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PPARγ expression by rambutan peel extract in obesity rat model-induced high-calorie diet

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ABSTRACT

Objective: To monitor the physiological characteristics and genes expression of obesity rat model after rambutan peel extract (RPE) treatment.
Methods: Twenty-four 12-week-old male rats were divided into 4 groups: normal, obesity, obesity treated with ellagic acid (O-EA) and obesity treated with RPE30 (O-RPE30). Physiological characteristics were monitored by measuring body weight, calorie intake, size of adipocyte and level of triglyceride. Peroxisome proliferator activated receptor gamma (PPARγ), CCAAT/enhancer-binding proteins α and fatty acid binding protein 4 (FABP4) expression were observed using immunohistochemistry, Western blotting and quantitative RT-PCR methods.
Results: Body weight gain of O-EA and O-RPE30 rats were lower than obesity group and size of adipocyte cells were smaller than obesity group ($P < 0.05$), but when we compared to normal group, those groups had higher body weight gain and larger adipocyte cells. The level of triglycerides, protein expression of PPARγ and mRNA level of *FABP4* genes were significantly downregulated on O-EA and O-RPE30 compared to obesity group ($P < 0.05$). Our results indicated that RPE had potential substance as inhibitor of body weight gain, declining of size of adipocyte, level of triglycerides, PPARγ expression and mRNA level of *FABP4* gene on obesity rat model.
Conclusions: RPE have anti-obesity activity by inhibiting body weight gain, declining size of adipocyte, decreasing triglyceride, PPARγ expression and mRNA level of *FABP4* gene on obesity rat model.

1. Introduction

High fat and carbohydrate diet has an important role in the stimulation of pre-adipocytes maturation and the up-regulation of adipogenesis genes [1,2]. High carbohydrate and fat diet will stimulate some growth factors to be released, such as platelet derivate growth factor, vascular endothelial growth factor and insulin like growth factor-1 (IGF-1) [3–7]. IGF-1 has an important role in pre-adipocytes maturation both *in vivo* and *in vitro* [8,9]. Activation of IGF-1 stimulates mitogen-activated protein kinase (MAPK) and genes cascade pathway which have role in adipogenesis [1,10,11]. The activation of IGF-1 will induce the activation of extracellular regulated kinase 1-2 (ERK1-2) and MAPK members. These proteins will initiate the expression of CCAAT/enhancer binding protein β (C/EBPβ) that is important to induce the expression of CCAAT/enhancer binding protein α (C/EBPα)

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and peroxisome proliferator activated receptor γ (PPARγ) [12]. The expression of C/EBPα and PPARγ provide the expression of fatty acid binding protein 4 (FABP4), lipoprotein lipase and 1-acylglycerol-3-phosphate O-acyltransferase proteins that have the same role in pre-adipocytes maturation [13]. Obesity is the condition when adipocyte tissue is increased by cell proliferation or cell enlargement [14]. Obesity is related to high intake of fat and carbohydrate that enter into body. Inhibition of adipocyte maturation is important way to prevent obesity development. Early process inhibits MAPK activity by specific compound to protect adipogenesis [15,16]. Various specific compounds from plants can reduce the activity of various lipase enzymes, reduce MAPK activity and can be used for related disease treatment [17]. Rambutan peel extract (RPE) compound has potential as anti-hyperglycemic and anti-acid synthase [18,19]. RPE compounds have the ability to reduce MAPK and ERK1-2 activity. Reductions of ERK1-2 activity are linked to the expression of C/EBPα and PPARγ which are the key of adipogenesis. RPE extract reduces IGF-1 and IGF-1R expression on obesity rat model [19,20]. Regulation of adipogenesis genes is the key in obesity prevention [21]. This research focusing on monitoring body weight, calorie intake, size of adipocyte cells, level of triglyceride and expression of PPARγ, C/EBPα and FABP4 of visceral fat by using immunohistochemistry (IHC), Western blotting and quantitative RT-PCR (qRT-PCR) analyses of obesity-induced high calorie diet rat model.

2. Materials and methods

2.1. Preparation of experimental animal

Rats were obtained from a laboratory animal provider in Bandung, Indonesia. They were treated with normal and high calorie diet after weaning until the 12th week. The composition of normal food was 63% carbohydrate, 3% fat, 13% protein, 21% vitamin and mineral (manufactured by PT Comfeed, Indonesia) and obesity model rat was treated with 74% carbohydrate, 6% fat, 20% protein, vitamins, minerals and 1% fiber (manufactured by PT Phokphand, Indonesia). Rats obesity level was measured according to Lee index [22]. The rats were maintained at Biosciences Laboratory, Brawijaya University.

