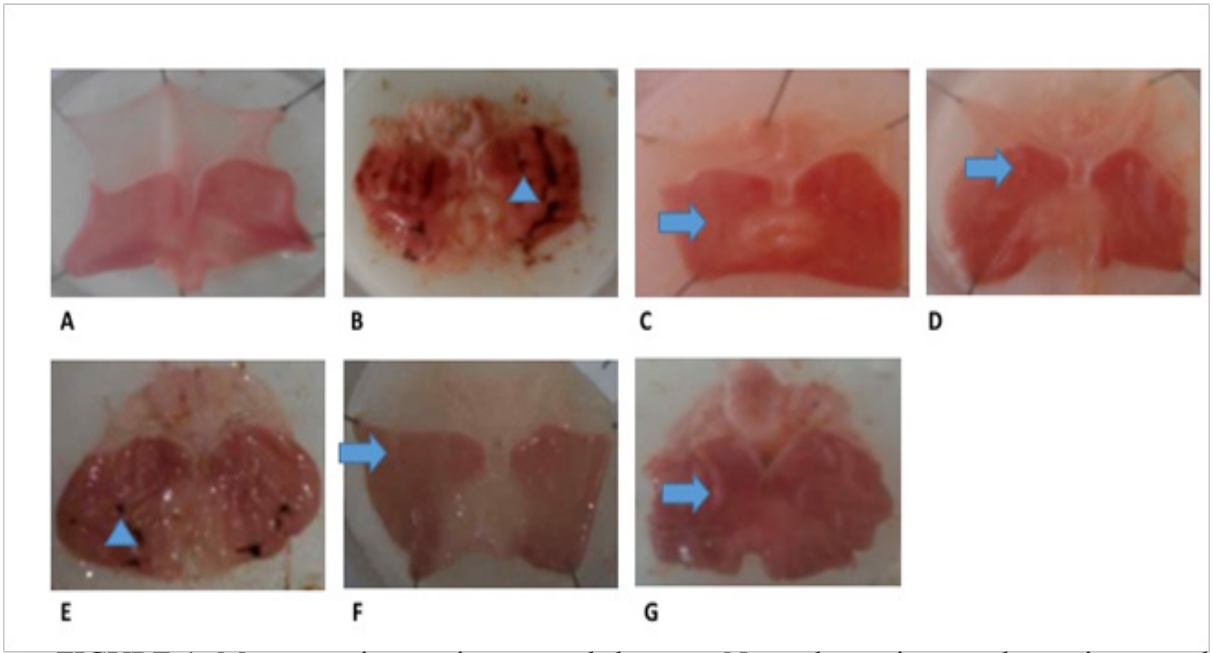

FIGURE 1. Macroscopic gastric mucosal damage, Normal gastric was shown in control group (CA); mucosal damage in the form of spot ulcer was shown in ethanol 3rd day (ED_3) (C), ethanol 5th day (ED_5) (D), aspirin 6th day (AD_6) (F) and aspirin 8th day (AD_8) (G); severe mucosal damage (ulcer with bleeding) was shown in ethanol 1st day (ED_1) (B) and aspirin 4th day (AD_4) (E).

Normal gastric mucosa can be seen in the control group (CA). All of the rats in the group showed normal gastric mucosa. Most of the rats from several groups showed mild gastric mucosal damage with spot ulcers. Most of the rats from groups ED_1 and AD_4 showed severe gastric mucosal damage (ulcer with bleeding or exsanguinations) (TABLE 7).

TABLE 7. Score of gastric mucosal damage macroscopically

Group	Modus
CA	0
ED_1	4
ED_3	2
ED_5	2
AD_4	4
AD_6	2
AD_8	2

Interpretation: 0=normal, 1= red colour, 2=spot ulcer, 3=bleeding, 4= ulcer with bleeding, 5= perforation

Microscopically, the assessment of gastric mucosal damage was done by summing up the score of percentage of lesion area and ulcer depth which can be seen in TABLES 3 and 4. Gastric mucosal damage could be in the form of erosion or ulcer. Erosion is epithelial lesion of gastric mucosa; and ulcer is the absence of gastric mucosa (epithelium, lamina propria and muscularis mucosa) and sometimes the lesion could reach the tunica muscularis.

The microscopic score of gastric mucosal damage can be seen in TABLE 8. The lowest mean of the score was 1.000 ± 0.866, which appeared in the CA and AD_8 groups, that shown as erosion (FIGURES 2A and 2G). The erosion also appeared in the ED_3, ED_5, and AD_6 groups, but the lesion was deeper and accompanied by low level of bleeding (FIGURES 2C, 2D, and 2F). Compared to the CA and AD_8 group, the mean of the score of gastric mucosal damage was higher (1.833±0.577). The most serious damage appeared in the ED_1 and AD_4 groups. These groups also showed ulcers with heavier bleeding (FIGURES 2B and 2E). The highest mean score of gastric mucosal damage was also shown by these groups (4.667±0.577 for ED_1 and 4.000±1.000 for AD_4). Kruskal Wallis test found that there was difference in the means in the treatment groups ($p=0.000$; $p<0.05$). Further tests using Mann Whitney found that the mean difference can be seen in all of the groups compared to the CA except AD_8 (TABLE 8).

TABLE 8. Mean of the microscopic score of gastric mucosal damage

Group	Mean score ± SD
CA	1.000 ± 0.866
ED_1	4.667 ± 0.577
ED_3	1.833 ± 0.577
ED_5	1.833 ± 0.577
AD_4	4.000 ± 1.000
AD_6	1.833 ± 0.577
AD_8	1.000 ± 0.866

Interpretation: severity of gastric mucosa microscopically was 0.5 to 6 (mild to severe)

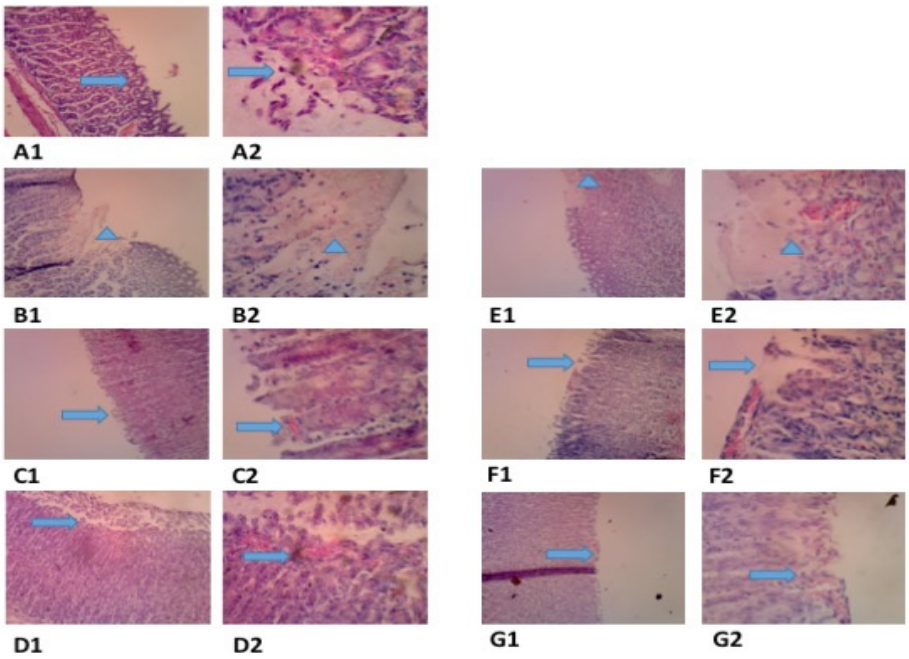

FIGURE 2. Microscopic appearance of gastric mucosal damage. A. control group (CA); B. ethanol 1st day group (ED_1); C. ethanol 3rd day group (ED_3); D. ethanol 5th day group (ED_5); E. aspirin 4th day group (AD_4); F. aspirin 6th day group (AD_6); G. aspirin 6th day group. CA and AD_8 shown the lightest erosion (2A and 2G). ED_3, ED_5 and AD_6 shown deeper erosion with light bleeding (2C, 2D and 2F). ED_1 and AD_4 shown ulcer with heavier bleeding. The number after alphabet in the figure means: 1 for low magnification and 2 for high magnification. The ulcer showed by arrow head and the erosion showed by arrow.

COX-2 expression

The mean score of COX-2 expression can be seen in TABLE 9. The data showed abnormal distribution after Shapiro Wilktest. In order to test the mean difference among the groups, Kruskal Wallistest was performed and showed that some of the groups have different means of COX-2 expression score ($p=0.048$; $p<0.05$). Further, Mann Whitney tests were done to see the differences among the groups.

TABLE 9. COX-2 expression on induced gastric mucosal damage tissue

Group	Mean score
CA	2.667 ± 0.577
ED_1	6.000 ± 0.000
ED_3	5.667 ± 0.577
ED_5	5.334 ± 0.577
AD_4	6.000 ± 0.000
AD_6	5.334 ± 0.577
AD_8	5.000 ± 0.000

Interpretation: 0-2=negative expression, >2-5= weak expression, >5-8= strong expression

The CA and AD_8 groups showed weak COX-2 expression and the other groups shown strong expression (FIGURE 3). Mann Whitney tests shown that the mean score of COX-2 expression in the CA group was lowest and significantly different from all of the treatment groups. The ED_1 and AD_4 groups had the highest mean score of COX-2 expression compared to ED_3, ED_5, AD_6 and AD_8 (TABLE 9).

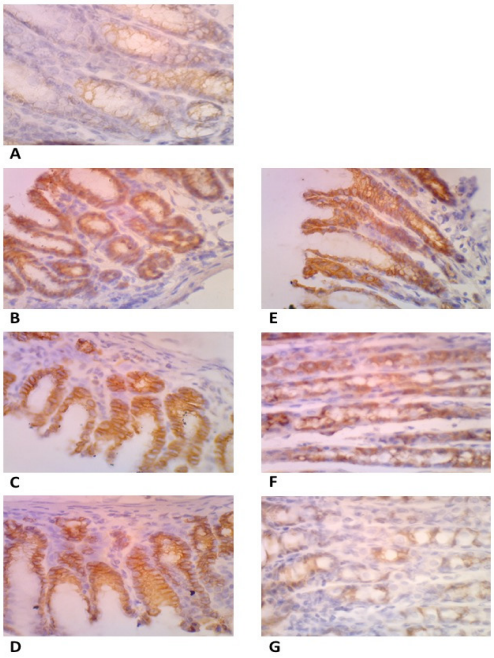

FIGURE 3. COX-2 expressions on gastric mucosa. A. Control group (CA); B. Ethanol 1st day group (ED_1); C. Ethanol 3rd day group (ED_3); D. Ethanol 5th day group (ED_5); E. Aspirin 4th day group (AD_4); F) Aspirin 6th day group; G. Aspirin, 8th day group (AD_8). Control group (CA) and aspirin 8th day group shown weak COX-2 expression and the other (ED_1, ED_3, ED_5, AD_4 and AD_6) shown strong COX-2 expression. 400x magnification

iNOS expression

Shapiro Wilk test found abnormal distribution on iNOS expression data. The mean difference tested by using Kruskal Wallis shown that some of the groups had different means of iNOS expression score ($p=0.009$; $p<0.05$). Further, Mann Whitney test was done to see the differences among the groups.

iNOS expression in ethanol or aspirin induced gastric mucosal damage can be seen in FIGURE 4. The CA group shown negative iNOS expression (FIGURE 4A) with the lowest mean score (1.334±0.577), which represents the value for negative expression. The AH8 grouphad low iNOS expression (FIGURE 4G). The other groups (ED_1, ED_3, ED_5, AD_4, and AD_6) shown strong iNOS expression.

Mann Whitney test shown that all of the mean scores of iNOS expression of the treatment groups were significantly different with the CA group. Even though the other treatment groupshad different means of iNOS expression compared to control (CA), it can be divided into 3 categories. The highest mean score of iNOS expression was shown by the ED_5 group (6.000 ± 0.000), followed by a cluster of ED_1, ED_3 and AD_6 groups and the next category was a cluster of AD_4 and AD_8 groups (TABLE 10).

FIGURE 3. iNOS expressions on gastric mucosa. A. Control group (CA); B. Ethanol 1st day group (ED_1); C. Ethanol 3rd day group (ED_3); D. Ethanol 5th day group (ED_5); E. Aspirin 4th day group (AD_4); F) Aspirin 6th day group; G. Aspirin, 8th day group (AD_8). Control group (CA) and aspirin 8th day group shown weak COX-2 expression and the other (ED_1, ED_3, ED_5, AD_4 and AD_6) showed strong COX-2 expression. 400x magnification

TABLE 10. iNOS expression on induced gastric mucosal damage tissue

Group	Mean score ± SD
CA	1.334 ± 0.577
ED_1	5.334 ± 0.577
ED_3	5.667 ± 0.577
ED_5	6.000 ± 0.000
AD_4	4.000 ± 0.000
AD_6	5.667 ± 0.577
AD_8	4.334 ± 0.577

Interpretation: 0-2= negative expression, >2-5= weak expression, >5-8= strong expression

DISCUSSION

Gastric damage is caused by the imbalance of defensive and invasive factors for gastric acid production and result in over production.[2] In this research, induction material such as 100% ethanol and aspirin as NSAID drugs were used to induce the gastric mucosal damage. Ethanol and aspirin are able to cause very severe mucosal damage compared to other material such as indomethacin and hydrochloric acid (HCl). Previous study on indomethacin induced gastric mucosal damage shown that the gastric mucosal cells were unchanged after induction, while the induction using 80% ethanol and aspirin caused gastric mucosal cells hypertrophy.[16]

The most severe gastric mucosal damage found in this study was ulcers with bleeding (in groups ED_1 and AD_4). High ethanol concentration can induce ulcers in a relatively short time, therefore, the ulcer already could be seen on the first day after induction. Contrary to this pattern, aspirin can induce ulcers at low dosage and needs longer time, hence, the ulcer was gradually seen on the fourth day after induction. It has been explained in the previous study that the use of aspirin at lower dosage (75-300 mg) will gradually activate various disturbances of the digestion channel, such as dyspepsia, erosion of gastric wall, and ulcer with bleeding leading to perforation.[15] Moreover, another study stated that lesions of gastric mucosa caused by NSAID including aspirin will attain the peak on the third day after induction and decrease in the 10th day.[17]

Gastric ulcers are caused by local inflammation, which is initiated by the imbalanced production of mucus, bicarbonate and gastric acid resulting in gastric mucosa irritation. Further, the decreasing mucus production causes pepsin penetration to mucosal epithelium.[18] Broken epithelium and continuous gastric acid production trigger inflammatory cytokine production, which activates macrophages. Macrophages will produce MCP-1 that causes the macrophages to induce the production of IL-1β and TNF-α. This process causes the activation of the cytokine networking to trigger neutrophils migration from circulation to the inflammation area.[8]

More specifically, the gastric mucosal damage caused by ethanol occurs due to free radical production as the result of metabolism and local inflammation in the mucosa. Ethanol has a firm and easy penetration nature to the mucosal gastric layers. Various damages cause the accumulation of free radicals that trigger the imbalance of the production of ROS and antioxidant defense.[19] This condition promotes oxidation stress accompanied by the reduction of PG resulting in the COX-2 activation to induce PG production.[20]

The process of gastric mucosal damage induced by aspirin needs longer time until the onset. Therefore, the ulcer appeared on the 4th day after induction. Aspirin induced gastric ulcer was initiated by COX-1 inhibition by aspirin that causes the decreasing of PG production and results in the disruption of blood flow in the mucosa.[8] Mucosal erosion and ischemic injury as the consequences of the condition will induce COX-2 activation to increase the production of PG.[21]

The lighter spot ulcers appeared in groups ED_3, ED_5, AD_6 and AD_8. It seems that the healing process of mucosa was already started. The healing process in ethanol groups occurred earlier. Previous study explained that the mucosal healing

process happened on the 3rd day up to 18th day or more.[22,23] It involves the role of COX-2 as a mucosal gastro-protective factor. During inflammation, COX-2 will induce the production of PG that plays a role in ulcer healing process, angiogenesis, and increasing production of mucus and bicarbonate.[21] Angiogenesis is an important process in the regeneration of blood vessels which are involved in oxygen and nutrient supply during microcirculation repair.

Inflammation and restoration of mucosa involves the role of COX-2 and iNOS. One study by Masayuki *et al*.[21] explained that iNOS has a positive cell presence in the ulcer area with un-finished reepithelization. iNOS supplies new epithelial cells, covers the epithelial tissue eroded by the ulcer, and also acts as a barrier toward any pathogens. Several studies also mentioned that iNOS was a factor of angiogenesis that plays a role in microcirculation repair. Gastric mucosal damage that is represented by epithelial damage and the accumulation of acid reflux production would stimulate the production of inflammatory cytokines, including TNF-α and also induce the neutrophil infiltration. TNF-α and neutrophil infiltration would stimulate production of angiogenesis factors such as vascular endothelial growth factor (VEGF) and iNOS.[22,24,25]

During the angiogenesis process, endothelial cells undergo proliferation, migration, and regeneration to create new functional blood vessels.[25] Angiogenesis is started on the 1st day after the onset of mucosal damage, increases on the 3rd day and decreases on the 10th day. It is in line with iNOS expression, which is increased on the 3rd day after the onset of mucosal damage and starts to decrease on the 10th day.[22] This study found similar results that iNOS expression in the ethanol groups appeared on the 1st day after induction, when the gastric ulcer already occurred, while in the aspirin groups, iNOS expression can be observed on the 4th day after induction, at the same time with the onset of the ulcer.

The iNOS expression in ethanol induced gastric ulcer continues to increase until the 5th day since the onset of the ulcer (ED_5 group), while aspirin induced gastric ulcer showed increasing iNOS expression on the 3rd day after onset (AD_6 group) and started to decline on the 5th day after onset (AD_8 group). It is assumed that the process of gastric mucosal damage in ethanol induced ulcers is still occurring until the 5th day after onset, while in aspirin induced ulcers it already starts the recovery process on the 5th day after onset. From the explanation above, iNOS is an important factor for the healing process of gastric mucosal damage, therefore iNOS expression can be observed during the ulcer and the healing process with different levels of expression related to the level of damage and the stage of the healing process.

