UDC 57

THE EFFECTS OF QUERCETIN TOWARDS REACTIVE OXYGEN SPECIES LEVELS AND GLUTATHIONE IN TOXOPLASMA GONDII PROFILIN-EXPOSED ADIPOCYTES IN VITRO

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ABSTRACT

Toxoplasma gondii (*T. gondii*) has been found to potentially cause adipocyte dysfunction by activating the inflammatory pathways through its profilin. In response to inflammation, adipocytes produce Reactive Oxygen Species (ROS). To scavenge ROS, endogenous or exogenous antioxidants are required. Glutathione (GSH) is one of enzimatic antioxidant that abundant in all of body cells. Quercetin, an exogenous antioxidant, can be widely found in natural products. This research aims to explore the effects of quercetin towards ROS and GSH stimulated from *T. gondii* profilin-exposed adipocytes. To achieve this, adipocytes were exposed to 20 µM *T. gondii* profilin and treated with four doses of quercetin; 31.25, 62.5, 125, and 250 µM. The results showed that quercetin significantly reduced the ROS levels ($p < 0,001$) and significantly increased GSH ($p < 0,001$) in *T. gondii* profilin-exposed adipocytes compared to untreated cells, with an effective dose of 62.5µM. This study implies that quercetin might be a promising candidate for development of antioxidant treatment interventions to prevent toxoplasmosis-mediated adipocytopathy.

KEY WORDS

obesity, *Toxoplasma gondii* profilin, ROS, GSH, quercetin

Due to its high prevalence, obesity is a major global health issue, including in developing countries and children (Ng M, et al. 2014). The increasing number of obese patients demands more research about pathogenesis of obesity to identify more effective novel intervention targets. Life style interventions and body weight reduction alone have shown unsatisfying results, especially because of the problems of discontinuity (Reever GM, et al. 2013). Several studies described infection through inflammatory pathways as a potential cause of obesity, further defined as *infectobesity* (Vasilakopoulou A and Roux C W 2007; Hedge V and Dhurandar NV 2013). The revelation of infectious agents playing roles in obesity leads to the idea of specific treatment modalities for obese people due to infection (Hedge V and Dhurandar NV 2013).

One of the potential pathogens involved in obesity pathogenesis is *Toxoplasma gondii* (*T. gondii*). This apicomplexan protozoa infected 30% of population around the globe (Reever GM, et al. 2013). As an intracellular parasite, *T. gondii* can infect all nucleated cells, including adipocytes (Toulah, Fawzia H, et al. 2011). As other members of the apicomplexan phylum, the actin cytoskeleton change during gliding movement is essential for *T. gondii* during host cell invasion. Profilin is an important component for actin polymerisation during actin-dependent gliding movement of *T. Gondii* (Plattner, Fabienne et al. 2008). Profilin-like protein of *T. gondii* is also an immunogenic element that stimulates inflammatory pathway through its recognition by the endosomal pattern recognition receptor (PRR), Toll-like receptor 11 (TLR 11) (Andrade WA. et al. 2013; Susanto, et al. 2014; Yarovinsky F 2014; Iskandar A et al. 2016).

As a response to inflammation, cells will generate of reactive oxygen species (ROS). ROS production leads to random and unregulated intracellular oxidation, which in turn triggers oxidation of iron, intracellular lipids, proteins, and DNA, resulting in vast intracellular

molecular damage. Many diseases potentially arise as the consequence, such as neurodegenerative diseases, atherosclerosis, aging process, and metabolic syndrome (Holmström, Kira M and Toren F. 2014).

To scavenge ROS and intracellular damage, antioxidants are needed. Body cells are equipped with endogenous enzimatic antioxidant. Glutathione (GSH), a cystein protein contain tripeptide, has an important role in cellular redox (Holmström, Kira M and Toren F. 2014).

Synthetic antioxidants are commercially available, but the safety and toxicity risks of synthetic antioxidants are higher than natural antioxidant (Ebrahimzadeh, et. al. 2008). Flavonoid is one of the most well recognized exogenous natural antioxidants, which is produced outside the body. Quercetin, a type of flavonoid, can be widely found in natural products, such as onion, cherry, tomato, broccoli, apple, green tea, black tea, grape, or blueberry (Fazel S et al. 2015) ; Ratnawati R and Hernowati TE. 2015). Previous studies showed that quercetin inhibits proliferation and differentiation of pre-adipocyte culture by decreasing the expression of adipocytokines, such as CCAAT/enhancer binding protein alpha (C/EBPα) and sterol regulatory element binding protein 1c (SBREP-1c) (Ratnawati R and Hernowati TE. 2015).

This study aims to explore the potency of quercetin to scavenge ROS free radicals and to stimulate GSH endogenous antioxidant by exposure of *T. gondii* profilin-exposed adipocytes. *T. gondii* profilin can be recognised by TLR 11 and stimulate inflammatory pathway that has causal relationship with adipocytopathy. The results of this study could be implied in development of potential antioxidant treatment interventions to prevent toxoplasmosis-mediated adipocytopathy.