2.2. RPE preparation

The RPE extract was prepared from 0.5 g dried rambutan peel and 10 mL of distillated aquadest then homogenized using centrifuge (10000 r/min, 5 min). Supernatant was filtered using 0.45 μm Millipore. Rats were treated using RPE 30 mg/kg body weight (based on previous research) from 50 mg/mL stock solution. Volume of RPE that used for oral administration was 2 mL.

2.3. Treatment

Rats were divided into 4 groups: non-treatment rats as normal control, non-treatment obesity rats, obesity rats with ellagic acid (O-EA) treatment as positive control and obesity rats with RPE 30 (O-RPE30) mg/kg body weight treatment. RPE was given orally once every two days, for 12 weeks. The study was approved by institutional ethic committee (Brawijaya University Research Ethics Committee).

2.4. RPE evaluation on calorie intake, body weight, weight of visceral fat and level of triglycerides

Calorie intake was determined (g/rat/day). The calorie intake was calculated [food intake multiplies total calorie diet (kcal)]. The animal body weights were recorded weekly. The body weight gain number was calculated from final body weight minus initial body weight. Twelve weeks after treatment, the rats were dissected under low concentration of ether anesthesia. Blood was collected at centrifuge tubes, stored at room temperature (RT) until serum and blood separated. And then, this collected tube was centrifuged at 3000 r/min at RT for 15 min. Serum samples were frozen for biochemical analysis. Visceral fat was taken from the dorsal side and weighted. Visceral fat was divided into two parts. First part was washed in phosphate buffered saline (PBS) and fixed in 4% of paraformaldehyde for IHC and the second part was prepared for immunoblotting analysis. Visceral fat was washed in PBS-diethylpyrocarbonate for total RNA isolation preparation.

2.5. Expressions of C/EBPα, PPARγ and FABP4

2.5.1. IHC

Adipocytes tissue was stained using hematoxylin-eosin. C/EBPα, PPARγ and FABP4 expressions were observed using IHC staining [23]. Rabbit C/EBP primary antibody (no.cat. SC61), rabbit PPARα primary antibody (no.cat. SC3456), and goat FABP4 primary antibody (no.cat. SC18601) (Life Span, BioScience) were dissolved in 2% bovine serum albumin (BSA) (1:1500) and incubated for 1 h. The secondary antibody we used was anti-rabbit immunoglobulin G (IgG) and anti-goat IgG (Lifespan Bioscience) that were dissolved in 2% BSA (1:2000) then incubated for 1 h. The adipocytes tissue was visualized by hypothalamic regulatory peptides-conjugated secondary antibody substrate (Zymed). The slides were washed three times for 10 min using PBS. The slides were dried and mounted with Canada balsam (Sigma) and then observed using Olympus 1000 microscope. Adipocytes that expressed *PPARγ* gene and genes related *C/EBPα* and *FABP4* were indicated by nucleus with brown color. Each slide was observed in 5 areas, each area seen 50 cells. Cells which expressed PPARγ, C/EBPα and FABP4 were calculated. Average of cells which expressed PPARγ, C/EBPα and FABP4 were presented in graph.

2.5.2. Western blotting

The visceral fat tissues were pulverized using mortar and pestle, then added 500 μL extract buffer (1 mmol/L phenylmethanesulfonyl fluoride in dimethylsulfoxide, 50 mmol/L pH 7.4 KH_2PO_4, 0.5% Nonidet P.40, d-dH_2O). The solution was centrifuged at 4 °C and 10000 r/min for 15 min. Protein from visceral fat was separated using 12.5% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membrane (Millipore, Billerica, MA) based on a wet transfer (Bio-Rad). Rabbit C/EBPα primary antibody, rabbit PPARγ primary antibody, and goat FABP4 primary antibody (Lifespan Bioscience) were dissolved in 2% BSA (1:1500) and incubated overnight at 4 °C, then washed with Tris-buffered saline. The secondary antibody, anti-rabbit IgG and anti-goat IgG (Lifespan Bioscience) was dissolved in 2% BSA (1:2 000), and then was visualized by Western Blue secondary antibody substrate (KLP). Membrane with specific probe was observed using ChemiDoc-

Table 1
Specific primer design for one-step qRT-PCR.