CONCLUSION

The damage process of ethanol induced gastric ulcers occurred faster than the one induced by aspirin. Contrary to this pattern, the recovery process of aspirin induced ulcers happened faster than the one induced by ethanol. It was also shown by the decreasing of iNOS expression in the aspirin 8th day group (5th day after onset), while the iNOS expression in the ethanol 5th day group (5th day after onset) was still increasing. The highest COX-2 expressions in both ethanol or aspirin induced gastric ulcer groups were shown at the onset, 1st day for ethanol and 4th day for aspirin.

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Profile of traumatic brain injury (TBI) in relation with maxillofacial and thoracic injury Dr. Hasan Sadikin General Hospital, Bandung

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ABSTRACT

Traumatic brain injury (TBI) has a relation with concomitant injuries, which are mostly maxillofacial and thoracic injury. This study aimed to know which injury was common in TBI patients and the difference in the severity of TBI when patients were associated with one or both types of injuries. This was a descriptive retrospective study. The data used was medical records from Department of Neurosurgery, Dr. Hasan Sadikin General Hospital, Bandung, Indonesia from the period of August 2015 to July 2016. Total sampling method was used in this study. The variables were patients with TBI, maxillofacial injury, thoracic injury, accident related mechanism and non-accident related mechanism and the Glasgow Coma Score (GCS). The total cases for this study were 47 patients. The highest case was males with 37 cases and 10 for females. Accident related trauma had 23 cases whereas non accident related trauma had 24 cases. The total cases of maxillofacial injury were 32, thoracic were 6 cases whereby for both injuries were 9 cases. Patients with mild TBI were 28 cases, moderate TBI were 13 cases, and severe TBI had 6 cases in total. The rate of TBI was higher in single injury which was the maxillofacial injury. However, the thoracic and both injuries combined had higher severity of TBI compared to maxillofacial injury.

ABSTRAK

Cedera otak traumatika (COT) dapat disertai dengan cedera maksilofasial dan cedera thoraks. Penelitian ini bertujuan untuk mengetahui pasien dengan COT yang memiliki keparahan yang lebih tinggi dari cedera *multiple* dan perbedaan dalam keparahan COT ketika pasien dikaitkan dengan satu jenis cedera atau kedua cedera. Penelitian ini merupakan penelitian deskriptif retrospektif. Data yang digunakan adalah rekam medis pasien dari Departemen Bedah Saraf, Rumah Sakit Hasan Sadikin dari periode bulan Agustus 2015 hingga Juli 2016. Sampel diperoleh adalah sebanyak 47 pasien berdasarkan *total sampling*. Variabel dalam penelitian ini adalah pasien dengan cedera traumatis otak, cedera maksilofasial, cedera thoraks, mekanisme terkait dengan kecelakaan, mekanisme yang tidak terkait dengan kecelakaan dan *Glasgow Coma Scale* (GCS). Total kasus untuk penelitian ini adalah 47 pasien. Kasus cedera kepala terbanyak terjadi pada laki laki, yaitu 37 kasus dan 10 sisanya pada perempuan. COT akibat kecelakaan ada 23 kasus dan bukan akibat kecelakaan ada 24 kasus. Total kasus cedera maksilofasial adalah 32 dan untuk thoraks adalah 6 dan untuk jenis cedera keduanya adalah 9 kasus. Pasien dengan cedera COT ringan ada 28, kasus COT sedang ada 13 dan COT berat memiliki 6 kasus. Kasus COT kebanyakannya terjadi pada kasus cedera maksilofasial. Namun, jika cedera toraks dan dua cedera terkombinasi maka tingkat keparahan COT menjadi lebih tinggi dibandingkan dengan cedera maksilofasial.

***Keywords*:** traumatic brain injury -maxillofacial - thoracic injury – accident – Glasgow Coma Scale

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INTRODUCTION

Traumatic brain injury (TBI) have a correlation with certain types of injuries and mostly are those with multiple injuries non related to neurological problems.[1] A situation exists when the physical loads overload the brain is known as TBI. It means that there is an accident which gives an impact that causes a high level of stress that cannot be handled by the brain until it damages it in the form of brain injury.[2] A study was conducted by the National Automotive Sampling System-Crashworthiness Data System and Crash Injury Research Engineering Network to investigate the injuries affecting which part of the body organs from a road accident. Th most affected body organs are the head and thorax.[3]

Thoracic injuries patients commonly have been linked with TBI.[4] Thorax-only blast injury apparatus and a jugular-only blast injury apparatus was constructed to see if the wave from a thoracic injury would transmit to the brain and how the direct wave at the cranium causes the brain injury.[2] Maxillofacial injuries are most commonly related to TBI as well. The brain is affected because its position is located near to the orbital and zygomatic areas.[5] Out of 1.4 million of Americans from the year 1995 to 2001 have been diagnosed with TBI, more than one million was spotted with mild TBI with a Glasgow Coma Score (GCS) of 14 to 15, with a score of 9 to 13 for moderate TBI and 3 to 8 for severe TBI. American Congress of Rehabilitation Medicine and The Centers for Disease Control and Prevention has defined that there is no abnormality in neuroimaging of TBI and TBI is solely diagnosed based on clinical symptoms.[6] This study was conducted to evaluate TBI patients with highest rate of concomitant injuries and the difference in the severity of TBI when the cases were associated with one or both types of injuries.

MATERIALS AND METHODS

Subjects

This was a descriptive retrospective study using the medical record of patients from the Department of Neurosurgery, Dr. Hasan Sadikin General Hospital, Bandung during the period of August 1st, 2015 to July 31st, 2016 who met the inclusion and exclusion criteria. Patients aged from 18 to 65 years who diagnosed with TBI associated with maxillofacial or thoracic or both injuries were involved in this study. Whereas, patients who not meet into this age criteria, or not diagnosed with TBI but have concomitant injuries or vice versa were excluded from the study. The protocol of the study has been approved by the Health Research Ethics Committee Faculty of Medicine, Universitas Padjadjaran/Dr. Hasan Sadikin General Hospital, Bandung.

Data collected

The data of patients included traumatic brain injury, maxillofacial injury, thoracic injury, accident-related mechanism and non-accident related mechanism were gathered. The Glasgow Coma Score was calculated to classify TBI severity into mild, moderate and severe.

Statistical analysis

Data were presented as mean ± standard deviation (SD) or frequency or percentage and the then analyzed descriptively.

RESULTS

A total of 47 patients with TBI associated with either maxillofacial, thoracic or both injuries and classified based on age and gender are presented in TABLE 1. The highest range of age collected from the data between the age of 18 to 27 years old for both males and females.

TABLE 1. Frequency of TBI associated with concomitant injuries based on age and gender (n = 47)

Age (years)	Frequency	Mean ± SD	TBI + Maxillofacial		TBI + Thoracic		TBI + Both injuries	
			(n)	(%)	(n)	(%)	(n)	(%)
Male								
• 18-27	14							
• 28-37	9							
• 38-47	7	35±12.77	24	51.1	6	12.8	7	14.9
• 48-57	5							
• 58- 67	2							
Female								
• 18-27	5							
• 28-37	1							
• 38-47	2	33±14.35	7	14.9	2	4.2	1	2.1
• 48-57	1							
• 58-67	1							

Accident related trauma had 23 cases and mostly were due to motorcycle accidents. Most of them were diagnosed with TBI and maxillofacial injury. Car crash contributed to the highest TBI associated with both maxillofacial and thoracic injuries (FIGURE 1).

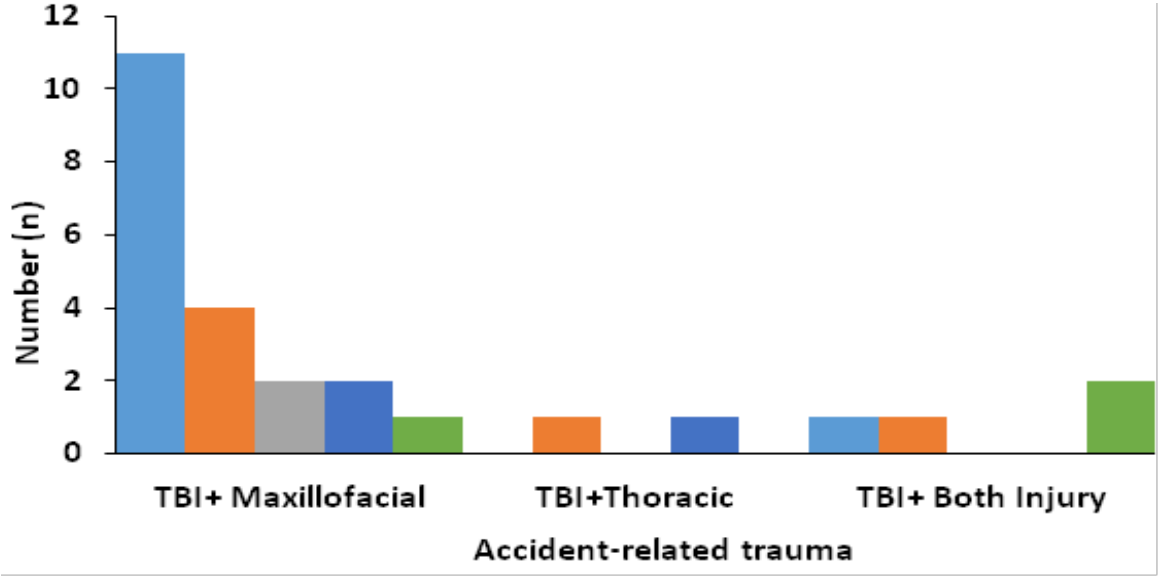

FIGURE 1. TBI associated with concomitant injury based on accident-related trauma

On the other hand, non-accident–related trauma had 24 cases in total. There were no cases reported that sports caused traumatic brain injury in the data. Most patients were diagnosed TBI due to fall either from bike, staircase, or ladder. The mechanism of injury was unknown for 7 cases (FIGURE 2).

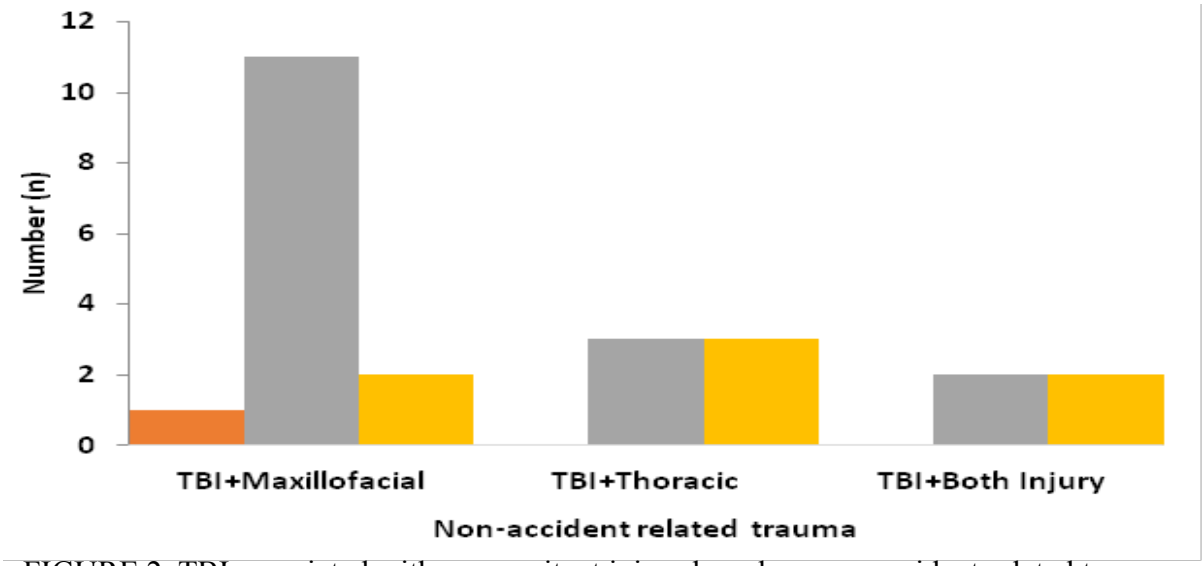

FIGURE 2. TBI associated with concomitant injury based on non-accident related trauma

Based on the previous data (TABLE 1, FIGURE 1 and 2), TBI with maxillofacial, thoracic, or both injuries can be classified into mild, moderate, or severe TBI. Most TBI patients had maxillofacial injury, (32 cases), and several patients had thoracic (6 cases) or both (9 cases) injuries (TABLE 2). The highest severity of TBI evaluated based on GCS reported in the period of August 2015 to July 2016 was TBI with GCS from 14 to 15 (28 cases), whereas moderate TBI with GCS from 9 to 13 was 15 cases and severe TBI with GCS from 3 to 8 was 8 cases.

TABLE 2. Frequency of TBI with concomitant injury based on its severity evaluated by GCS

Glasgow Coma Score	TBI+ Maxillofacial		TBI+ Thoracic		TBI+ Both Injuries	
	(n)	(%)	(n)	(%)	(n)	(%)
Mild (n=27)	21	44.6	2	4.3	5	10.5
Moderate (n=13)	9	19.1	2	4.3	2	4.3
Severe (n=6)	2	4.3	2	4.3	2	4.3

TABLE 3 shows the percentage of each injuries based on the GCS severity cases. It is understood that TBI patients with maxillofacial injury had the highest percentage for mild TBI. However, it is demonstrated that more patients with thoracic injury and both injuries had severe TBI than patients with mild and moderate TBI.

TABLE 3. Percentage of TBI severity based on different types of injury

Types of injury	Mild (%)	Moderate (%)	Severe (%)
TBI + Maxillofacial	75.0	69.2	33.3
TBI + Thoracic	7.1	15.4	33.3
TBI + Both Injuries	17.9	15.4	33.3
Total	100.0	100.0	100

Note : (mild, n=28; moderate, n=13; severe, n=6)

DISCUSSION

TABLE 1 shows the frequency of traumatic brain injury associated with concomitant injuries based on age and gender. It proved that males (n=50), which comprised of 79% of total cases, had higher rate of concomitant injuries than females (n=13), which were 21% of total cases. A study conducted in Qatar[7]causes, and outcome of TBI in adolescents and young adult population in Qatar. Method. This was a retrospective review of all TBIs admitted to the trauma center between January 2008 and December 2011. Demographics, mechanism of injury, morbidity, and mortality were analyzed in different age groups. Results. A total of 1665 patients with TBI were admitted; the majority were males (92% reported that patients who were diagnosed with TBI were mostly males. They had risk of almost four times higher than females, as most of the males in Qatar had head injury since adolescence. It was also reported that the highest age group that was diagnosed with TBI and had concomitant injury falls under the age category of 20 to 29 years[2] old. Compared to this study, it happed almost under the age group of 18 to 27 years[2] old.

As can be seen in TABLE 2, the accidents related to motorcycles[2] are the highest, which was 9 out of 23 cases (39.1%), compared to other accident–related trauma. This is in agreement with a previous study conducted by Aladelusi *et al*.[8] where most of their patients were motorcyclists compared with other accident-related patients. In addition, only 15% of that motorcyclists wore helmet on road and this seemed to contribute to most of the maxillofacial injury. A study in the United States[3] proved that car crash caused injuries in both the head and thorax as also found in this research, where 2 out of 3 cases of car crash contributed to TBI with both maxillofacial and thoracic injuries compared to individual injuries. A research conducted in Austria[1] showed that for non-accident related trauma, fall had the highest rate compared to sports related, violence, others and unknown causes of traumatic brain injury. This also occurred in this study (TABLE 3), where fall had 16 cases out of 24 cases and contributed up to 66.7%, 47.8% of which were maxillofacial injury. Eight cases had unknown mechanism of injury and 1 case was violence case. The previous study from Austria[1] and Qatar[7] do not support this research where the accident-related trauma cases were higher than the non accident-related trauma cases in their respective countries, although a study from USA[9] has stated that fall was their most common mechanism of injury, and hence supporting this study.

The study conducted by Meaney *et al*.[10] reported that in public, most people with TBI were diagnosed with mild TBI. This was similar to this study. Out of 47 cases, 28 cases (59.4% of all cases) were reported with mild TBI. Moderate TBI was seen in fewer cases (13 cases or 27.7% of all cases), and the least, 6 cases (12.9% of all cases) were severe TBI patients. Patients with TBI associated with maxillofacial injury were the highest cases diagnosed with mild TBI. The same result has been reported in a previous

study conducted ini Nigeria[8] with 35% of all cases. However, a study performed by Arslan *et al*.[11] reported that more patients with TBI associated with maxillofacial injury were diagnosed with severe TBI, which was 17 out of 24 cases. This could be due to the causal differences as most patients were admitted because of violence. Severe TBI patients were equally associated with maxillofacial, thoracic, or both injuries combined as there were 2 out of 6 cases for each type of injury. On the other hand, the study conducted by Veysi *et al*.[12] reported that the severity of TBI started from moderate to severe TBI (based on severity of the thoracic injury). Therefore it is different from this study that showed an equal number of cases for each type of severity. In this study, TBI associated with both injuries (maxillofacial and thoracic injuries) was mostly diagnosed with severe TBI with 33.3% of all cases, while for mild TBI was 17.9% of all cases and moderate TBI was 15.4% of total cases. A study performed in Australia[13] has mentioned that patients with TBI and concomitant injury might undergo worse TBI outcome than those diagnosed with TBI only due to the involvement of cytokine that cause the TBI pathobiology. It partially in agreement with this study, as severe TBI was the most common diagnosis and followed by mild and moderate TBI. The limitation in this study was the lack of primary data.

CONCLUSION

In conclusion, TBI can be associated with concomitant injuries. Patients are most commonly associated with either maxillofacial, thoracic, or both injuries in an accident-related or non accident-related trauma. The rate of TBI is higher in single injury which is the maxillofacial injury. However, the thoracic and both injuries combined has higher severity of TBI compared to maxillofacial injury.