MATERIALS AND METHODS OF RESEARCH

Experimental design. This research used true experimental study using adipocyte culture that exposed to profilin *T. gondii* and treated by quercetin. Samples were divided into 6 groups. Each group contains four replication samples each, namely : Negative control (maturated only); Positive control (maturated and 20 μM *T. gondii* profilin exposed); Q 31.25 (maturated, 20 μM *T. gondii* profilin and Quercetin 31.25 μM exposed); Q 62.5 (maturated, 20 μM *T. gondii* profilin and Quercetin 62.5 μM exposed); Q 125 (maturated, 20 μM *T. gondii* profilin and Quercetin 125 μM exposed), and Q 250 (maturated, 20 μM *T. gondii* profilin and Quercetin 250 μM exposed). The ROS level was measured using flowcytometry method. The GSH level was measured using ELISA method.

Adipocyte culture. Adipocyte culture was developed from adipose tissue of 1 month old wistar rats. Adipose tissue was collected from peritoneal and retroperitoneal regions. The tissue was munched mechanically by scalpel or scissors and digested enzimatically by type 1 collagenase (Worthington).

The obtained cells were maintained in a culture flask and nourished using Dulbecco's Modified Eagle Medium (DMEM) (Gibco©) containing sodium bicarbonate, L-glutamine, antibiotics (100U/ml Pennicillin and 100 mg/ml Streptomycin (MP Biomedicals, LCC)), and supplemented with 10% of heat-inactivated fetal bovine serum (Gibco©).

Adipocyte culture were kept at 37° C, 5% CO_2 environment. Culture media were changed every 48 hours until confluency was achieved (Ratnawati R and Hernowati TE. 2015; Zhu S et al 2010). After the cells in the culture flask were confluent, the researchers then subcultured the cells into 12-well culture plates. The cells in the culture plates achieve the same treatment as in the culture flask.

Profilin and quercetin exposure. All of the confluent groups of pre-adipocyte culture were maturated by 0,1 μM dexamethasone, 0,5 mM isobutylmethylxanthine, and 0,1 μM insulin (Ratnawati R and Hernowati TE. 2015). Simultaneously with maturation process, some of the cell cultures were exposed to 20 μM *T. gondii* profilin (MyBioSource) (positive control and four quercetin treatment groups) and quercetin (Sigma) (four quercetin treatment groups) dissolved in DMEM then incubated for 48 hours (Ratnawati R and Hernowati TE. 2015; Mochamad R, et al. 2013).

ROS level measurement. After 48 hours of incubation, adipocytes were harvested enzimatically by trypsin-EDTA, stained with 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescence dye (Genecopoeia), and analysed by flowcytometry with an appropriate emmision length of fluorescein isothyocyanate (FITC) (Wang X and Roper M G. 2014).

GSH level measurement. Culture media after 48 hours of incubation was examined using ELISA method. Furthermore, the ELISA method was read by ELISA reader with 450 nm emmision length.

Statistical analysis. ROS levels and GSH were presented as mean and standard error of the mean (SEM) of four independent replications. Homogen and normal data (p>0,05) were analysed by ANOVA test. ANOVA test was performed to assess differences between groups. When the data were not homogen and normal, the researchers then used Kruskal Wallis test. Furthermore, Mann Whitney test was used to compare the mean between two groups. Spearman correlation test was conducted to evaluate correlation between quercetin dose and ROS levels. Pearson correlation test was used to evaluate correlation between quercetin dose and GSH level.

Ethical statement. All procedures involving animals were in accordance with the ethical standards of Faculty of Medicine, Brawijaya University (No 99/EC/KEPK-PSPD/03/2017).

RESULTS OF STUDY

ROS level. The results showed that the mean of ROS level in positive control group (70.87%) was higher compared to negative control (20.49%). The decline in ROS levels were observed in all quercetin treatment groups (Q 31.25= 12.37%, Q 62.5 = 11.40%, Q 125 = 11.50% and Q 250 = 3.94%) compared to negative or positive control groups (Figure 1).

Since the data were not homogenously and normally distributed, Kruskal Wallis test was performed to assess association between quercetin dose and ROS levels. Kruskal Wallis test showed that the significant difference appeared in at least one group (p = 0,004). Mann Whitney test showed the significant differences between negative and positive control, negative control and Q 250, positive control and the other groups, Q 31.25 and Q250, Q 62.5 and Q 250, and Q 125 and Q 250.

Spearman correlation test displayed strong correlation between quercetin dose and ROS levels (correlation value of -0.723). Negative value indicated that the higher the quercetin dose was, the lower the ROS levels would be. Significant correlation was observed between different doses of quercetin treatment and ROS levels of ROS in *T. gondii* profilin-exposed adipocytes (p <0,001).