Genes	Sequence
PPARγ	PPARγ-F-5′-TCTGGGAGATCCTCCTGT T-3′
	PPARγ-R-5′-CAATCGGATGGTTCTTCGGA-3′
C/EBPα	C/EBPα-F-5′-CGACTTCTACGAGGCGGAG -3′
	C/EBPα-R-5′-TGGCTTTATCTCGGCTCTTG-3′
FABP4	FABP4-F-5′-GGACCTGGAAACTCGTCTCC-3′
	FABP4-R-5′-GGACCTGGAAACTCGTCTCC-3′
EF	ER-F-5′-ACCACCAGCTTGTCACCATC-3′
	ER-R-5′-CCAGGAAGAGCTTCACTCAAAGCTT-3′

imaging (Bio-Rad) and counted the specific protein bands by Quantity One software.

2.6. RNA isolation from visceral fat

Total RNA extraction was using NucleoSpin® RNA Macherey-Nagel (Germany). About 30 mg visceral fat had been pulverized using mortar and pestle. The extraction was added 500 µL RA1 buffer and 3.5 µL β-mercapto-ethanol. The pulverized fats were centrifuged at 13 000 r/min for 1 min. Lysate was filtered using a violet ring and placed on the collecting tube then centrifuged at 15 000 r/min for 1 min. The supernatant was transferred in a 1.5 mL tube, then added 350 µL of 70% ethanol and homogenized. Lysate was transferred in column blue ring and centrifuged at 15 000 r/min for 30 s then was added 350 µL membrane desalting buffer and centrifuged at 15 000 r/min for 1 min. DNAase reaction mixture was added to the center of silica membrane and incubated around 15 min at RT. The silica membrane was washed 3 times, first added 200 µL buffer RA2, second added 600 µL buffer RA3 and finally added 250 µL buffer RA3, centrifuged at 15 000 r/min for 1 min and discarded flow-through. NucleoSpin® RNA II column (light and blue ring) was placed into collection tube (1.5 mL) then added 60 µL buffer elution (RNase-free H_2O) and centrifuged for 1 min at 15 000 r/min. RNA total concentration was measured using NanoDrop spectrophotometer (ND2000).

2.7. qRT-PCR

qRT-PCR was designed using rat specific primer (Table 1). The procedure of q-RT was based on one-step RT-PCR (Sigma–Aldrich) kit. Concentration of RNA for qRT-PCR was 10 ng/µL.

2.8. Statistical analysis

One way ANOVA was used for statistical analysis of data. Least significant difference test was used for determining the significance. A probable value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of the RPE on calorie intake, body weight, level of triglyceride and diameter of adipocyte

Obesity rat model consumed 30% calories more than control rat. O-EA calories intake was similar to RPE30 obesity rat model. Body weight significantly increased in rat fed high calorie diet compared to control group (fed normal diet). Obesity rats with RPE treatment had reduced body weight gains significantly, and it was similar to EA treatment ($P < 0.05$). Level of triglycerides on obesity rat model was different compared to normal rats, O-EA and O-RPE30 ($P < 0.05$). Level of triglyceride was shown on obesity-RPE30 rat model. As expected, the microanatomy slides of visceral fats shown the adipocytes in obesity rats were significantly larger. After RPE treatment, adipocytes appeared smaller in obesity rats (Figure 1). Data of the RPE effect on calorie intake, body weight, level of triglyceride, and adipocyte size were shown in Table 2.

3.2. IHC and Western blotting

The expression profile of protein density of C/EBPα, PPARγ and FABP4 was shown in Figure 2. PPARγ expression on obesity rat model was significantly higher than both of O-EA and O-RPE30 rat models ($P < 0.05$). RPE30 treatment on obesity rat model showed the inhibition of PPARγ expression compared with obesity rat model. It had the similar expression profile to control and O-EA treatment. C/EBPα expression was not different among all the treatments except normal. FABP4 expression on obesity rat model showed higher expression compared with O-EA and O-RPE30.