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Factors influencing plateletpheresis donations in Dr. Sardjito General Hospital, Yogyakarta, Indonesia

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ABSTRACT

Despite its life saving potential, regrettably, blood transfusion has yet to be optimally applied in Indonesia. Funding difficulties and both public and professional lack of knowledge hinders its progress. More lives can be saved by using a safer, more proper, and specialized blood transfusion procedure. Plateletpheresis, one method to obtain platelet products, requires a different donation procedure than that of whole blood-derived platelet. High quality plateletpheresis product donation will positively impact the transfusion efficacy and platelet recovery of the recipient, improving patient's clinical state. This study analyzes factors from both the donor and donation procedure that influence the quality of plateletpheresis product. The study analyzes data of plateletpheresis donations from blood transfusion service and plateletpheresis transfusions from medical records at Dr. Sardjito General Hospital, Yogyakarta, Indonesia, within the period of August 2012 to January 2013 using cross sectional design. Forty-four plateletpheresis donations were obtained during the study. All donors were male with the following mean values; age 31.9 ± 9.9 years, weight 70.2 ± 10.2 kg, body mass index (BMI) 24.7 ± 3.2 kg/m2, hematocrit 44 ± 3.2 %, and procedure time 84.2 ± 19.2 min. The median value of platelet yield was $3.2x10^{11}$ ($2.1x10^{11}$ – $4x10^{11}$). The median value of pre-donation platelet count was $248.5x10^3$/µL ($204x10^3$/µL – $391x10^3$/µL) and the mean value of product volume was 275 ± 22.9 mL. The results showed that pre-donation platelet count ($r = 0.329$; $p < 0.05$) and product volume ($r = 0.661$; $p < 0.05$) positively correlated to the yield of platetetpheresis products. However, the yield of plateletpheresis products was not correlated to the post-transfusion platelet count ($r = 0.327$; $p > 0.05$). In conclusion, pre-donation platelet count and product volume of plateletpheresis influence the yield of plateletpheresis. However, the yield is not correlated to the post-donation platelet count. Thus, other clinical factors should be considered.

ABSTRAK

Meskipun memiliki potensi besar dalam menyelamatkan manusia, transfusi darah belum diterapkan secara optimal karena terbatasnya pendanaan serta pengetahuan masyarakat dan tenaga medis di Indonesia. Lebih banyak jiwa dapat diselamatkan dengan menggunakan prosedur transfusi darah yang lebih aman, tepat, dan spesifik. Plateletferesis, salah satu metode untuk mendapatkan produk trombosit, membutuhkan prosedur donasi yang berbeda dibanding transfusi darah konvensional. Produk donasi plateletferesis yang berkualitas akan berdampak positif terhadap efikasi transfusi dan pemulihan trombosit penerima, sehingga dapat memperbaiki keadaan klinis pasien. Penelitian ini bertujuan untuk menganalisis faktor-faktor dari donor dan prosedur donasi yang mempengaruhi kualitas produk plateletferesis. Penelitian ini menggunakan rancangan potong lintang

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untuk menganalisis data donasi plateletferesis dari unit pelayanan transfusi darah (UPTD) dan data transfusi plateletferesis dari rekam medis di Rumah Sakit Umum Pusat Dr. Sardjito, Yogyakarta, Indonesia periode bulan Agustus 2012 hingga Januari 2013. Didapatkan 44 sampel penelitian. Semua donor adalah laki-laki dengan nilai rata-rata usia 31,9 ± 9,9 tahun, berat badan 70,2 ± 10,2 kg, indeks massa tubuh (IMT) 24,7 ± 3,2 kg/m2, hematokrit 44 ± 3,2%, dan waktu prosedur 84,2 ± 19,2 menit. Nilai media hasil produk plateletferesis adalah $3,2x10^{11}$ ($2,1x10^{11}$ - $4x10^{11}$). Nilai median hitung platelet pra-donasi adalah $248,5x10^3$/µL ($204x10^3$/µL - $391x10^3$/ µL) dan nilai rerata volume produk adalah 275 ± 22,9 mL. Hasil penelitian menunjukkan bahwa jumlah platelet pra-donasi (r = 0,329; p <0,05) dan volume produk (r = 0,661; p <0,05) berkorelasi positif dengan hasil produk plateletferesis. Namun demikian, hasil produk plateletferesis tidak berkorelasi dengan hitung trombosit pasca transfusi (r = 0,327; p> 0,05). Dapat disimpulkan, jumlah platelet pra-donasi dan volume produk plateletferesis mempengaruhi hasil produk plateletferesis. Namun, hasil produk plateletferesis tidak berkorelasi dengan hitung platelet pasca-donasi. Faktor klinik lainnya harus dipertimbangkan dalam plateletferesis.

Keywords: platelet – plateletpheresis – pre-donation – post-donation - platelet count-product

INTRODUCTION

Millions of people all over the world are saved by blood transfusions annually.[1] To obtain an optimal results in blood transfussion, it is important to provide a safe method of blood transfusion in accordance to the standard and comprehensive workflow to obtain an optimal result in blood transfusion, thus increasing the quality of care.[1,2] Recently, blood transfusions use specific blood components that meet certain requirements. For example, the need for red blood cells, granulocytes, platelets and blood plasma that contain specific proteins and clotting factors. To reduce the adverse effects of transfusions, a clear guideline for the transfusion of specific blood components is needed.[3] One blood component that is commonly used in transfusions is platelets, through a procedure known as plateletpheresis.

Plateletpheresis is a blood donation process of removing whole blood from the donor, separating only the platelets, and then returning the remaining blood components (erythrocytes, leucocytes and blood plasma) to the donor. Patients requiring platelet transfusions can receive platelet products through platelet concentrates or a plateletpheresis. Over the years, plateletpheresis has been considered superior to platelet concentrates.[4,5] One study reveals plateletpheresis components are considered to be fresher than platelet concentrates, since plateletpheresis components are specially collected for specific patients.[3] Another study shows plateletpheresis provide better platelet increments, and cause less alloimmunization.[5] The major advantages are also that there is a reduced donor exposure and a therapeutic dose can be collected from a single donor with special characteristics, such as HLA-matched, PIA1 negative, or IgA-deficient.[6] Data from the American Red Cross also showed a lower risk of transfusion reaction due to bacterial contamination from plateletpheresis rather than to platelet concentrates.[7,8]

Although gradually increasing, plateletpheresis is still quite new in Indonesia and rarely performed. This pattern is caused by several factors; the aphaeresis machine is quite expensive, and there are limited aphaeresis donors due to the pre-donation terms and conditions that have to be fulfilled by the donors. This fact needs to be attended to, as it is expected that

plateletpheresis can provide optimal results in every donation in terms of efficiency and efficacy. The plateletpheresis product quality is influenced by two aspects: the donor itself and the donation procedure.[4] To the author's knowledge, unfortunately, there have been no studies done in Indonesia that analyze factors influencing the plateletpheresis product quality. A high quality of plateletpheresis product donation will positively impact the transfusion efficacy and platelet recovery of the recipient, improving his or her clinical state and outcome. It is thus essential to further elaborate the profile of plateletpheresis donation.

MATERIALS AND METHODS

Design of the study

This cross sectional study was conducted at the blood transfusion service unit (*Unit Pelayanan Transfusi Darah*/UPTD) and the Medical Records Department (*Instalasi Rekam Medis*/IRM) Dr. Sardjito General Hospital, Yogyakarta. The samples used in this study were 44 data sets of plateletpheresis donations within the period August 2012 to January 2013. The study began with collecting data of plateletpheresis donations in UPTD within the period of August 2012 to January 2013.

Data collection

We assessed data from the donors characteristics including: age, gender, body weight, BMI (body mass index), hematology profiles (hematocrit and pre-donation platelet count); as well as aspects of the donation procedure, which include the duration of donation procedure and volume of plateletpheresis products; and from the aspects of the product which include the yield of plateletpheresis. After that, we collected data of recipients who received transfusions by donors that have been recorded previously and within the timeframe specified in the IRM. The data of recipients that were taken in the medical record specified the increase in the number of platelets after transfusion (platelet increment) to assess the efficacy of the transfusion.

Statistical analysis

The collected data were then analyzed and compared to determine whether there was a relationship or correlation between the variables using the Statistical Package for Social Science (SPSS). The Spearman's rho correlation test was used to evaluate the correlation between pre-donation platelet count and the yield of plateletpheresis, the age of the donor and the yield of plateletpheresis, weight of the donor and the yield of plateletpheresis, hematocrit value and the yield of plateletpheresis. The one-way ANOVA was used to evaluate the differences between the means of the three age groups. A p value <0.05 was considered to be significant.

RESULTS

Forty-four plateletpheresis donations were obtained during the study. All donors/ subjects were male. The age range of donors ranged from 18 to 55 years with a mean age of 31.95 ± 9.92 years (TABLE 1).

TABLE1. The characteristic of donors by age

Age group (years)	Number of subjects	Percentage (%)
< 30	18	40.9
30 – 40	17	38.6
> 40	9	20.5
Total	44	100

The mean values of donors' basic characteristics were as follows weight was 70.2 ± 10.2 kg, BMI was 24.7 ± 3.2 kg/m^2, and hematocrit was 44.05 ± 3.29 % (TABLE 2).

TABLE 2. The basic characteristics of donors

Characteristic	Mean ± SD
Age (years)	31.95 ± 9.92
Weight (kg)	70.2 ± 10.24
BMI (kg/m^2)	24.7 ± 3.2
Hematocrit (%)	44.05 ± 3.29

The median value of the yield of plateletpheresis was $3.2x10^{11}$, with a maximum value of 4×10^{11}, and the minimum value of 2.19×10^{11}. The distribution of the median value of the yield of plateletpheresis in each donor is shown in FIGURE 1.

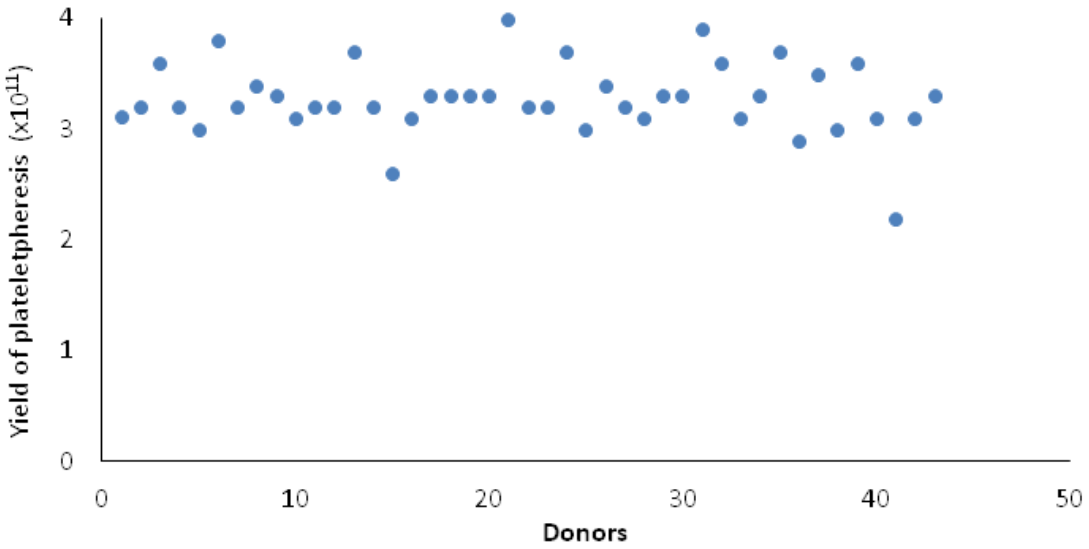

FIGURE 1. Distribution of the median value of the yield of plateletpheresis in donors

The median value of pre-donation platelet count in donors was $248.5 \times 10^3/\mu L$ with the maximum value of $391 \times 10^3/\mu L$, and minimum value of $204 \times 10^3/\mu L$. The distribution of the median value of the pre-donation platelet count in each donor is shown in FIGURE 2.

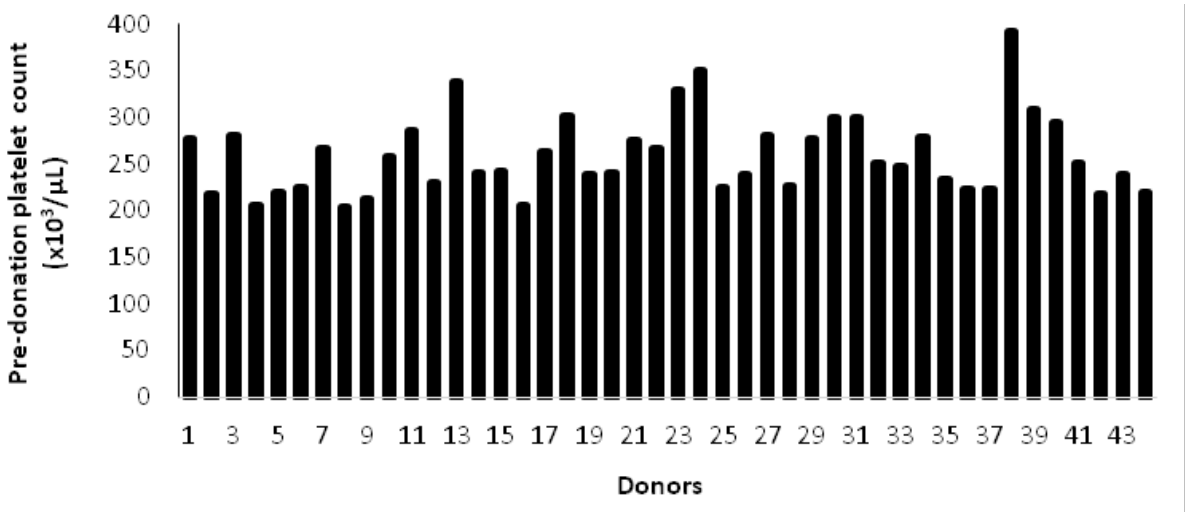

FIGURE 2. The distribution of the median value of the pre-donation platelet counts in donors

From the result of Spearman's rho correlation test, it was found that the correlation between pre-donation platelet count and the yield of plateletpheresis was statistically significant ($p < 0.05$). The value of Spearman's rho coefficient correlation of 0.329 indicated that the direction of the correlation was moderately positive. Therefore, the pre-donation platelet count was positively correlated with the yield of plateletpheresis and was statistically significant.The mean value of plateletpheresis in the age group of less than 30 years was $3.35 \pm 0.26x10^{11}$, while in the age group of 30 to 40 years, the mean value was $3.17 \pm 0.39x10^{11}$, and in the age group of above 40 years, the mean value was $3.32 \pm 0.23x10^{11}$ (TABLE 3).

TABLE 3. Distribution of the yield of plateletpheresis based on age group

Age (years)	Number of Subjects	Mean ± SD ($x10^{11}$)
< 30	18	3.35 ± 0.26
30 – 40	17	3.17 ± 0.39
> 40	19	3.32 ± 0.23

The result of Spearman's rho correlation test showed that the correlation between the age of the donor and the yield of plateletpheresis was not significant ($p > 0.05$). The value of Spearman's rho coefficient correlation of -0.137 indicated that the direction of the correlation was negative. Thus, it can be concluded that there was no correlation between age of the donor and the yield of plateletpheresis. We also performed one-way ANOVA correlation test to determine whether there were any statistically significant differences between the means of the three age groups. It was found that there was no difference between donor age groups and the yield of plateletpheresis compared to one another ($p = 0.238$).

Spearman's correlation test results showed that the correlation between weight of the donor and the yield of plateletpheresis was not significant ($p > 0.05$). The value of Spearman's rho coefficient correlation test of 0.033 indicated that the direction of the correlation was positive. Thus, we concluded that there was no correlation between weight and the yield of plateletpheresis.Similar correlation tests were performed on the Body Mass Index (BMI) variable. It was found that the correlation between BMI of the donors and the yield of plateletpheresis was not significant ($p > 0.05$). The coefficient correlation value of 0.027 indicated that the direction of the correlation was positive. Thus, we concluded that, there was no correlation between BMI and yield of plateletpheresis.

In the hematocrit variable, there was no significant correlation ($p > 0.05$) in the result of Spearman's rho. The value Spearman's rho coefficient correlation test of -0.156 indicated that the direction of the correlation was negative. Thus, we concluded that there was no correlation between the hematocrit value and the yield of plateletpheresis. From the aspect of the donation procedure, we obtained the mean value of the product volume of platetelpheresis was 275 ± 2.9 mL and the mean value of the duration of plateletpheresis was 84.2 ± 19.2 minutes. The distribution of the mean value of the product volume in each donor is shown in FIGURE 3, and the distribution of the mean value of the duration of plateletpheresison each donor is shown in FIGURE 4.