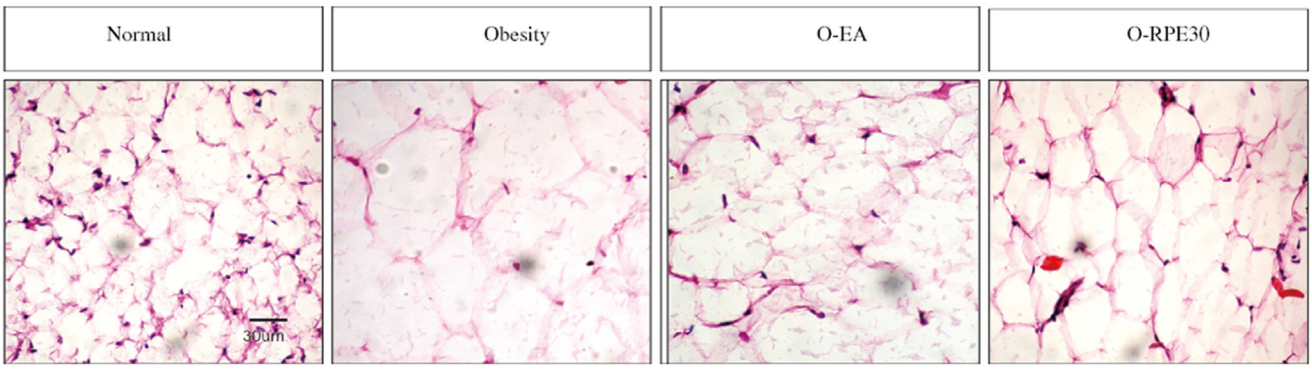

Figure 1. Size of adipocyte on visceral fat stained with hematoxylin eosin (scale: 30 µm, magnification: 400×).

Table 2

Calorie intake, body weight, size of adipocyte and level of triglycerides on normal, obesity, obesity model rat treated EA and RPE30.

Parameters	Calorie intake (kcal)	Body weight (g)			Size of adipocyte (μm)	Level of triglyceride (mg/dL)
		Initial	Final	Gain		
Normal	50.02 ± 2.90[a]	265.35 ± 5.61	327.55 ± 3.20	62.20 ± 2.41[a]	33.41 ± 1.10[a]	78.04 ± 21.04[b]
Obesity	78.68 ± 6.87[b]	334.05 ± 8.25	440.43 ± 4.61	106.35 ± 3.64[c]	104.19 ± 3.09[d]	118.20 ± 20.42[c]
O-EA	71.36 ± 6.70[bc]	368.00 ± 4.41	449.85 ± 9.82	81.85 ± 5.23[b]	75.85 ± 0.34[b]	76.64 ± 18.72[b]
O-RPE30	63.69 ± 2.55[c]	345.87 ± 3.43	428.16 ± 1.12	82.89 ± 2.31[b]	90.53 ± 1.53[c]	42.36 ± 11.96[a]

The data represent the mean ± SD for six rats per group. The different abjad indicate significance compared another ($P < 0.05$).