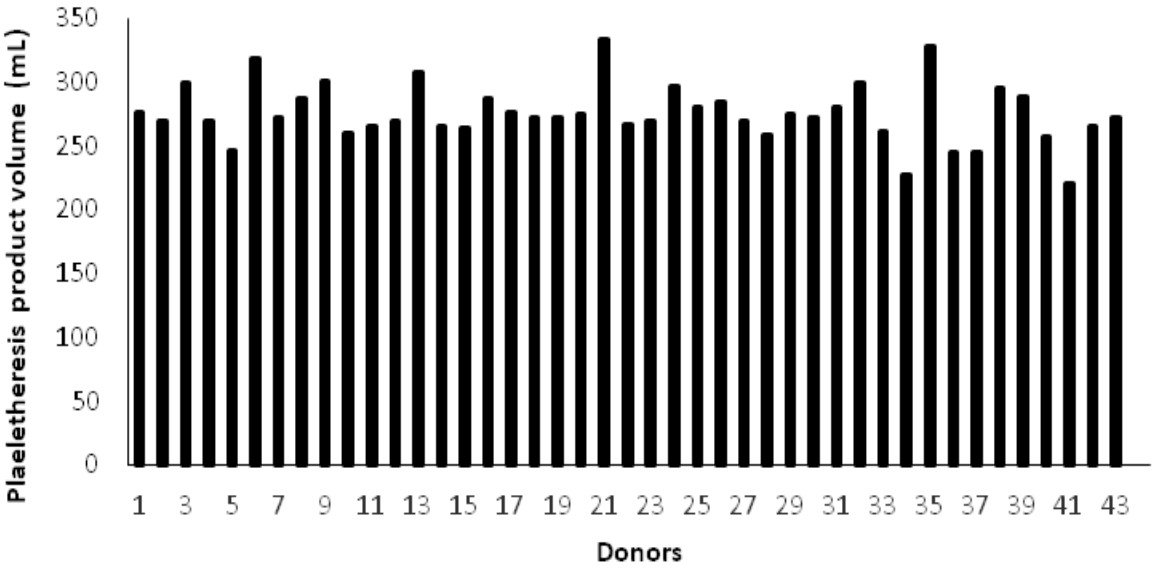

FIGURE 3. Distribution of the mean value of the product volume of plateletpheresis in donors

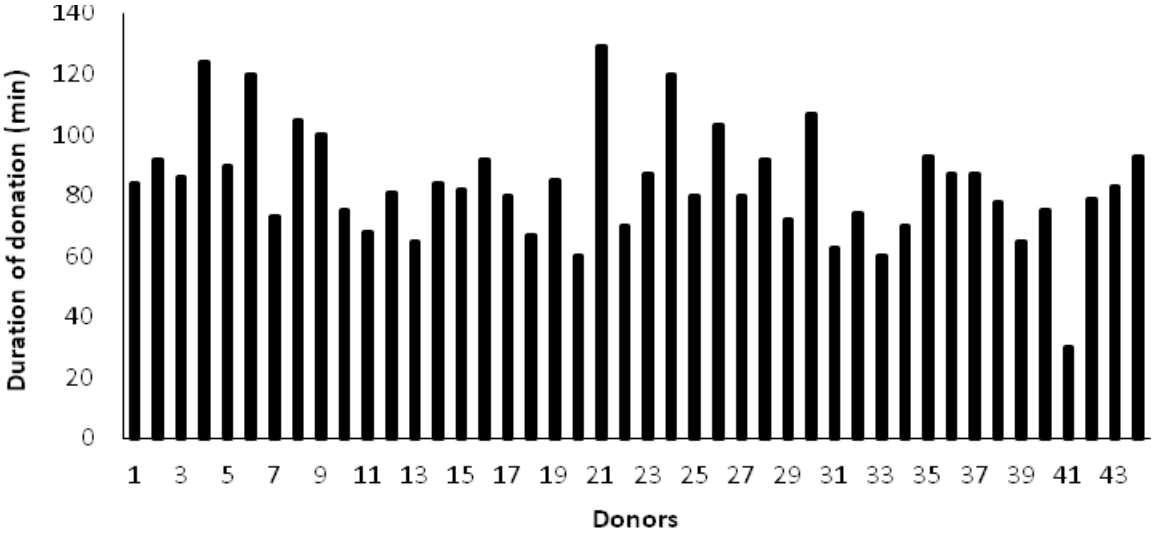

FIGURE 4. Distribution of the mean value of the duration of plateletpheresis in donors

The result of Spearman's rho correlation test showed that the correlation between the product volume of plateletpheresis and the pre-donation platelet count was not significant ($p > 0.05$). The value of Spearman's rho coefficient correlation test of 0.211 indicated that the direction of the correlation was positive. Thus, we concluded that there was no correlation between the product volume of plateletpheresis and the pre-donation platelet count. The next result of Spearman's rho showed that the correlation between the product volume of plateletpheresis and the yield of plateletpheresis was significant ($p < 0.05$). The value of Spearman's rho coefficient correlation test of 0.661 indicated the moderately positive correlation. Thus, we concluded that the product volume of plateletpheresis was moderately positively correlated with the yield of plateletpheresis and was statistically significant.

From the donation procedure variable,

the results of Spearman's rho correlation test found that the correlation between the duration of plateletpheresis and the yield of plateletpheresis was not significant ($p > 0.05$). The value of Spearman's rho coefficient correlation test of 0.224 indicated that the direction of the correlation was positive. Thus, we concluded there was no correlation between the duration of the plateletpheresis and the yield of plateletpheresis.We examined the recipient data in the medical records that contain the increase of post-transfusion platelet count. In the medical records, there were only 27 recipients who received transfusions from the donors. This limited number then affected the reduced number of samples when we were analyzing the correlation between the increased yield of plateletpheresis and the post-transfusion platelet count.

With these limitations, we analyzed the correlation between the yield of plateletpheresis of the donor and the increased number of platelets after transfusion (platelet increment) of the recipient. Normality test showed that the distribution of the yield of plateletpheresis was not normal despite performing data transformation. Thus, we performed Spearman's rho correlation test. From the result of Spearman's rho correlation test, we found that the correlation between the yield of plateletpheresis and the increase number of platelet after transfusion (platelet increment) of the recipient was not significant ($p > 0.05$). The value of Spearman's rho coefficient correlation test of 0.327 indicated that the correlation was positive. Thus, it can be concluded that there was no correlation between the yield of plateletpheresis of the donor and the increase number of platelets after transfusion (platelet increment) of the recipient.

DISCUSSION

Our study shows similar findings with previous studies, that the pre-donation platelet count is positively and statistically significantly correlated with the yield of plateletpheresis (TABLE 4). Pre-donation platelet count is the main factor that is believed to influence the yield of plateletpheresis. The higher the number of pre-donation platelet count, the higher the yield of plateletpheresis.[6]

TABLE 4. Comparison with other studies

Correlation	Goodnough et al.	Rajendra et al.	Das et al	Guerero-Rivera et al. grup 1	Guerero-Rivera et al. grup 2	Enein et al.	Patel et al.	This study
Pre-donation PC	Direct correlation	0.506 (<0.05)	0.51 (<0.05)	0.554	0.758	0.512	0.302 (<0.05)	0.329 (<0.05)
Pre-donation Hb	-	-0.1 (>0.05)	0.05 (>0.05)	Inverse correlation	Inverse correlation	0.306	-0.001 (>0.05)	-
Pre-donation Hct	-	-	-	-	-	-	-0.018 (>0.05)	-0.213
Donor's weight	-	0.18	-	-	-	-	0.023 (>0.05)	0.033 (>0.05)
Duration	-	-	-	-	-	-	-0.047 (>0.05)	0.224 (>0.05)
Volume	-	-	-	-	-	-	0.158 (<0.05)	0.661 (<0.05)

Several studies mentioned that age was a factor that influences the yield of plateletpheresis. One study recently concluded that age was negatively and significantly correlated with the yield of plateletpheresis – the older the donor, the lower the yield of plateletpheresis.[9] However, in our study, there is no correlation between age and yield of plateletpheresis (TABLE 4.).Weight and BMI are several factors from the donor that are believed can influence the yield of plateletpheresis.[10] Body mass index is an indicator of the amount of tissue mass (muscle, fat, and bone) in an individual. A study showed that BMI has a positive correlation with the yield of plateletpheresis.[9] However, another study found that weight is not correlated with the yield of plateletpheresis.[10] The BMI reflects weight and body mass in line with the blood volume. Someone with greater blood volume has a tendency to produce higher platelet count.[11] However, in our study, body weight and BMI are not correlated with the yield of plateletpheresis (TABLE 4).

Another factor of the donor that influences the yield of plateletpheresis is the hematocrit value. Hematocrit indicates the proportion or percentage of erythrocyte components contained in blood components as a whole – the higher the hematocrit value, the smaller the other blood components, including platelets. One study revealed that the hematocrit value is negatively and statistically significant correlated with the efficiency of plateletpheresis collection.[12] Other studies find no correlation between pre-donation hematocrit value with the yield of plateletpheresis.[5] Similarly, in our study, there is no correlation between the hematocrit value and the yield of plateletpheresis. The guidelines issued by the American association of blood banks (AABB) stated that the pre-donation hematocrit value of the donor has to achieve at least 38%, and in our study, the mean hematocrit value was 44 ± 3.29%.

All subjects in our study were male. Thus, we could not analyze the correlation between gender and the yield of plateletpheresis. One study found that women produced a higher yield of plateletpheresis than men.[10] This result may be due to the prevalence of iron deficiency in women, leading to low hemoglobin levels. Low levels of hemoglobin, then, as mentioned above, result in an increase in the number of platelets, which then increases the yield of yield plateletpheresis.[6] Again, from the donor aspect, one interesting study found that donors with low hemoglobin level would produce a high yield of plateletpheresis and vice versa. That result was because low hemoglobin level provides a high level of pre-donation plasma volume that produces a high yield of plateletpheresis.[8] However, another study found that hemoglobin level had no correlation with the yield of plateletpheresis.[14] This research was unable to assess the correlation of the hemoglobin level with the yield of plateletpheresis because there was no history or information regarding the pre-donation hemoglobin level of the donors.

From the aspect of plateletpheresis procedure, it is mentioned that the product volume of plateletpheresis is correlated to the yield of plateletpheresis. Based on FDA guidelines, the volume of plateletpheresis' products (excluding anti-coagulant) should not exceed 500 mL (or 600 mL of the donor with a body weight greater than or equal to 80 kg). One study revealed that the product volume of plateletpheresis correlated positively and was statistically significant to the yield of plateletpheresis showing that the higher the product volume of plateletpheresis, the higher the yield of plateletpheresis.[9] The study also found that the product volume of plateletpheresis is one of the main factors of the procedural aspects that influence the yield of plateletpheresis. Similarly, in our study, the product volume of plateletpheresis has a positive and statistically significant correlation with the yield of plateletpheresis. Thus, our study is in line with the existing studies.

In this study, we also analyzed

the correlation between pre-donation platelet count and the product volume of plateletpheresis. There is no correlation between the number of pre-donation platelet count and the product volume of plateletpheresis. However, few studies analyzed the pre-donation platelet count with the yield of plateletpheresis, instead of the product volume of plateletpheresis. Another aspect of the procedure that is considered in a few studies to have a correlation with the yield of plateletpheresis is the duration of the donation procedure (plateletpheresis). One study mentioned that a high yield of plateletpheresis can be obtained from a high pre-donation platelet count and less duration of plateletpheresis.[12] In this study, we find no correlation between the duration of plateletpheresis and the yield of plateletpheresis. It is explained in several studies that the duration of the procedure varies from 20 to 120 min, depending on the product being donated, as well as the number of cells, the size of the donor, vascular access, technical factors, and other limitations on the procedure.[14,15]

In our research, there is no correlation between the increase number of post-transfusion platelet count (platelet increment) and the yield of plateletpheresis. This result might be due to a limitation in our study, which used a variable of Absolute Count Increment (ACI), instead of Corrected Count Increment (CCI). ACI, in this case, is the increase in the number of platelets after transfusion. Some studies show a positive correlation between the yield of plateletpheresis and the efficacy of transfusion using CCI variable. Limitations of this study might also include the unavailability of height and weight data of the recipient in some medical records, which is required for the calculation of the body surface area and CCI.

CONCLUSION

It can be concluded that the pre-donation platelet count and the product volume of plateletpheresis influence the yield of plateletpheresis. Optimization of the yield of plateletpheresis, which is influenced by pre-donation platelet count and the production volume of plateletpheresis, is an emerging issue in blood transfusion services. However, the yield of plateletpheresis itself is not correlated with the number of post-transfusion platelet count. Thus, other clinical factors must be considered. It is necessary to consider other indicators such as CCI, to assess the efficacy of the transfusion. Therefore, further research with more samples should be conducted.

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Outcomes of surgical management of intracerebral hemorrhagic stroke at a tertiary care center in Yogyakarta, Indonesia

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ABSTRACT

Hemorrhagic stroke remains a significant cause of morbidity and mortality worldwide. However, the role of surgical treatment for hemorrhagic stroke remains controversial. Previous studies had proposed surgery can prevent herniation, lowering intracranial pressure (ICP) and reducing mass effect and cellular toxicity. Outcome in previous studies are conflicting. Studies concerning outcomes of surgical management of hemorrhagic stroke in Indonesia are limited. This study aimed to compare the outcome of hemorrhagic stroke treatment between surgical evacuation and conservative management in Dr. Sardjito General Hospital, Yogyakarta, Indonesia. Retrospective cohort study was conducted. Eighty spontaneous intracerebral hemorrhagic (ICH) stroke patients involved in this study during January 2014 to August 2015. Of whom 36 (45%) male and 44 (55%) female. There were 45 and 35 patients underwent surgical treatment and conservative management, respectively. Surgical treatment group survival was 74%, whereas the survival in the conservative group was 26%. There was a statistically significant difference between surgery group and conservative group ($p<0.001$; CI 95% 2.24-15.69). Furthermore, there was association of GCS less than 8, high systolic BP, intraventricullar hemorrhage (IVH) and length of stay (LOS) with poor outcome. Analysis also showed surgical treatment, lower GCS score and hypertension associate with unfavorable Glascow outcome scale (GOS). In conclusion, patients with hemorrhagic stroke who are surgically indicated might have an advantage of hematoma evacuation and better outcome.

ABSTRAK

Stroke perdarahan masih merupakan penyebab signifikan morbiditas dan mortalitas di seluruh dunia. Kaidah pembedahan terhadap kebanyakan pasien stroke perdarahan masih dianggap kontroversial. Teori rasional untuk evakuasi hematoma berpatokan pada konsep prevensi herniasi, menurunkan tekanan intrakranial, dan menurunkan dampak hematoma pada jaringan sekitarnya dengan mengurangi efek massa atau toksisitas seluler. Penelitian yang menganalisis hasil penanganan pasien stroke perdarahan di pusat pelayanan bedah saraf di Indonesia masih terbatas. Penelitian ini bertujuan untuk menganalisis hasil penanganan pasien stroke perdarahan yang menjalani terapi pembedahan atau konservatif di Rumah Sakit Umum Pusat (RSUP) Dr. Sardjito. Penelitian ini merupakan penelitian kohort retrospektif yang membandingkan hasil klinis pada pasien stroke perdarahan yang menjalani pembedahan dan tidak di RSUP Dr. Sardjito, yang merupakan pusat rujukan kasus stroke perdarahan di provinsi DI Yogyakarta dari bulan Januari 2014 sampai dengan Agustus 2015. Dilakukan analisis terhadap data pasien meliputi umur, jenis kelamin, Glascow outcome scale (GOS), penanganan dan hasil dari penanganan yang diberikan. Pengumpulan data dilakukan di Instalasi Catatan Medis RSUP Dr. Sardjito. Dari 45 pasien yang dilakukan operasi, 74% pasien selamat. Dari 35 pasien yang diindikasikan untuk dilakukan operasi, tetapi tidak menjalani operasi, 26% pasien selamat. Terdapat hubungan yang bermakna antara terapi bedah dengan hasil klinis (p

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<0,001; CI 95%: 2,24-15,69). Terdapat hubungan antara nilai GCS kurang dari 8, tingginya tekanan darah sistolik, perdarahan intraventrikular dan lama perawatan terhadap keluaran yang lebih buruk. Terdapat hubungan juga antara terapi bedah, GCS rendah, dan tekanan darah sistolik terhadap nilai GOS yang lebih buruk. Dapat disimpulkan bahwa pasien dengan stroke hemoragik yang diindikasikan dengan pembedahan kemungkinanmemiliki keuntungan evakuasi hematoma dan hasil yang lebih baik.

Keywords: intracerebral hemorrhagic stroke – surgical treatment – conservative management – outcomes

INTRODUCTION

Stroke remains a leading cause of morbidity and mortality around the world. There are two types of stroke, i.e. ischemic (non-hemorrhagic) stroke and hemorrhagic stroke. Over 80% of stroke are non-hemorrhagic type, hemorrhagic stroke only accounted 15-22% of all mortality caused by stroke.[1] Regardless of the small percentage, the hemorrhagic stroke has higher mortality rate than ischemic stroke. The role of surgical evacuation for hemorrhagic stroke remains controversial. The rational theory for the evacuation of hematoma based on the concept of prevent herniation, lowering the intracranial pressure and prevent the pathophysiology of hematoma impact on the surrounding tissue by reducing the effects of cellular toxicity mass or blood products.[2-4]

In previous studies, the outcomes of stroke treatment are inconsistent. Randomized controlled trials comparing surgical treatment with conservative treatment showed no significant benefit. Two prospective randomized controlled trials, and three meta analysis to compare the outcomes between conservative treatment and surgical treatment of intracerebral hemorrhage (ICH) also showed similar results. Randomized trials to compare between surgery and conservative management did not demonstrate a clear benefit for surgical intervention. Moreover, the generalizability of the results of these trials can be questioned, because patients at risk for herniation were likely excluded and the largest and most recent studies had high rates of treatment group crossover from conservative management to surgery.[2]

Studies concerning the outcomes of surgical management of hemorrhagic stroke in Indonesia are limited. This study aimed to compare the outcomes of intracerebral hemorrhagic stroke treatment between surgical evacuation and conservative management in Dr. Sardjito Hospital, Yogyakarta, Indonesia.

MATERIALS AND METHODS

Subjects

This was a retrospective cohort study comparing of the clinical outcome of intracerebral hemorrhagic stroke patients who underwent surgery and conservative treatment in the Department of Neurosurgery, Dr. Sardjito General Hospital, Yogyakarta, Indonesia, within the period of January 2014 to August 2015. Data were collected form patient's medical record. Patient's sociocemographic and clinical characteristics, the therapy, and follow up of their outcome were collected. After treatment, patient's outcome (recover or death), length of stay and Glasgow outcome scale (GOS) were identified.

Patients included in the study were patients who diagnosed with spontaneous ICH. Patients with subarachnoid hemorrhage or traumatic ICH were excluded in this study. Patients who were surgically indicated but the family refused to do were considered as conservative management group.

Protocol

To compare sociodemographic characteristics of ICH patients, the gender, age, area of residence were identified

(TABLE 1). Patients were divided by gender (male and female), age at the time of admission and area of residence (same area as the medical institution or other area).

Clinical characteristics are factors related to medical treatment received by the patients; and included diagnosis, whether surgery was performed, initial blood pressure and Glasgow coma scale (GCS), and length of stay (LOS). Glasgow outcome scale (GOS) were examined after discharge from hospital when patients went to follow up to hospital. Nontraumatic intracerebral hemorrhage (I61.0-I61.9) were classifiable under ICD-10 codes. Surgery was classified as "performed" in case in which there was a recorded day of a main operation that was performed clearly for treatment purposes and not for diagnostic or exploratory purposes or to treat complications (the organization for economic cooperation and development also classifies medical and surgical categories based on whether surgery is performed). The length of stay was calculated by counting the number of days that passed between admission and discharge days.

TABLE 1 Variable observed of ICH patients

Variable	Measure	
Independent Variable		
Sociodemographic		
• Sex	0. Male	1. Female
• Age	Patient's age	
• Area	0. Same	1. Other
Clinical characteristics		
• Management	0. Surgery	1. Conservative
• Length of stay	Days stay in hospital	
• Diagnosis	0. IVH	1. other
• Hypertension	0. Yes	1. No
• Glasgow coma scale (GCS)	0. ≤ 8	1. > 8
Dependent Variable		
• Outcome	0. Recover	1. Death
• Glasgow outcome scale (GOS)	0. Favorable	1. Unfavorable

Statistical analysis

The t test and Chi-square test were performed for comparative analysis of ICH patient characteristics such as sociodemoraphics, clinical conditions and managements, depending on outcome of treatment as recover or death and GOS, divided into favorable (GR/MD) or unfavorable (SD/V/D) outcome. Log transformation was performed for the systolic BP and LOS, as the average and median values exhibited asymmetric distribution. The level of significant was set as $p < 0.05$. Statistical analysis was performed using SPPS for Windows version 24.

RESULTS

A total 83 patients were recruited between January 2014 to August 2015. Among 83 stroke patients, the majority had ICH (80 or 96%), followed by subarachnoid hemorrhage (SAH) (2 or 3%) and malignant neoplasm of cerebrum (1 or 1%). Only patients diagnosed with IHC were included, so total sample analysed was 80 patients. TABLE 2 shows the frequency of sample based on hospitalization period and TABLE 3 summarizes all characteristics measured.

TABLE 2. Number of sample data based on hospitalization period

Period	Frequency	(%)
January-December 2014	45	54.2
January-August 2015	38	45.8
Total	83	100

TABLE 3. Characteristics of patients

Variable	N	%	CI 95% (%)
Sex			
• Male	36	45	34.1-55.9
• Female	44	55	44.1- 65.9
Age (mean ± SD years)	59.4 ± 13.51		56.4-62.3
Province			
• Yogyakarta	62	76	66.6-85.3
• Central Java	14	18	6.4-21.6
• East Java	2	3	-0.7- 6.7
• West Java	1	2	-1.1-5.1
• Unknown	1	1	-1.2-3.2
Therapy			
• Surgery	45	56	45.1-66.8
• Conservative	35	44	33.1-54.8
Outcome			
• Recover	46	58	47.1-68.8
• Death	34	42	31.2-52.8
Length of stay (mean ± SD days)	12.9 ± 9.87		10.7-15.0
Diagnosis			
• ICH unspecified	46	58	47.1-68.8
• ICH intraventricular	19	24	14.6-33.4
• ICH hemisphere	9	11	4.1-17.9
• ICH multiple localized	4	5	0.2-9.8
• ICH cerebellum	2	2	-1.1-5.1
Glasgow Coma Scale			
• ≤ 8	54	68	57.8-78.2
• >8	26	32	21.8-42.2
Blood pressure (mean ± SD mmHg)			
• Systole	153.4 ± 11.88		150.8-156.0
• Diastole	87.6 ± 6.79		86.1-89.1
Hypertension (>140 mmHg)			
• Yes	62	78	68.9-87.1
• No	18	22	12.9-31.1
Glasgow outcome scale (GOS)			
• Favorable	32	40	29.3-50.7
• Unfavorable	48	60	46.3-70.7

TABLE 4 shows in ICH patients, surgical treatment group had better outcome than conservative group (p <0.001). Compared with patients who survived, died patients showed higher initial systolic BP (p=0.009), poorer initial lower GCS (p<0.001), shorter LOS (p<0.001) and had slighty different outcome whether diagnosis was ICH with IVH or other ICH (p =0.03). Similarly, when compared with favorable GOS scoring, surgical treatment had better recovery outcome than conservative treatment (p<0.001), lower GCS score (p<0.001) and higher initial systolic BP (p =0.03).

TABLE 4. Outcome of hemorrhagic stroke treatment between surgical evacuation and conservative management

Variable	Outcome				GOS			
	Recover (n=46)	Death (n=36)	p	OR/MD (CI 95%)	Favorable (n=32)	Unfavorable (n=48)	p	OR/MD (CI 9 5%)
Sex*								
• Male	18	18	0.220	0.57 (0.23-1.40)	12	24	0.270	0.60 (0.24-1.49)
• Female	28	16			20	24		
Age (mean ± SD mmHg)**	57.67 ± 13.02	61.62 ± 14.02	0.190	-3.94 (-10.00-2.12)	58.00 ± 13.77	60.25 ± 13.41	0.470	-2.25 (-8.46- 3.96)
GCS*								
• ≤ 8	20	34	<0.001	0.37 (0.26-0.52)	7	47	<0.001	0.006 (0.001-0.051)
• > 8	26	0			25	1		
Systole (mean ± SD mmHg)**	15.43 ± 10.55	157.32 ± 12.56	0.009	-6.88 (-12.04- -1.73)	149.94 ± 10.31	155.65 ± 12.41	0.034	-5.70 (-10,98 - -0.43)
Diagnosis*								
• IVH	7	12	0.037	0.33 (0.11-0.96)	4	15	0.054	0.31 (0.09-1.06)
• Other	39	22			28	33		
LOS (mean ± SD days)**	17.24 ± 10.12	7.09 ± 5.68	<0.001	10.15 (6.31-13.99)	13.13 ± 4.29	12.79 ± 12.31	0.860	0.33 (-3.53-4.19)
Therapy*								
• Surg	34	11	<0.001	5.92 (2.24-15.69)	26	19	<0.001	6.61 (2.29-19.08)
• Cons	12	23			6	29		

*: X^2 analysis; **: t-test analysis; GCS: glasgow coma scale; LOS: length of stay; Surg: surgery; Cons: conservative; SD: standard deviation; IVH: intraventricular hemorrhage; GOS: Glasgow outcome scale; OR: odds ratio; MD: mean difference

TABLE 5 shows whether surgery was done or not, GCS score less than 8 tend to got surgery than GCS score more than 8 (p =0.002). There were no association between sex, age, systolic BP and LOS with treatment choices for patients. Moreover, there were no association between sex, age, GCS, systolic BP and LOS with wether diagnosis was ICH with or without IVH.

TABLE 5. Factors associated with therapy and diagnosis

Variable	Therapy				Diagnosis			
	Surgery (n=45)	Conservative (n=35)	p	OR/MD (CI 95%)	IVH (n=19)	Other (n=61)	p	OR/MD (CI 9 5%)
Sex*								
• Male	17	19	0.140	0.51 (0.21-1.25)	12	24	0.068	2.64 (0.91-7.66)
• Female	28	16			7	37		
Age (mean ± SD mmHg)**	60.82 ± 14.46	57.46 ± 12.13	0.272	3.36 (-2.56-9.28)	61.05 ± 12.96	58.82 ± 13.72	0.533	2.23 (-4.81-9.27)
GCS*								
• ≤ 8	24	30	0.002	0.19 (0.06-0.58)	16	28	0.075	3.23 (0.85-12.30)
• > 8	21	5			3	23		
Systole (mean ± SD mmHg)**	152.69 ± 12.35	154.23 ± 11.38	0.569	-1.54 (-0.68- 3.82)	148.95 ± 10.34	154.74 ± 12.07	0.063	-5.79 (-11.54- -0.40)
LOS (mean ± SD days)**	14.44 ± 8.83	10.97 ± 10.87	0.119	3.47 (-0.91-7.86)	13.11 ± 11.41	12.87 ± 9.44	0.928	0.24 (-4.95-5.43)

*: X^2 analysis; **: t-test analysis; GCS: glasgow coma scale; LOS: length of stay; SD: standard deviation; OR: odds ratio; MD: mean difference; IVH: intra-ventricular hemorrhage.

DISCUSSION

In this study, we found a better outcome (reduce mortality and better GOS) in hemorrhagic stroke patients underwent surgery compared to those who were conservatively managed. For over 50 years, there have been clinical trials of surgical clot evacuation aimed at reducing the mass effect.[5,6] Since the first clinical trial of surgical ICH evacuation[5], there have been multiple small trials but the result was conflicting.[6] To clarify this, large surgical trial (the Surgical Trial in Intercerebral Hemorrhage (STICH) trial, n=1033) have been conducted, but failed to demonstrate any benefit of clot evacuation.[7] Sub-analysis of the STICH I trials results indicated that superficial hematoma (<1 cm from cortical surface) lobar hemorrhage might benefit from clot evacuation, perhaps because reducing surgical trauma than deep hemorrhage patients. Additional subgroup analysis suggested that the risk for a poor outcome was increased for patients who presented as comatose (GCS score ≤8).[2]

The STICH II trial addressed the question of whether early surgery would be beneficial for conscious patients with superficial lobar hemorrhage of 10 to 100 mm^3 within 1 cm of the cortical surface and without IVH and who were admitted within 48 hours of ictus. STICH II trial resulted again no evidence of significantly improved outcome compared to medical treatment.[8]

However, recent report, American Heart Association/American Stroke Association (AHA/ASA) guideline for spontaneous ICH management 2015 has suggested a possible role for craniectomy in ameliorating increased ICP caused by ICH.[2] The guideline suggest that surgery give benefit for preventing herniation, reducing ICP and decreasing the pathophysiological impact of the hematoma on surrounding tissue by decreasing mass effect or the cellular toxicity of blood products. Whether surgery has the benefit on ICH still controversial, other approaches currently being tested use minimally invasive surgery in combination with hematoma lysis methods. In the minimally invasive surgery plus rtPA for intercerebral hemorrhage evacuation (MISTIE) trial (NCT00224770), a minimally invasive approach is being used with t-PA to assist evacuation[9] and this has recently been reported to reduce perihematomal edema.[10]

Several nonrandomized studies have suggested that patients with cerebellar hemorrhages >3 cm in diameter or patients in whom cerebellar hemorrhage is associated with brainstem compression

or hydrocephalus have better outcomes with surgical decompression.[11-14] A recent meta-analysis has suggested that u-PA, an alternate thrombolytic, is superior to t-PA for IVH clot evacuation.[15]

To understand underlying causes and natural history of ICH, several studies have been conducted, as well as preclinical studies involving animal models. Spontaneous ICH (not related to trauma), most frequently occurs secondary to hypertension.[7] This similar found in our study, which higher blood pressure associated with mortality and morbidity. However, ICH may also result from bleeding associated with amyloid angiopathy, tumors, hemorrhagic conversion of ischemic stroke, dural venous sinus thrombosis, vasculitis and vascular marformations.[16,17]

This study showed that higher systolic BP associated significantly with morbidity and mortality, whereas sex and age did not. Seventy-eight percent patients had hypertension when diagnosed with ICH. Broderick *et al.*[14] and Zazulia *et al.*[18] reported that post – hematoma enlargement causes a midline shift and accelerates neurological deterioration. Mendelow *et al.*[7] revealed that nontraumatic ICH most frequently occurs secondary to hypertension, with up to 70% of patients with ICH having history of hypertension. Ruiz-Sandoval *et al.*[17] also reported that hypertension is significant contributory factor for ICH and associated with morbidity and mortality. AHA/ASA guidelines for the management of spontaneous ICH 2015 recommended for ICH patients presenting with systolic BP between 150 and 220 mmHg and without contraindication to acute BP treatment, acute lowering of SBP to 140 mHg is safe (Class I, Level of Evidence A).[2] It is also mentioned in Neurosurgery National Guideline for Healthcare that SBP>180 mmHg or MAP>130 mmHg with or without increased ICP should give anti-hypertension drug to lowering SBP.[19] This guideline was used in Neurosurgery Department, Dr. Sardjito General Hospital, Yogyakarta as its clinical standard work pathway.

STICH trial found that comatose (GCS score ≤8) patients had increased the risk for a poor outcome[7]. Patients tended to be in a coma (GCS score ≤8), did not normalize with medical management. Our observations are consistent with previous study mention above, which comatose patients had higher mortality and unfavorable outcome.

Current recommendations for management of ICH with IVH or hydrocephalus are for ICP monitoring when GCS less than 8 and for ventricular drainage, if there is decreased level of consciousness. Moreover, AHA/ASA recommended patients with a GCS score of ≤8, those with clinical evidence of transtentorial herniation, or those with significant IVH or hydrocephalus might be considered for ICP monitoring and treatment. A CPP of 50 to 70 mm Hg may be reasonable to maintain depending on the status of cerebral autoregulation (Class IIb; Level of Evidence C).[4] In clinical standard work pathway used in Dr. Sardjito General Hospital, neurological deficit, increased ICP, hydrocephalus >24 hours and brain stem compression are an indication for surgical evacuation.

The location of an ICH is important in determining the outcome and potential treatment. Depending on location, bleeding from an ICH may extend into ventricular system. Such intraventricular hemorrhage occurs in ~40% of ICH patients and predictor of poor outcome. Hanley[20] reported that spontaneous ICH with IVH is about 42%-55% of cases. IVH is an independent predictor of worse outcomes with mortality rates of 29-78%, compared to 5-29% for ICH without IVH. This finding was lower in our study, whereas ICH with IVH was account for 24%. However, ICH with IVH was an independent factor associated with higher mortality.

In this study, the LOS for all patients was 12.9 days. Patients who underwent surgery stayed for 1.5 days longer (1.3 times, 14.4 days) than patients who did not (10.9 days). Although there was no different for LOS in surgery or conservative group, however, there was significant difference

for LOS between died or recover group. Kim *et al.*[4] reported who targeted 700,056 cases based on system data from the Korean Hospital Discharge In-Depth Injury Survey, that average LOS being 28.9 days; patient underwent surgery stayed in the hospital on average 9.2 days more (1.4 times, 35.1 days) than patient who did not (25.9 days). However, factors associated with shorter length of stay was not discused in this study.

The limitation of this study was we could not find clear cause of death of hemorrhagic stroke patients, whether hemorrhagic stroke only or other comorbid disease that was diagnosed during hospitalization period.

CONCLUSION

In conclusion, patients with hemorrhagic stroke who were surgically indicated might have an advantage of hematoma evacuation. The further and more targeted study is needed by incorporating the factors and causes of deaths related to surgical treatment, so that we can obtain more detailed data.

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The role of Malassezia sp., sebum level and trans epidermal water loss (TEWL) toward the dandruff severity between hijab and non hijab wearing subjects

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ABSTRACT

Dandruff is a common symptom in adolescence, which possibly due to increasing of *Malassezia sp.* colonization, sebum level as well as skin hydration. Wearing hijab is predicted to increase the humidity and sebum level lead to increase the number of *Malassezia sp*. and dandruff severity. A case-control study was conducted on 19 female with dandruff who wear hijab and 19 female with dandruff who do not wear hijab, age between 18 and 27 years old. The dandruff severity was defined clinically using a scoring system (0-100), only subjects with minimum score of 28 will be included in this study. The sebum and trans epidermal water loss (TEWL) levels were measured with Sebumeter and Tewameter from Courage Khazaka. The *Malassezia sp.* was examined using microscopic examination of the squama and culture in the Saboraud medium. There were no significant differences found between hijab and non-hijab groups for dandruff severity. No difference found between two groups for sebum level, *Malassezia sp.* number microscopically, and *Malassezia sp*. colonization. Only TEWL level in hijab group that was found to be higher than non-hijab group. Wearing hijab does not increase the dandruff severity, sebum level and colonization number of *Malassezia sp*. Wearing hijab is found to increase the TEWL level.

ABSTRAK

Ketombe merupakan gejala umum pada masa remaja, yang disebabkan oleh meningkatnya coloni *Malassezia sp*., tingkat sebum serta hidrasi kulit. Penggunaan jilbab diperkirakan menyebabkan peningkatan kelembaban dan sebum yang akan meningkatkan jumlah *Malassezia sp.* dan tingkat keparahan ketombe. Sebuah studi kasus-kontrol dilakukan pada 19 wanita berketombe yang memakai jilbab dan 19 wanita berketombe yang tidak memakai jilbab, usia antara 18 dan 27 tahun. Keparahan ketombe didefinisikan secara klinis menggunakan sistem penilaian (0-100), hanya subjek dengan skor minimal 28 yang akan dimasukkan dalam penelitian ini. Tingkat sebum dan *trans epidermal water loss* (TEWL) diukur dengan Sebumeter dan Tewameter dari Courage Khazaka. *Malassezia sp.* diperiksa menggunakan pemeriksaan mikroskopik dari skuama dan kultur dalam medium Saboraud. Tidak ada perbedaan signifikan yang ditemukan antara kelompok hijab dan non-hijab untuk keparahan ketombe. Tidak ada perbedaan yang signifikan ditemukan antara dua kelompok untuk tingkat sebum, jumlah *Malassezia sp*. secara mikroskopis, dan koloni *Malassezia sp*. Ditemukan tingkat TEWL kelompok berhijab lebih tinggi daripada kelompok non-hijab. Memakai jilbab tidak meningkatkan keparahan ketombe, tingkat sebum, serta jumlah kolonisasi *Malassezia sp.* Memakai hijab meningkatkan tingkat TEWL.

Keywords: dandruff severity - *Malassezia sp* - sebum - TEWL - hijab

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INTRODUCTION

Dandruff, a mild form of seborrhoic dermatitis, is a common complain. It is estimated that half of adolescence population from various race and ethnicity are facing this problem.[1,2] The high prevalence of dandruff can be witnessed by many anti dandruff products on the market. However nowadays, there are still few medical interventions aimed at this problem.[3] The pathogenesis of dandruff is still unclear. Some theories are stated by experts such as dandruff caused by commensal bacterial and fungal such as *Malassezia sp.*, high level of sebum, high level of humidity and individual predisposition with the disturbed of skin barrier.

Hijab is commonly used by moslem women in Indonesia. Its importance showed with the finding of various products that are aimed for hair treatment of hijab users. In Indonesian NA-DFC (The National Agency of Drug and Food Control) page, we can find 17 products registered for hair treatment of hijab users and 350 registered products for dandruff.[4] The scalp of hijab wearing are considered to be more humid which is predicted to raise the dandruff risk through making a better environment for the growth of *Malassezia sp.* and lowering the barrier of stratum corneum. Indonesia has tropical climate with high humidity through out the year. Therefore, it is need to proof whether dandruff severity, as well as sebum and TEWL levels which reflects skin barrier and the number of *Malassezia sp*. on hijab wearing female play an important role. This data is needed for the correct choice of scalp treatment products type for hijab users who have dandruff.

MATERIALS AND METHODS

This study was conducted at Department of Dermatology and Venereology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. This study was a part of a clinical study of antidandruff shampoo products, and conformed to the ethical guidelines of the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.

Subjects

The subjects of this study were female who wear hijab as case group and subjects who do not wear hijab as control group. The subjects were matched according to age. The subject included total of 73 female, age between 18 and 40 years old with dandruff severity between 28-56 of Squire method or considered as mild seborrhoic dermatitis. All subjects have signed the informed consent to join the study. The study was started after all subjects underwent "wash-out" period using neutral shampoo without active ingredients for two weeks.[5] The inclusion criteria were healthy women age 18-40 years, aged matched between hijab and non hijab subjects. The exclusion criteria were pregnancy and lactating. The sample size were determined statistically as 31.