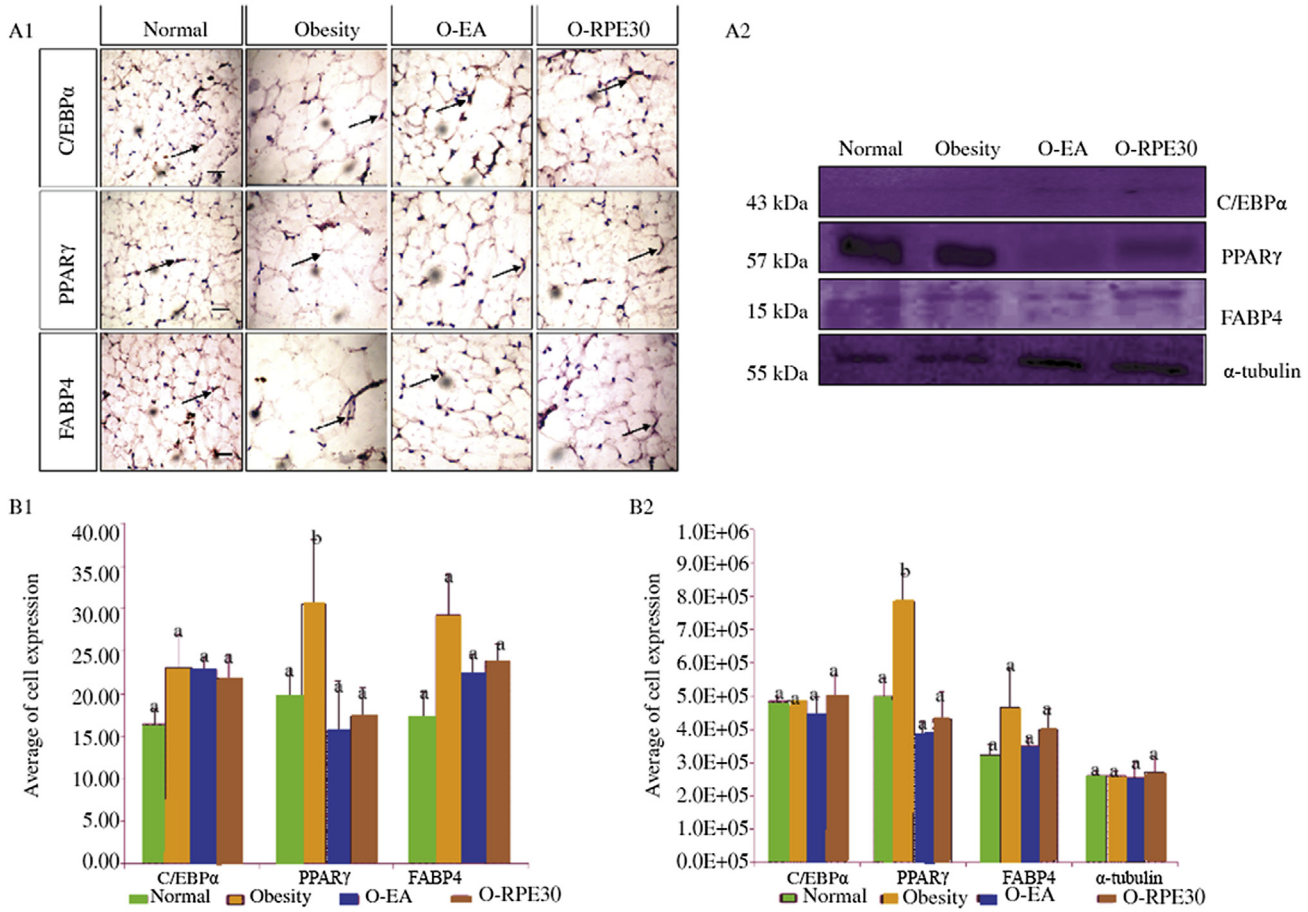

Figure 2. IHC and Western blotting of C/EBPα, PPARγ and FABP4.

A1: The expressions of C/EBPα, PPARγ and FABP4 on visceral fat detected by IHC (scale: 30 μm, magnification: 400×); A2: The expressions of C/EBPα, PPARγ and FABP4 protein detected by Western blotting; B1: The average cell expressions of C/EBP, PPARγ and FABP4 on visceral fat; B2: The mean density of protein C/EBPα, PPARγ and FABP4. [a,b]: Indicate significance ($P < 0.05$).

3.3. qRT-PCR

mRNA gene C/EBPα level on obesity rat model was similar to O-RPE30 rat model, but 12% lower than EA treatment (Table 3). There is no difference of PPARγ mRNA level in all treatment (Table 4). There was a decrease in mRNA level on *FABP4* gene, on O-EA and O-RPE30 compared to obesity. mRNA level on O-EA treatment decreased about 40% and 30% on O-RPE30 respectively (Table 5).

Table 3

Level of mRNA *C/EBPα* gene synthesized by using primers QF1/QR1 on visceral fat detected by qRT-PCR, normalized by EF gene. Mean ± SD %.

Samples	Mean ± SD
Normal	66.4 ± 7.1
Obesity	100.7 ± 17.2
O-EA	88.4 ± 59.9
O-RPE30	10.2 ± 28.6

Table 4

Level of mRNA *PPARγ* gene synthesized by using primers QF1/QR1 on visceral fat detected by qRT-PCR normalized by EF gene. Mean ± SD %.

Samples	Mean ± SD
Normal	115.3 ± 3.4
Obesity	126.6 ± 72.2
O-EA	131.7 ± 66.6
O-RPE30	127.1 ± 45.5

Table 5
Level of mRNA *FABP4* gene synthesized by using primers QF1/QR1 on visceral fat detected by qRT-PCR normalized by EF gene. Mean ± SD %.

Samples	Mean ± SD
Normal	50.6 ± 59.8
Obesity	100.0 ± 2.0
O-EA	60.4 ± 51.7
O-RPE30	70.0 ± 35.5

4. Discussion

Adipogenesis process involves various cellular activation pathways [1,24]. MAPK pathway such as ERK1-2 activation may lead to main adipogenesis genes expression, such as C/EBPα, PPARγ and FABP4 [25]. IHC and immunoblotting had shown the same result which indicated high expression of PPARγ in the obesity rat model group [1].

Growth hormone is one factor that can trigger the transcription of *IGF-1* gene for adipocyte differentiation. It initiates and stimulates cell on pre-adipocyte phase that has mitogenic effect on the clonal expansion. Pre-adipocyte secretes insulin growth factor-binding proteins which is important in modulating the IGF-1 action on adipogenesis process [11]. IGF-1 is a ligand that plays an important role in insulin synthesis regulation, mitogenic factors, cell growth and differentiation through MAPKase pathways [21,26,27].

PPARγ expression was increased in obesity rat model and was not followed by the expression of C/EBPα and FABP4. It may be caused by C/EBPα which plays a role in PPARγ activation. Two group researchers proved that C/EBPα was needed before the adipocyte-specific gene expression [10,28]. The activity of C/EBPα will be declined following the increase of *PPARγ* gene expression. C/EBPα mediates the cell at the arrest cycle and will be declined as PPARγ begins to be expressed. Expressions of C/EBPα and FABP4 had no difference in obesity rat model, O-EA and O-RPE30 [29]. The expression of FABP4 is increased in obesity rat model and decreased in O-EA, O-RPE30 and control. FABP4 plays its role at the end of adipogenesis process and carries the triglycerides from outside to cytoplasm of the cell. FABP4 is a protein that carries the free fatty acid that expresses in adipocyte [30]. The ability of RPE to act as anti-hyperglycemic and anti-obesity agents has been reported *in vitro* and *in vivo* [18]. The RPE and geraniin isolated from RPE possess alpha-glycosidase, alpha amylase, and α-reductase inhibitory activities. RPE at an oral dose of 100 mg/kg could prevent inflammation in dark agouti rats [31]. Many researches found RPE is a safe treatment to be used in therapy [32].

O-EA treatment rat model has low expression of PPARγ. EA inhibits the adipocyte formation in 3T3-L1, PPARγ and lipid accumulation for 37% [33,34]. EA is a synthetic compound that has been widely used as anti-obesity and anti-lipogenic that inhibits ERK1-2. EA can block the cell cycle on G1/S phase [33]. Treatment using RPE 30 mg/kg body weight decreases PPARγ level which is similar to normal and O-EA. The declined level of PPARγ caused by the phytochemical extract of rambutan peel activity which has the ability to bind IGF-1 and IGF-1R has a role in ERK-1 activation pathways. Adipogenesis regulation is activated mainly due to the bond of chemical compound with IGF-1 receptor that declined the activity of ERK1-2 [6,35]. The low expression of IGF-1 may be affected by the level of EA in RPE that has the same characteristic with epigallocatechin-3-gallate, one of green tea chemical compound that is able to inhibit IGF-1 and Ras/Raf/MAPK signaling pathways [21]. Some active compounds that inhibit the activity of IGF-1 and its receptor are able to inhibit the MAPK signaling cascade that lead to low expression of ERK1-2 and PPARγ. RPE (30 mg/kg body weight) decreases the expression of ERK1-2 and PPARγ, approaching the level of normal rat.

Gene expression is controlled in many steps, because it involves the processes through regulation of transcription, post transcription and translation [36]. Expressions of PPARγ reduce on O-EA and O-RPE30 rat models, but expression of C/EBPα is not different in all of the treatments. EA is a hydrophilic compound in rambutan peel and it may bind the surface-cell receptor like IGF-1R and indirectly can decrease the mRNA level of C/EBPα and PPARγ [30]. This research has the same result as research of pomegranate which can weaken adipocyte through controlling mRNA level of MAPKs. EA of pomegranate causes lipolysis on fat tissue and fatty acid oxidation on liver [37].

The result of this research showed that RPE30 is more effective than EA, due to its chemical compounds from RPEs and synergistic work that led to a greater effect than EA treatment alone [38]. RPE is an alternative way which can be used in obesity prevention. The intensive researchers focused on adipocyte have a chance to find a potential medicine against obesity and insulin resistance [6]. The main factor that caused obesity is the increasing adipocyte, so inhibition of the formation of fat cells is an important way to prevent obesity.

Rambutan peel is a waste material that has a potential to regulate obesity by preventing the adipocyte formation through normal *PPARγ* genes on obesity rat model. Chemical compounds of RPE have anti-obesity activity by inhibiting body weight gain, declining size of adipocyte and decreasing triglyceride. It also decreases the expression of PPARγ and level of mRNA *FABP4* gene.

Conflict of interest statement

We declare that we have no conflict of interest.

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