Scoring dandruff severity

The clinical examination to defined dandruff severity (according to Squire) was conducted by dermato-venereologist.[6] The degree of dandruff severity was defined according to dandruff size that attached on the scalp. Using comb and hair clip, strand of hair was separated to see and score the dandruff severity at ten scalp regions. The scores were averaged to defined the dandruff severity of the subject. Sebum level measurement was conducted on four predefined spots using the Sebumeter Courage Khazaka, and TEWL measurement was conducted using the Tewameter Courage Khazaka, the result scores then were averaged.

Microscopic examination

Microscopic examination of *Malassezia sp.* was conducted by attached an adherence tape that was cut with the size of 1 cm^2 on squama in the the dandruff regions for 5

seconds. The tape then released and attached on object glass and stained with KOH Parker, then seen under microscope with 1000 magnification. Spores number count per view field (VF) was conducted at 10 VF and the results were then averaged. *Malassezia sp.* colonization was conducted by scrapping skin using scalpel no. 15 and a transparent plastic with a hole in its middle (diameter of 3 mm). The skin scraps then were put on the object glass and covered with deck glass and transported to the laboratory to be cultured. The scrap result then was put inside eppendorf tube contained 1 mL of NaCl and then vortex for 1 minute. Using oshe that was dipped in the eppendorf tube, we performed swab on SDA medium + cycloheximide + chloramphenicol + olive oil then it was put inside incubator with temperature between 32 and 37 ^{o}C and kept between 3 and 10 days. After 3 - 10 days, the growth colony number were counted using computer programme Image J.

Statistical analysis

The differences of dandruff severity, sebum level, TEWL, and *Malassezia sp.* number between hijab and non-hijab groups were tested with statistical programme using the t-test. The correlation between dandruff severity, sebum level, TEWL, and *Malassezia sp.* number were statistically analyzed using the Pearson test, with significance was set at $p < 0.05$. The tests were conducted using statistical computer programme.

RESULTS

Characteristics of subjects

Thirty eight female subjects suffered from dandruff, consisted of 19 hijab wearing subjects and 19 non hijab wearing subjects with matched age were included in the study. The mean age for hijab wearing subjects was 23.9 ± 3.4 years old, and for non hijab wearing subjects was 24.1 ± 2.7 years old.

Dandruff severity, sebum level, TEWL, and *Malassezia sp*.

Hijab wearing subjects had lower dandruff severity (37.2) compared to non hijab wearing subjects (47.5), however it was statistically not significant ($p>0.05$). Sebum level of hijab wearing subjects (21.3) was found to be lower than non hijab wearing subjects (26.1) eventhough the difference was not statistically significant ($p>0.05$). Skin barrier measurement of hijab wearing subjects showed a higher TEWL level (19.2) compared to non hijab wearing subjects (15.8) which was statistically significant ($p<0.05$). The average number of *Malassezia sp.* per microscopic field view of hijab wearing subjects was 8.5, slightly higher than non hijab wearing subjects (8.4), however this difference was not significant. The colonies number of *Malassezia sp.*on subjects with dandruff who were wearing hijab (63.28) was higher than non hijab wearing subjects (12.56), however it was not statistically significant ($p>0.05$). More detailed information regarding the difference of dandruff severity, TEWL and sebum levels were presented in TABLE 1.

TABLE 1. The comparison of dandruff severity, sebum level, TEWL level, number of *Malassezia sp.* and colonization number of *Malassezia sp.* between hijab and non hijab wearing subjects

Variable	Hijab (mean ± SE)	Non-hijab (mean ± SE)	p
Dandruff severity	37.28 ± 2.46	47.50 ± 6.39	0.15
Sebum level (μg/cm2)	21.34 ± 3.21	26.12 ± 5.35	0.45
TEWL level (g/m2/h)	19.20 ± 0.87	15.82 ± 1.06	0.02
Number of Malassezia sp.	8.53 ± 2.21	8.44 ± 2.68	0.97
Colonization of Malassezia sp.	63.28 ± 26.47	12.56 ± 5.34	0.07

The correlation between dandruff severity and sebum level, TEWL, and *Malassezia sp*.

Analysis of all dandruff subjects found a weak negative correlation and statistically not significant between dandruff severity and sebum level (r = - 0.217; p = 0.205). A weak negative correlation and not statistically significant was also found between dandruff severity and TEWL level (r= - 0.163; p= 0.342). There was also a weak negative correlation and not significant found between dandruff severity and colonies number of *Malassezia sp.* (r= - 0.240, p= 0.164). A weak negative correlation and not statistically significant was also found between colonies number of *Malassezia sp.*and dandruff severity (r = -0.201; p=0.241).

More detailed information regarding the correlation between dandruff severity, sebum and TEWL levels and colonies number of *Malassezia sp*. of hijab and non hijab wearing subjects were presented in TABLE 2.

TABLE 2. The correlation between dandruff severity with sebum and TEWL levels of hijab and non hijab wearing subjects

The correlation between dandruff severity with	Hijab R=	Non-hijab R=
Sebum level	-0.15	-0.28
TEWL level	-0.25	-0.02
Number of *Malassezia sp.*	-0.34	-0.23
Malassezia sp. colonization	-0.27	-0.21

Correlation with p > 0.05

The correlation between TEWL and *Malassezia sp.*

Correlation test between TEWL level and colonies number of *Malassezia sp.*of hijab wearing subjects showed a moderate power of positive correlation but it was not statistically significant (r=0.423; p=0.08). A weak negative correlation and not statistically significant (r=- 0.226; p=0.367) was found in non hijab wearing subjects

DISCUSSION

Our study found that female population wearing hijab have no difference in term of dandruff severity with non hijab wearing subjects. Neither significant difference found for sebum level between hijab and non hijab wearing subjects nor for colonies number of *Malassezia sp*. Only an increase of TEWL level was found. It showed that there was decline of skin barrier on hijab wearing subjects with dandruff. In our study, a positive correlation with moderate power was found between TEWL level and colonization of *Malassezia sp*. TEWL is one of objective measurement to define the epidermal barrier power. The TEWL values are varied between different locations of human body surfaces and on each location. There are also different dominant factors that influence the TEWL level. Study by Liu *et al*. found that the dominant factor influenced TEWL on forehead area was temperature and humidity. The dominant factor on arm area that was covered by clothes, and on palm area the dominant factor was environmental air pressure.[7] TEWL level could increase with combination of pressure and fabric type. The fabric that is made from polyester can increase the TEWL level twice time than cotton fabric.[8] The use of hijab give risk to increase the pressure on scalp. The type of fabric used could increase the TEWL level, eventhough it is not closely related with dandruff severity. Our study showed a correlation of dandruff severity

with colonization of *Malassezia sp*. The increasing level of TEWL was a sign of reduction instratum corneum barrier that has risk to cause various of scalp disorders. The selection of appropriate fabric type and model of hijab that not cause much pressure on scalp are recommended.

There was a weak negative correlation which is not statistically significant between sebum level, TEWL level and dandruff severity in both hijab and non hijab wearing subjects. A study of subjects which consisted of 59 male and female with dandruff in Shanghai with age range between 18 and 60 years old, also found a negative correlation between dandruff severity and sebum level.[8]The controversy of sebum and *Malassezia sp.* role in dandruffis still exist until now. There are some factors that predicted to play a role in dandruff pathogenesis, included individual susceptibility, sebum level, and colonization of *Malassezia sp*. A study of scalp lipid showed that squalene peroxide was thought to be the factor that play a role indandruff pathogenicity to pass the skin barrier, besides the existence of *Malassezia sp.* which is thought to be the source of squalene peroxide.[9]A study in Chinese ethnic by Xu also found a negative correlation between dandruff and sebum and water levels in scalp, and there was no significant correlation found with *Malassezia sp.*. However there was a strong correlation found with increased number of Staphylococcus, and decreased number of Propionebacterium. It seems that the balance interaction between host and commensal bacteria plays a significant role in dandruff pathogenesis.[10,11]

Our study also showed that the sebum in our dandruff subjects hijab or non hijab still within normal level compared to the data of Thailand population (15 μg/cm^2),[12] while the TEWL from our study higher than normal level (13 g/m^2/h).[13] These findings support that dandruff as a mild form of seborrhoic dermatitis is a manifestation of skin barrier disfunction, which showed increasing of TEWL. Dandruff is divided in two types i.e. dry dandruff in which the scalp appears dry, with the flakes small and few, and oily dandruff related to abundant sebum production, in which flakes are thicker, larger and stuck together, forming oily, damp and yellowish patch or the real seborrhoic dermatitis.[13-15] The subjects of our study were female with dry dandruff. Selection of subjects with mild dandruffis probably caused normal level of sebum which consequently unable to show the correlation between sebum level and dandruff severity. The duration of hijab use was not considered in our study, as well as model and fabric type of hijab and individual sensitiviy factors that influenced dandruff severity such as immune response, neurogenic factor, emossional stress, and nutritional factor.

CONCLUSION

There is no difference of dandruff severity, sebum level and colonization number of *Malassezia sp.* between women wearing hijab and not wearing hijab. However, TEWL level is found to be higher in hijab wearing subjects.

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Cervical cancer screening coverage in urban and rural areas in Southeast Sulawesi: its determinants

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ABSTRACT

Cervical cancer is a woman's health problem that is still widespread throughout the world, especially in developing countries such as Indonesia. The high incidence of cervical cancer is related with early detection program. Visual inspection of acetic acid (VIA) is used as an alternative screening method because it is easier, cheaper and its effectiveness is not much different from the Pap test. There are differences on coverage of VIA test in urban and rural areas. This study aimed to identify determinants of cervical cancer screening coverage in urban and rural areas. The study was a cross sectional study. Total of 372 women who did the VIA test in urban areas of Kendari and rural areas of South Konawe in January to June 2016 were included in this study. Coverage of cervical cancer screening was examined in relation to the level of education, knowledge, distance of health facilities and family support. The data was analyzed using Chi-square test with a significance value $p < 0.05$. The majority of respondents were the age groupof 31-40 years old, low parity, and first intercourse at ≥ 17 years old. There were significant difference of education level (p=0.000), knowledge (p=0.000) and distance of health facilitiesin urban and rural areas (p=0.000). There was no significant differences between family support in urban and rural areas (p=0.224). In conclusions, education level, knowledge, and distance of health facilities are determinants of cervical cancer screening coverage in urban and rural areas in Southeast Sulawesi.

ABSTRAK

Kanker cervik adalah masalah kesehatan perempuan diseluruh dunia, terutama di Negara berkembang seperti Indonesia. Resiko tinggi terhadap kanker cervik berkaitan dengan program deteksi dini. *Visual inspection of acetic acid* (VIA) digunakan sebagai metode pemeriksaan alternatif karena mudah, murah, dan efektif, tidak berbeda jauh dari tes Pap. Terdapat perbedaan pada cakupan VIA yang digunakan di daerah urban dan pedesaan. Penelitian ini bertujuan untuk mengidentifikasi faktor penentu dari cakupan deteksi kanker cervik di daerah urban dan pedesaan. Penelitian ini merupakan penelitian *cross sectional.* Sebanyak 372 perempuan yang melakukan VIA test di Kendari (daerah urban) dan Konawe Selatan (daerah pedesaan) pada rentan bulan Januari hungga Juni 2016 berpartisipasi pada penelitian ini. Cakupan pemeriksaan kanker cervik diperiksa keterkaitannya antara tingkat pendidikan, pengetahuan, dukungan keluarga, dan jarak tempat tinggal dengan fasilitas kesehatan. Analisis data dilakukan dengan uji *Chi-square* dengan nilai signifikansi $p<0.05$. sebagian besar responden berumur 31-40 tahun, paritas rendah, dan berhubungan intim pertama kali pada ≥ 17 tahun. Hasil menunjukkan adanya perbedaan signifikan pada tingkat pendidikan (p=0.000), pengetahuan (p=0.000) dan jarak antara tempat tinggal dan fasilitas kesehatan (p=0.000). Tidak terdapat perbedaan yang signifikan pada dukungan keluarga pada daerah urban maupun pedesaan (p=0.224).

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Kesimpulan yang dapat diperoleh adalah tingkat pendidikan, pengetahuan, dan jarak tempat tinggal dan fasilitas kesehatan menentukan cakupan pemeriksaan kanker servik pada daerah urban dan pedesaan di Sulawesi Selatan.

Keywords: distance of health facilities - education - knowledge – cervical cancer - determinants

INTRODUCTION

Cervical cancer is a woman's health problem that is still widespread throughout the world, especially in developing countries such as Indonesia. In 2012, cervical cancer was the fourth most common cancer among women in worldwide. Around 528,000 new cases of cervical cancer were reported in 2012 and around 85% of cases occurred in developing countries. Around 266,000 women died of cervical cancer and around 87% of these deaths occurred in developing countries.[1] The high incidence of cervical cancer were related with early detection program. The incidence of cervical cancer in developed countries was fewer than in developing countries, due to public awareness for early detection and prevention program.[2-4]

The majority of cervical cancer case (about 70%) is diagnosed at an advanced stages (stage IIb or more).[5] The majority of cervical cancer patients come to the clinic when symptoms have appeared and usually advanced stage.[2,4] The treatment of advanced cervical cancer is more difficult, expensive, unsatisfied results and low life expectancy.[6]

The problem of cervical cancer in developing countries is associated with early detection program that still encountered many obstacles.[2,4] Some problems that were found such as facilities for cervical cancer screening, culture, family support, attitude, knowledge and education. Those problems affect difficulty to diagnose early cervical cancer.[2,4,7-9] Inadequate health care and public health infrastructure, competing health priorities, and poverty prevent gaining traction of prevention programs.[10]

Cytology-based screening strategies have been effective in reducing rates of cervical cancer in the United States and other developed countries. Cytology-based screening is infeasible in low-resource settings, because they need skilled personnel, laboratory equipment, transporting and storing of cervical specimens.[11]

Visual inspection of acetic acid (VIA) is used as an alternative screening test of cervical cancer especially in developing countries. Effectiveness of VIA is not much different from Pap test. Moreover, it is easier and cheaper than the Pap test. The VIA test can be performed in primary health care due to the very simple equipment needed. In addition, the results can be immediately provided because it does not require repeated request, wider coverage, and no expert screener is required to examine the samples.[4,11,12]

There are differences on coverage of VIA test in urban and rural areas in Southeast Sulawesi. There were 7.56% of women who conducted VIA test in Lepo-Lepo Healthcare in Kendari City. While in Landono Healthcare in South Konawe District, there were 26.3% of women who conducted VIA test.[13] This study aimed to identify determinants of cervical cancer screening coverage with VIA test in urban and rural areas in Southeast Sulawesi. The data would be used to plan constructively steps in preparing early detection programs of cervical cancer with VIA test. For community health services, this study is conducted as suggestion to make activities that improve service quality and increase promotion and preventive efforts.

MATERIALS AND METHODS

Sample specificity

This study was a quantitative research with cross sectional design. The population was women of childbearing age who used VIA test in Lepo-Lepo Healthcare in Kendari City and Landono Healthcare in South Konawe District, Southeast Sulawesi. The study was conducted in a period of January to June 2016. Samples were selected by consecutive sampling and were previously provided by informed consent. The inclusion criteria were patients aged 20-50 years old, married, and never had a hysterectomy. The exclusion criteria were incomplete questionnaires. Protocol of the study has been approved by the Research Ethics Committee of the Faculty of Medicine, Halu Oleo University, Southeast Sulawesi.

Data collection

Determinants of cervical cancer screening covered education background, knowledge, distance of health facilities and family support. The cervical cancer screening was the women who comes and performs cervical cancer screening by VIA test. Education was the level of formal education that has been followed. Knowledge was the ability to answer correctly related to the questions on knowledge of cervical cancer. Distance of health facilities was distance from women's house to service facilities of VIA test according to her perception. Family support was a family attitude given to perform early detection cancer with VIA test.

Statistical analysis

Data were presented as frequency and analyzed by SPSS for Windows version 16. The data were analyzed by Chi-square test with a significance value $p < 0.05$.

RESULTS

A total of 372 respondents involved in this study. The characteristics of respondents are presented in TABLE 1. The largest age group of respondents was 31-40 years old. The majority of respondents were low parity and first sexual intercourse at the age of more than 17 years old. The majority of respondents were workers. The family incomes of respondents in urban areas were greater than in rural areas.

TABLE 1.Characteristics of respondents

Characteristics	Urban		Rural	
	n	%	n	%
Age (years old)				
• 20 – 30	33	17.8	60	32.3
• 31 – 40	81	43.5	69	37.1
• 41 – 50	72	38.7	57	30.6
Parity				
• Low Risk	174	93.5	129	69.4
• High Risk	12	6.5	57	30.6
The age of first intercourse				
• < 17 years old	21	11.3	36	19.4
• ≥ 17 years old	165	88.7	150	80.6
Occupation				
• Work	99	53.2	129	69.4
• Housewife	87	46.8	57	30.6
Family income				
• Less	21	11.3	117	62.9
• Enough	165	88.7	69	37.1

TABLE 2 presented the characteristics of respondents respondents from urban and rural areas consisted education, knowledge, distance of health facilities, and family support. The majority of respondents in urban areas had high education (56.5%), while respondents in rural areas had basic education (46.8%). There were significant differences between the education levels of respondents in urban and rural areas (p=0.000). The majority of respondents in urban and rural areas had sufficient knowledge, but the majority of respondents who had good knowledge were in rural areas (37.0%). There were significant differences between the knowledge of respondents in urban and rural areas ($p = 0.000$). Distance of health facilities in urban areas ware close distance (59.7%) while the distance in rural

areas was long distance (69.4%). There were significant differences between the distance of health facilities in urban and rural areas (p=0.001). Respondents' family support in urban areas was 93.5% and in rural areas was 87.1%. There was no significant difference between family support in urban and rural areas (p=0.224).

TABLE 2. Education, knowledge, distance of health facilities, and family support of respondents from urban and rural areas.

Characteristics	Coverage of VIA test				p
	Urban		Rural		
	n	%	n	%	
Education					
• Basic	15	8.00	87	46.8	
• Middle	66	35.5	75	40.3	0.000
• High	105	56.5	24	12.9	
Knowledge					
• Lack	33	17.7	12	6.5	
• Sufficient	141	75.8	105	56.5	0.000
• Good	12	6.5	69	37.0	
Distance of health facilities					
• Close distance	111	59.7	57	30.6	0.001
• Long distance	75	40.3	129	69.4	
Family support					
• Give support	174	93.5	162	87.1	0.224
• No support	12	6.5	24	12.9	
Total	186	100	186	100	

DISCUSSION

This study investigated women who live in urban and rural areas in the Southeast Sulawesi. The largest age group of respondents who performed VIA test were 31-40 years old. This result was similar to previous studies that found the percentage who do not perform cervical cancer screening in the last 5 years was higher in women aged 23-29 years old and 60-65 years old.[14]

The majority of respondents were low parity and first sexual intercourse at the age of more than 17 years old. Although it was classifiedas low risk, however the VIA test was still needed to be conducted. There are several risk factors of cervical cancer, such as having sexual intercourse at a young age and high parity.[1,2,15] Those who have been active sexually before the age of 16 years old have a risk of cervical cancer by 10-12 times greater than those aged 20 years old and over. Those who have children in the number above 5 will often experience trauma of the cervix, making it easier to change mucosal cells to be abnormal and closely related to the incidence of cervical cancer.[16]

VIA is used as an alternative screening and more encouraged, especially in developing countries. VIA is low-cost screening technologies.[17] Respondents come from various segments of community.[4] Respondents in this study have sufficient and less family income. This shows that the VIA test is an affordable examination. VIA test did not need much money and it was cheaper.[2,4] This is unlike the results of previous study which find that the higher income level would get, the better practice of Pap's smears would perform.[18] This condition is caused by Pap's smears test is more expensive than the VIA test. Economic factors contribute significantly to practice on VIA test. The ability to choose health care related to economic status. The higher income level of the community had, the better health practice of the community would get.[19] Previous research in five countries found that using the VIA test carried out together at the time of cervical examination can reduce the incidence of cervical cancer and reduce costs.[12,20]

The majority of respondents in this study were workers. The result was similar to the previous studies, it showed that employment did not affect VIA test.[7] It shows that the VIA test is easier to do because it can be done at primary health care, so that working women do not spend much time to leave their work.

In this study, most respondents in urban and rural areas have enough knowledge

about cervical cancer and VIA test. This study reported that rural respondents have more good knowledge of cervical cancer than urban respondents. Knowledge is something known or recognized. Knowledge is obtained by performing empirically and rationally, it can also be obtained through a reasonable and logical thinking mind. Knowledge is influenced by factors of education, media, and information.[18] Information and education can improve the knowledge so it contributes to the success of early detection program in rural areas. This result is similar to the previous studies that find the relationship between knowledge about cervical cancer with participation of VIA test.[7]

This study showed that respondents in rural areas had good knowledge of cervical cancer. It showed that health information and education had been successfully conducted in rural areas. Working area system of health center has made easier for officers to socialize and provide health information and education to the community. Although they have low education level, but good and intensive information both formal and informal will improve public knowledge. This study found that education level did not affect knowledge and VIA respondents.[7] Knowledge of urban communities may be derived from self-generated information, and it is because the urban communities are busier than rural communities, so health information and education from officers cannot beperformed intensively.

Coverage of VIA testis related to distance of residence and health center. VIA more accessible because it can be done in primary health center.[2,6] Practice is affected by the ease of access to health services. Access to health services is related to the distance of the health center. The closer the distance between recipients and service providers, the tendency to choose health services is higher.[2,4] Tran *et al*. found that women in rural areas were reluctant to screen for cervical cancer because remote medical centers required more time and cost for screening.[21]

Practice was influenced by family support. Family support affected the tendency to choose practice that were appropriate for family support.[8,18] This study found that the majorityof respondents in urban and rural areas had good family support. This result was similar to the previous studies that found the relationship between family support and interest in conducting VIA test.[8] Family support might be affected by environmental factors. The social environment has a profound influence on health status, such as health beliefs, traditions, social and cultural interaction. According to Blum, the environment is one of the determinants of health status. Social and cultural environment influences the health status and health practice. Cultural factors contribute to disparities in health care.[18]

Health practice can be influenced by internal and external factors. Similarly, the practice of cervical cancer screening is influenced by internal and external factors, which are predisposing factors, enabling factors and reinforcing factors.[18]

CONCLUSION

Based on the results study, we concluded that education level, knowledge, and distance of health facilities are determinants of cervical cancer screening coverage in urban and rural areas in Southeast Sulawesi. There are differences of education level, knowledge, and distance of health facilities for VIA test in urban and rural areas. There is no difference of family support for VIA test in urban and rural areas.

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Comparison of corneal endothelial cell count and intraocular pressure in pure-dispersive and dispersive-cohesive viscoelastic protection in phacoemulsification surgery

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ABSTRACT

There are so many aspects should be regarded when use viscoelastic device during phacoemulsification surgery. The advantages and disadvantages of pure-dispersive viscoelastic and dispersive-cohesive viscoelastic always require more our attention to use it conveniently. The purpose of the study was to compare between pure-dispersive viscoelastic versus dispersive-cohesive viscoelastic in phacoemulsification surgery in that of corneal endothelial cell count and intraocular pressure (IOP) change. This was a cross-sectional study involving 41 eligible patients who underwent phacoemulsification surgery by single operator. Data including characteristics of cataract patients, corneal endothelial cell count and IOP were taken before and after surgery. Data of characteristics of cataract patients were reported descriptively and compared using Anova and t-test. The mean change in corneal endothelial cell count on pure-dispersive viscoelastic group (71.99±71.20 cells/mm²) was lower than that on the dispersive-cohesive viscoelastic group (117.62±78.29 cells/mm²). However, it was not significantly different. The mean change in IOP on pure-dispersive viscoelastic group (0.75±1.626 mmHg) was significantly lower than that on dispersive-cohesive viscoelastic group (1.90±0.995 mmHg) (p=0.000). In conclusion, the increase of IOP in dispersive-cohesive viscoelastic group is higher than that on pure-dispersive viscoelastic group. However, there is no significant difference of the mean change in corneal endothelial cell on the both groups.

ABSTRAK

Banyak aspek yang harus diperhatikan ketika menggunakan peralatan viskoelastis selama tindakan bedah fakoemulsifikasi. Kelebihan dan kekurangan viskoelastis murni dispersif dan viskoelastis dispersif kohesif selalu memerlukan perhatian agar dapat digunakan dengan nyaman. Penelitian ini bertujuan untuk membandingkan viskoelastis murni dispersive dengan viskoelastis dispersif kohesif pada tindakan bedah fakoemulsifikasi dengan membandingkan perubahan jumlah sel endotel kornea dan tekanan intraokuler. Penelitian ini merupakan penelitian potong lintang yang melibatkan 41 pasien yang menjalani tindakan bedah fakoemulsifikasi yang memenuhi syarat oleh operator tunggal. Data yag meliputi karakteristik pasien katarak, jumlah sel endotel kornea dan tekanan intraokuler diambil sebelum dan sesudah pembedahan. Data demografi dilaporkan secara diskriptif dan dibandingkan dengan Anava dan uji t. Rerata perubahan jumlah sel endotel kornea pada kelompok viskoelastis murni dispersif (71,99±71,20 sel/mm^2) lebih rendah daripada kelompok viskoelastis dispersif kohesif (117,62±78,29 sel/mm^2), namun tidak berbeda nyata (p>0,05). Rerata perubahan tekanan intraoculer pada kelompok viskoelastis murni dispersif (0,75±1,626 mmHg) lebih rendah secara nyata daripada kelompok viskoelastis dispersif kohesif (1,90±0,995 mmHg) (p<0,05). Dapat disimpulkan, kenaikan tekanan intraokuler pada kelompok viskoelastis dispersif kohesif lebih tinggi daripada kelompok

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viskoelastis munri dispersif. Namun demikian, tidak terdapat perbedaan nyata rerata perubahan jumlah sel endotel kornea pada kedua kelompok.

Keywords: phacoemulsification - corneal endothelial cell - intraocular pressure – visco-elastic – pure-dispersive - dispersive-cohesive

INTRODUCTION

Age related cataract is believed to be the main cause of reversible blindness worldwide especially in developing countries.[1] It is estimated that blindness due to cataract is increasing by 1-2 million annually.[2] About 20 million individuals suffer from blindness due to cataracts[3] and 80% of them live in the developing countries.[4]

One of the most known treatment for cataract is phacoemulsification surgery. Phacoemulsification is safer, has faster rehabilitation, as well as less astigmatism and better postoperative vision compared to conventional extra capsular cataract extraction (ECCE).[5] It has largely replaced manual nucleus extraction (MNE) as the procedure of choice for cataract surgery.[6] However, there is an important factor in phacoemulsification procedure that needs to be considered when performing cataract surgery; the effect of viscoelastic, or also known as ophthalmic viscosurgical device (OVD) on the corneal endothelium.

There are two types of OVDs i.e. cohesive OVDs and dispersive OVDs. Both are used for different occasions. For example, a cohesive OVD could be selected to expand a small pupil, while a dispersive OVD could be used to protect an eye with a compromised corneal endothelium.[7] Eventhough some surgical strategies use two OVD types together in layers or serially,[8] the ability to protect endothelium and avoidance of intraocular pressure (IOP) spikes are the important factors that need to be considered in selecting an OVD.

The endothelium-protecting efficacy of an OVD can be evaluated in terms of postoperative measurements of endothelial cell density. The loss of endothelial cell during surgery or the postoperative phase can deteriorate at a faster-than-normal rate for at least 10 years thereafter.[9] If the normal endothelial cell density of ~2400 cells/mm2 falls below 300–500 cells/mm2, corneal edema can develop, and can be followed by bullous keratopathy.[10] Rheological properties indicate that a dispersive OVD, with its propensity to coat and protect intraocular tissues, might be a better choice for endothelial protection.

While we need OVDs capability to completely coat and protect intraocular tissues during surgery, an ideal OVD should also be able to be completely removed from intraocular tissues at the end of surgery. Residual OVD left in the eye can clog the trabecular meshwork, leading to a transient elevation in postoperative IOP.[11-13] This ocular hypertension sometimes need to be treated with IOP reducing medication, such as prophylactically in response to postoperative observations of IOP spikes to ≥30 mmHg[14] or ≥35 mmHg.[15] To avoid this complication, surgeons need to select an OVD that is conducive to complete removal. Rheological properties indicate that a cohesive OVD, with its propensity to be removed as a bolus, might be better than a dispersive OVD for avoiding IOP spikes.

The considerations of choosing the best OVDs sometimes work at cross purposes; no single OVD is a clear choice. The purpose of this study was to compare pure-dispersive viscoelastic and cohesive-dispersive viscoelastic in phacoemulsification surgery in term of corneal endothelial cell count and intraocular pressure change.

MATERIALS AND METHODS

Subjects

This was a cross-sectional study involving 40 eligible patients who underwent phacoemulsification surgery by single operator at the Department of Ophthalmology,

Dr. Sardjito General Hospital, Yogyakarta. Data including characteristics of cataract patients, corneal endothelial cell count and intraocular pressure were taken before and after surgery. Corneal endothelial cell count was determined using specular microscope and intraocular pressure was determined using non-contact tonometer. The study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.

Procedures

All the patients eligible to participate had to give inform consent and fill the questionnaire then followed by eye examination including visual acuity, anterior segment examination using slit-lamp, specular microscope and tonometry. Phacoemulsification surgery was performed by single operator. Measurements were performed two times i.e. before and after surgery.

Statistical analysis

Characteristics data of patients were reported descriptively. Proportion data was determined using Chi-square test. Comparison of corneal endothelial cell count and IOP change was calculated using Anova and t-test. All data analyses were performed with a commercial statistical software package (SPSS 16.0 for windows)

RESULT

A total of 41 (20 with pure-dispersive viscoelastic, 21 with cohesive-dispersive viscoelastic) patients were examined. The characteristics data of patients showed that both of two groups were similar in that of age, sex, grade of cataract, pre-op-corneal endothelial cell count but not in pre-op-intraocular pressure (TABLE 1.)

TABLE 1. Characteristics of subjects

Variable	Pure-dispersive viscoelastic (n= 20)	Cohesive-dispersive viscoelastic (n= 21)	p
Age (mean ± SD year)	61.05±13.7	64.76±12.9	0.378
Sex [n (%)]			
• Male	9 (45)	12 (57.1)	0.437
• Female	11 (55)	9 (42.9)	
Grade [n (%)]			
• 2	11 (55)	5 (23.8)	0.272
• 3	7 (35)	10 (47.6)	
• 4	2 (10)	6 (28.6)	
Endothelial count (mean ± SD cells/mm^2)	2961.05±178.21	2942.38± 133.00	0.705
IOP (mean ± SD mmHg)	17.40±1.93	15.61±1.68	0.003

IOP: intraocular pressure

TABLE 2 revealed that the mean change in corneal endothelial cell count on pure-dispersive viscoelastic group was 71.99±71.2 cells/mm², whereas that in the cohesive-dispersive viscoelastic group was 117.62±78.29 cells/mm^2. No significantly difference in the change of corneal endothelial of both groups was observed (p=0.056). The mean change in IOP on pure-dispersive viscoelastic group was 0.75±1.626 mmHg, whereas that in cohesive-dispersive viscoelastic group was 1.90±0.995 mmHg. Significantly difference in the change of IOP of both groups was observed (p=0.000).

TABLE 2. Change in that of corneal endothelial cell count and IOP pre- and post phacoemulsification surgery in both groups

Variable	Pure-dispersive viscoelastic (n= 20	Cohesive-dispersive viscoelastic (n= 21)	p*
Endothelial cell count (mean ± SD cells/mm^2)			
• Pre-	2961.05± 178.21	2942.38± 133.00	0.056
• Post-	2889.80±204.23	2824.80±165.60	
Change	71.99±71.2	117.62±78.29	
IOP (mean ± SD mmHg)			
• Pre-	17.40±1.93	15.61±1.68	0.000
• Post-	18.15±1.83	17.51±1.70	
Change	0.75±1.626	1.90±0.995	

IOP: intraocular pressure; *Anova

Change in that of corneal endothelial cell count and IOP in each group are presented in FIGURE 1 and FIGURE 2.

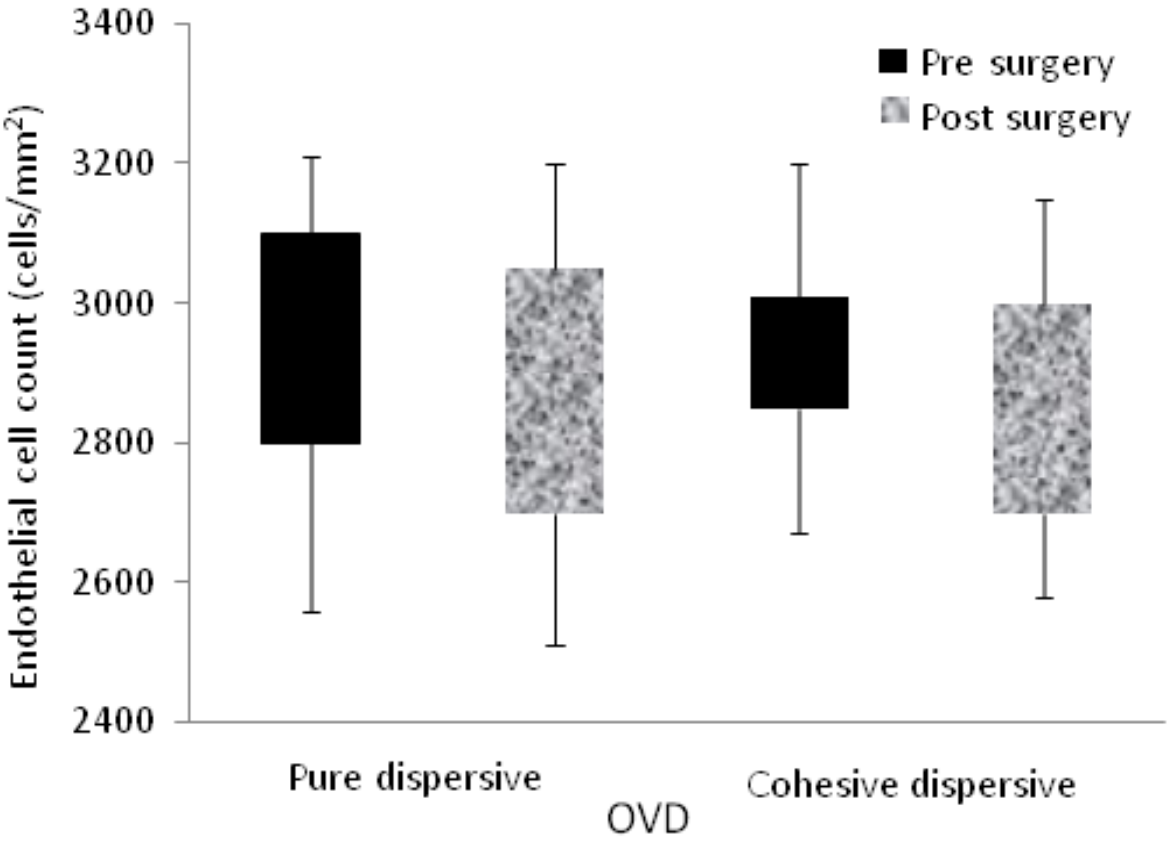

FIGURE 1. Change in the endothelial cells count pre- and post- phacoemulsification surgery in both groups

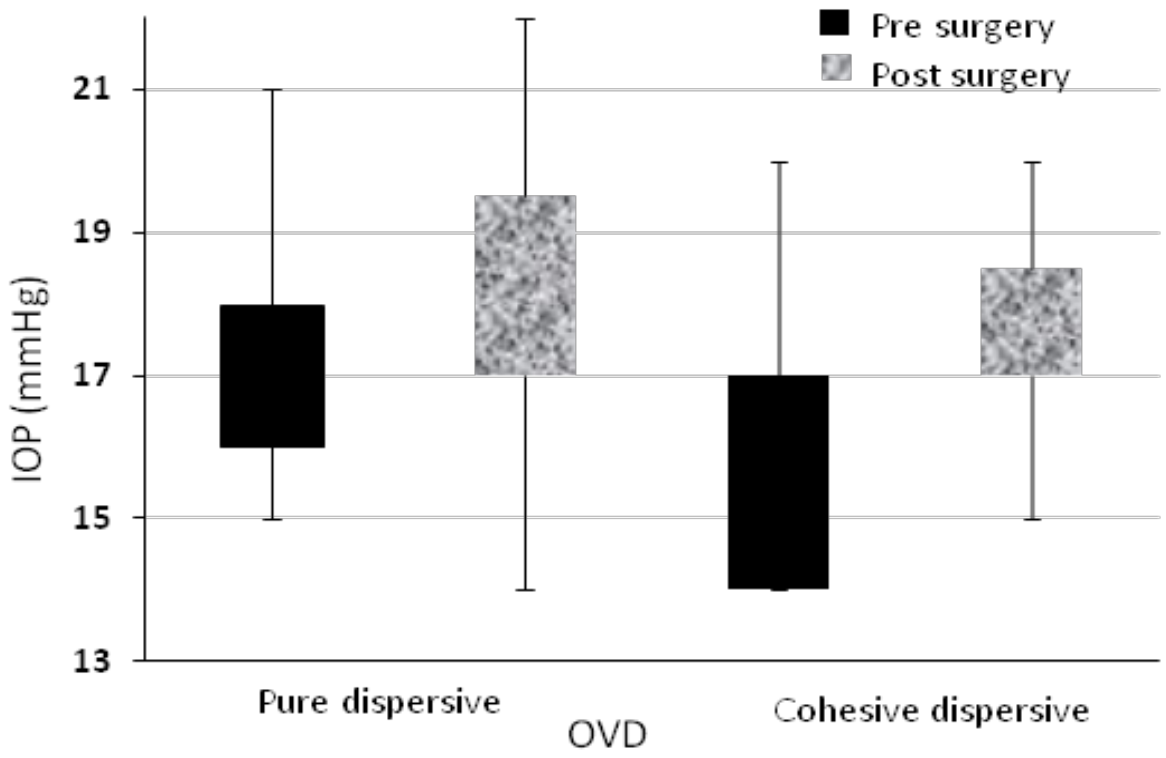

FIGURE 2. Change in the IOP pre- and post phacoemulsification surgery in both groups

DISCUSSION

OVDs facilitate cataract surgery by maintaining the depth and shape of the anterior chamber. This provides a workspace for the surgeon and viscous barrier that protects the delicate corneal endothelium from surgical instruments, from cataractous lens debris, and the intraocular lens during insertion.[7] Early OVDs were classified as either cohesive or dispersive, on the basis of objective rheological properties.[16] Cohesive OVDs are useful in creating and maintaining space in the anterior chamber.[7] Because cohesive OVDs tend to hold together as a mass, they are relatively easy to remove as a bolus at the end of surgery.[7] In contrast to cohesive OVDs, dispersive OVDs spread out when injected into the eye, making these substances less effective for maintaining space but more effective for coating and protecting intraocular tissues.[17] Irrigation/aspiration tends to pull away bits and fragments of dispersive OVDs, making these materials more difficult to remove at the end of surgery.[7,17] The different properties of cohesive and dispersive viscoelastics broaden the opportunities for a surgeon's selection of an OVD for cataract surgery.

The rheological properties of any OVD arise from the monomer type and polymer formulation of its constituents. In some cases, these constituents provide not only physical protection, but also chemical protection. Cohesive-dispersive viscoelastic contains two biologically relevant glycosaminoglycans: 1.6% hyaluronic acid (also found in connective tissues) and 4% chondroitin sulfate (also found in cartilage). Both chondroitin sulfate and hyaluronic acid are antioxidants. During an in vitro simulation of phacoemulsification, an OVD containing 3% chondroitin sulfate and 4% hyaluronic acid suppressed free radicals significantly more than an OVD containing 2.3% hyaluronic acid alone. The relative chemical and rheological protective effects of various OVDs are not yet fully understood.

With regard to the protection of endothelial cells in the current study, the percentage cell loss with the pure-dispersive viscoelastic group was lower than that with the cohesive-dispersive group although it was not significantly different. It is not clear

whether the endothelial protection provided by the OVDs was due to rheological properties, to chemical /antioxidant content, or to a combination of both. Protection could be related to endothelium-coating properties of the OVDs. Modi *et al*.[18] reported their two studies using animal eyes that a thin uniform layer of DisCoVisc OVD remained as a lining on the inner cornea after phacoemulsification and removal of OVD. It was suggested that this coating was indicative of the protective effects of the DisCoVisc OVD.

Rainer *et al*.[19] revealed the mechanism of postoperative IOP increase was not yet fully understood. A major reason for the postoperative IOP increased seems to be the amount of the remaining viscoelastic agent at the end of surgery. It was assumed that the remaining viscoelastic agent mechanically obstructs the trabecular outflow pathway and hence decreases the outflow facility. In order to avoid a postoperative IOP increase, a thorough removal of viscoelastic agent is vital. Surgical techniques for the removal of viscoelastic substances, especially from behind the IOL, have been described, but a complete prevention of a postoperative IOP increase could not be achieved with any technique.

Both of two types of viscoelastic must be removed out completely. However, it was nearly impossible to completely remove both viscoelastic agents without injuring the endothelium and other vulnerable structures of the eye. Assuming that the amounts of the remaining viscoelastic substances were similar in our study, the difference in postoperative IOP increase between the two viscoelastic agents might be explained by differences in their biophysical properties. The clearance of the viscoelastic agent through the trabecular meshwork is believed to be dependent upon the viscosity and molecular weight of the used materials. Theoretically, the lower the viscosity and the molecular weight of the viscoelastic agent, the faster is the clearance through the trabecular meshwork. In accordance with this theory, in our study "dispersive" which is less viscous and has a lower molecular weight than "cohesive" caused less IOP increasing. The lower viscosity of "dispersive" compared with "cohesive" may, however, have the disadvantage of poorer endothelial cell protection.

CONCLUSION

This study revealed that the increase of IOP in cohesive-dispersive viscoelastic group is higher than that in pure-dispersive viscoelastic group. However, the change in corneal endothelial cells is similar in both groups.

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Precocious puberty in McCune-Albright syndrome: a case report

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ABSTRACT

McCune-Albright syndrome (MAS) is a rare disease characterized by a triad of fibrous dysplasia, *cafe-au-lait* spots and peripheral precocious puberty. We reported a 5-year-8-month old girl with MAS who has been followed-up for 2 years and 8 months. She was referred to pediatric endocrinology clinic in our hospital for vaginal bleeding at age of 2 years 11 months. She had peripheral precocious puberty, i.e. increased estrogen level associated with very low gonadotropins, and *cafe-au-lait* spots on her face and was diagnosed as MAS. The patient was treated with estrogen receptor blocker (tamoxifen). She had no menses during the 2 years and 8 months of tamoxifen treatment. Her growth rate and bone maturation were also in normal ranges. However, at the end of tamoxifen treatment she had an episode of vaginal bleeding so that we had to change to other treatment modalities.

ABSTRAK

Sindrom McCune-Albright merupakan penyakit langka yang ditandai dengan trias displasia fibrosa, makula *cafe-au-lait* dan pubertas prekok perifer. Kami melaporkan anak perempuan usia 5 tahun 8 bulan yang telah kami amati selama 2 tahun 8 bulan. Pasien datang ke poliklinik endokrinologi di rumah sakit kami dengan keluhan perdarahan pervaginam pada usia 2 tahun 11 bulan. Ia mengalami pubertas prekok perifer yang ditandai dengan peningkatan kadar estrogen dan rendahnya kadar gonadotropin,, makula *cafe-au-lait*, dan didiagnosis dengan sindrom McCune-Albright. Pasien diterapi dengan penghambat reseptor estrogen (tamoxifen). Selama terapi, siklus menstruasi terhenti, kecepatan pertumbuhan dan maturasi tulang dalam batas normal. Namun pada akhir terapi dengan tamoxifen, pasien kembali mengalami perdarahan pervaginam sehingga kami harus mengganti dengan modalitas terapi yang lain.

Keywords: McCune-Albright syndrome - precocious puberty – tamoxifen – genetic disorders - gonadotropins

INTRODUCTION

McCune-Albright syndrome (MAS) is a genetic disorder characterized by abnormalities in skin pigmentation, endocrine system, and bone growth due to the GNAS (guanine nucleotide binding protein, alpha stimulating*)* gene mutations.[1,2] Prevalence of the disease ranged from 1:100.000 to 1:1.000.000 worldwide.[3] Classically, this syndrome presents with triad of fibrous dysplasia, typical skin pigmentation (*cafe-au-lait*), and endocrinopathies, including precocious puberty, hyperthyroidism, Cushing's syndrome, growth hormone excess, hyperprolactinemia, hyperparathyroidism, and/or rickets or osteomalacia.

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Precocious puberty was the most common endocrinopathy found in MAS. It occurs in 64-79% of girls and 15% of boys.[4] Aromatase inhibitors, e.g. testolactone, fadrazole, anastrozole, and letrozole, selective estrogen receptor modulators, e.g. tamoxifen, analogue of gonadotropin-releasing hormone (GnRH), and surgery were, so far, the suggested modalities to manage precocious puberty associated with MAS.[5] However, due to the rareness of the disease, the experience of using any of the above modalities are very limited. In this case report, we present a 2-years-8-month follow-up of tamoxifen use in a patient with peripheral precocious puberty of MAS.

CASE

A 2-years-11-month-girl, presented to pediatric endocrinology clinic at Sardjito General Hospital due to vaginal bleeding. The bleeding last for 2-3 days. On physical examination, we found breast enlargement (Tanner stage 2) and *cafe-au-lait* spots on her face. The child's height was 87,0 cm (plotted at -1.8 SD on the WHO child growth standard 2006). We observed low levels of gonadotropins, i.e. luteinizing hormone (LH) <0.1 mIU/mL (normal values: 2.4-12.6 mIU/mL, follicle-stimulating hormone (FSH) of 0.109 mIU/mL (normal values: 3.5-12.5 mIU/mL) and increased level of estradiol (635 pg/mL, normal values: 6-20 pg/mL). Bone age, assessed by Greulich-Pyle method, was equivalent to 2 years old. Pelvic ultrasound examination revealed post pubertal uterine size (2.57 x 2.67 x 1.73 cm), however, the ovaries were unvisualized. There was no history of pathological fracture. Based on the presence of peripheral precocious puberty and *cafe-au-lait* spots on the face, patient was diagnosed as MAS.

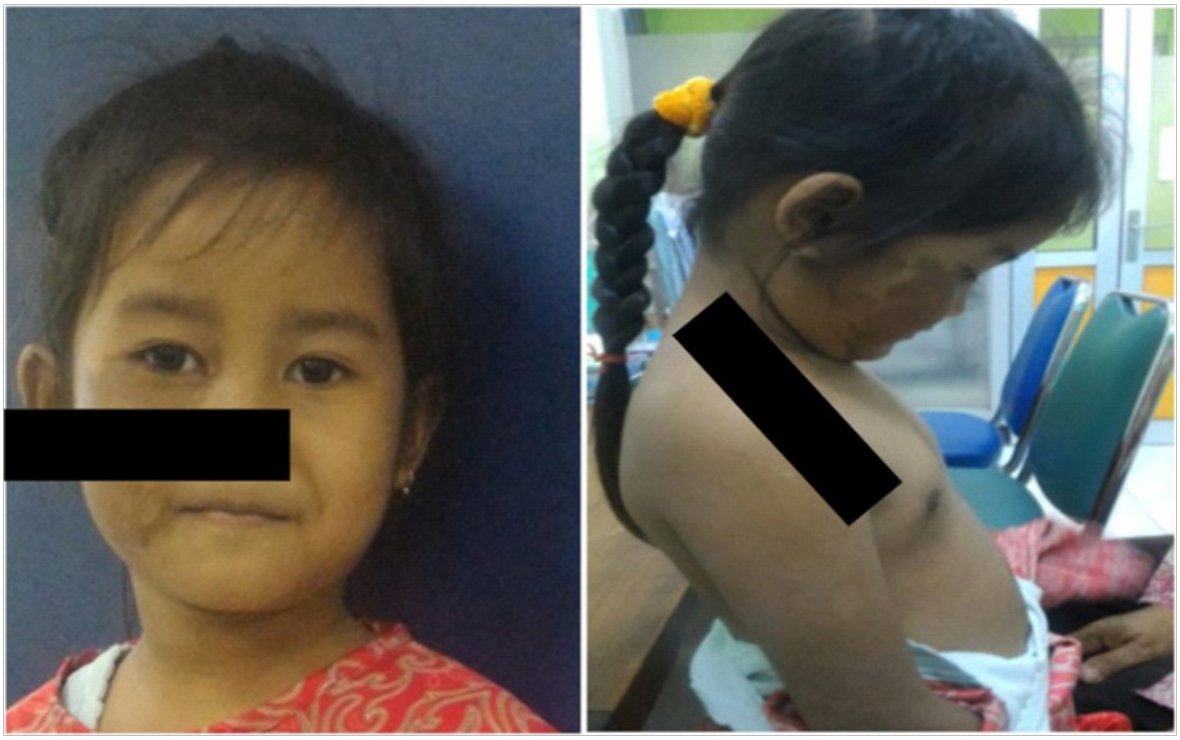

FIGURE 1. *Cafe-au-lait* skin pigmentation on the face

Treatment with tamoxifen (20 mg/day) was started immediately following the diagnosis. The menstrual bleeding was ceased shortly after the initiation of therapy. The serum estradiol level measured one year after the treatment declined to less than 5 pg/mL. Pelvic ultrasound examination carried out one year after the treatment showed smaller uterine size (2.71 x 2.21 x 1.20 cm). The ovaries were also unvisualized.

After 2 years and 8 months of regular treatment with single dose of tamoxifen 20 mg/day, the child had an episode of vaginal bleeding and breast enlargement, accompanied by pubic hair growth (Tanner stage 2). Evaluation of endocrine function observed low levels of gonadotropins (LH 0.1 mIU/mL, FSH 0.8 mIU/mL) and normal level of estradiol (5 pg/mL). Bone age was equivalent to 5 years old, which was still in-line for chronological age of 5 years and 8 months. On pelvic ultrasound examination, the uterine size increased compared to the last evaluation (3.79 x 2.17 x 1.96 cm), and the ovaries were still unvisualized.

The child has been growing well. Currently, at the age of 5 years and 8 months, she was 112.0 cm in height and 19.0 kg in weight (equal to -0.8 SD and -0.5 SD, respectively, on the WHO growth standard 2006), and has achieved Tanner stage of B2 and P2. The growth velocity was 7.5 cm/year during the last 2 years (95% confidence interval of growth velocity in pre-pubertal girls is 5.1-9.3 cm/year). During the observation, no other endocrinopathies that often accompany MAS (Cushing's syndrome, hyperprolactinoma, hyperthyroidism, and growth hormone excess) were observed. Thyroid ultrasound, and TSH and FT4 levels were normal. There were no bone abnormalities suggesting fibrous dysplasia. No pathological fracture was observed, but at the age of 4 years there was an open second metatarsal fracture on the left foot, coincidently due to motorcycle accident. The fracture completely healed without deformity or gait impairment after surgical treatment of open reduction and internal fixation.

DISCUSSION

MAS is characterized by a triad of fibrous dysplasia, *cafe-au-lait* spots, and precocious puberty. Other endocrinopathies such as hyperthyroidism, growth hormone excess, Cushing's syndrome, hyperprolactinemia, and rickets or osteomalacia may also occur, as well as other involvement of the liver, parathyroid, pancreas, and heart.[6] MAS is associated with mutation in the GNAS1 gene, which is mapped to chromosome 20q13.3. The protein product is involved in G-protein signaling.[2] The mutation in the GNAS gene occurs randomly during pregnancy and will result in mosaic of normal and mutated cells.[1,7] The manifestation and the severity of this disease depends on the number and the location of the cells expressing the mutated gene. The mutation influences activity of the related organ system at the level of interaction between hormones and receptor.[2]

Precocious puberty is the most common endocrinopathy found in MAS. Precocious puberty affects more girls than boys, characterized by premature vaginal bleeding. The precocious puberty was peripheral in origin, that is the hypothalamic-pituitary-gonadal axis was not active. The pathogenesis involves autonomous activation of ovarian tissue lead to estrogen hypersecretion. However, progression into central precocious puberty may also occur.[5,8]

Therapeutic options include the use of anti-estrogen (aromatase inhibitor, e.g. testolactone, fadrozole, anastrozole, andletrozole), estrogen receptor inhibitor (e.g. tamoxifen), analogue of GnRH (if there is a progression to central precocious puberty), and surgery (unilateral oophorectomy, cystectomy).[5] A multicenter study on the use of tamoxifen for precocious puberty in girls with MAS for one year has shown a decrease in vaginal bleeding episodes and significant improvements of growth velocity and bone maturation.[9]

We treated our patient with tamoxifen and observed good response during the first and second year of treatment. She had no menses, and her growth rate and

bone maturation were in normal ranges. However, on third year (after 2-years-and-8-month treatment) she suddenly had new episode of vaginal bleeding and breast enlargement, accompanied by pubic hair growth, despite regular treatment with tamoxifen. In fact, the patient had good adherence to the treatment, as we evaluated using the Medication Morisky Adherence scale (MMAS-8).[10] We decided to change to other treatment modalities since, to the best of our knowledge, there is no study so far on the safety of using higher dose of tamoxifen. Hormonal evaluation after the vaginal bleeding revealed no increase in gonadotropin level, therefore, progression to central precocious puberty can be ruled out.

Nunez *et al.*[11] reported that fadrazole was not effective for the treatment of precocious puberty and causing adrenal insufficiency. Meanwhile, Feuillan *et al.*[12] reported that testolactone may be used as a therapy for precocious puberty since it leads to a decrease in ovarian volume and estrogen concentration. However, one study[13], which evaluates the long-term therapy of testolactone, shows some patients have persistent symptoms of puberty, suggesting incomplete inhibitory effects of estrogen production. Other study[14] indicates that letrozole is an effective medication for precocious puberty in MAS. Recently, Estrada *et al.*[15] shows that letrozole has good long-term effect for precocious puberty in MAS in reducing episodes of menstruation and keeping the skeletal maturation and growth velocity so that predicted adult height can be achieved.

Based on those recent studies on the long-term effects letrozole in girls with precocious puberty in MAS, we changed the medication for our patient to letrozole following the failure of two-year-treatment with tamoxifen. We started the treatment with letrozole at dose of 1.25 mg/day since March 2017, and continuing to observe the result.

CONCLUSION

The diagnosis of MAS must be kept in mind in cases with gonadotropin-independent precocious puberty. Radiologic and laboratory assessments should be performed in order to investigate the presence of accompanying endocrinological and non-endocrinological disorders. A careful clinical observation and follow up of patients with mentioned clinical presentations is necessary.

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