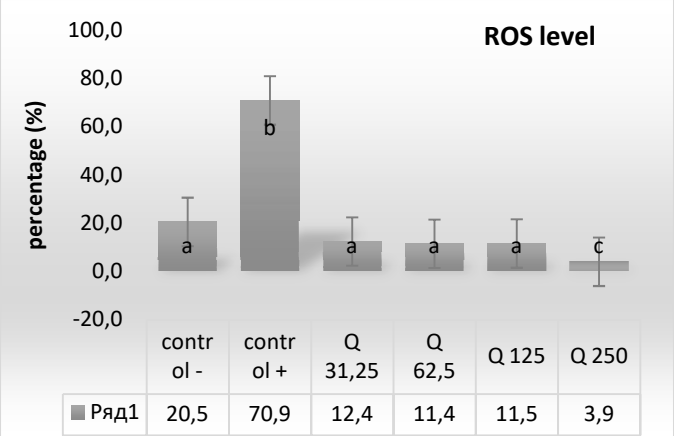

Figure 1 – ROS levels in quercetin-exposed treatment groups

Same notation showed no significant differences. Positive control, group with *T.gondii* profilin-exposed adipocytes only, was significantly higher than negative control (without exposure). All four treatment groups were significantly decreased than positive control groups. Among three different doses (Q 31,25, Q 62,5 and Q 250) were no significant differences. Q 250 has the lowest level of ROS among those groups.

GSH level. The results of this study showed that the mean of GSH level in positive control group (7.55µg/ml) was lower compared to negative control (5.42 µg/ml). GSH level increased in Q 31.25 (5,75 µg/ml), Q 62,5 (6,64 µg/ml), and Q 125 (8,15 µg/ml) compared to positive control. GSH level was decreased in Q 250 (7,29 µg/ml) compared to Q 125.

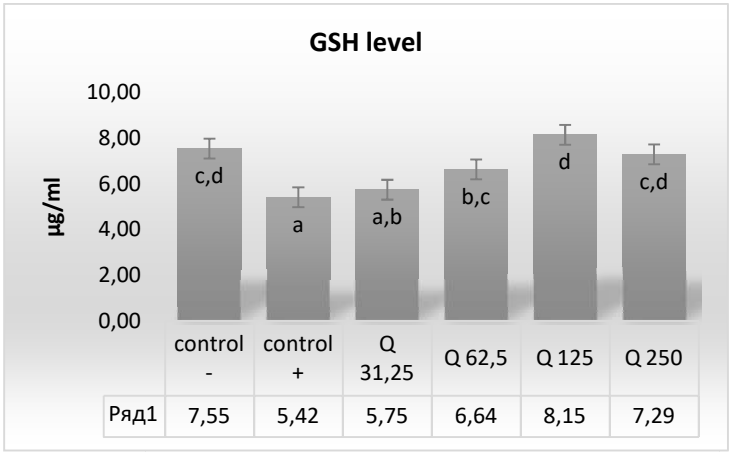

Figure 2 – GSH levels in quercetin – exposed treatment groups
Same notation showed no significant differences. Control - = negative control group (adipocyte culture without exposure), control + = positive control group (adipocyte culture with 20 µM *T. gondii* profilin exposure only), Q 31,25 (adipocyte culture with exposure of 31,25 µM quercetin and 20 µM *T. gondii* profilin), Q 62,5 (adipocyte culture with exposure of 62,5 µM quercetin and 20 µM *T. gondii* profilin), Q 125 (adipocyte culture with exposure of 125 µM quercetin and 20 µM *T. gondii* profilin), and Q 250 (adipocyte culture with exposure of 250 µM quercetin and 20 µM *T. gondii* profilin)

The data were homogenously and normally distributed ($p>0,05$). Then the researchers performed ANOVA test to assess association between quercetin dose and GSH levels. ANOVA test showed that significant difference was emerged in at least one group ($p<0,001$).

DISCUSSION OF RESULTS

During *T. gondii* infection, profilin acts as a pathogen associated molecular pattern (PAMP) recognised by the endosomal Toll-like receptor, TLR-11 (Yarovinsky F 2014). This recognition further stimulates the inflammatory pathway, eventually leading to the production of ROS (Furukawa S, et al. 2004).

This research used H2DCF-DA to measure ROS levels and observe quercetin potency as an antioxidant to scavenge ROS. The results showed there was significant difference between negative and positive control, which corresponds with the theoretical basis that profilin of *T. gondii*generates intracellular oxidative stress. The excessive ROS production stimulated by *T. gondii* profilin was unable to be neutralized by endogenous antioxidants alone (Marı M, et al. 2009), thus it stimulates intracellular molecular damage of lipids, proteins, and DNA. These damages in turn becomes the basic mechanisms of diseases, including obesity and metabolic syndrome (Furukawa S, et al. 2004); Holmström, Kira M and Toren F. 2014).

Quercetin, as an exogenous antioxidant, has the potency to scavenge free ROS radicals directly and stimulates increase endogenous antioxidant activity. Furthermore, quercetin was found to have anti-inflammation properties and can inhibit adipogenesis process (Fazel S et al. 2015; Ratnawati R and Hernowati TE 2015; Mochamad R, et al. 2013).

The results here showed significant differences between control and all doses of quercetin groups. These results confirmed the theorical basis that quercetin acts as an antioxidant in *T. gondii* profilin-exposed by significantly decreasing ROS levels (Fazel S, et al. 2015; Mochamad R, et al. 2013). Consistent with this results, a previous study by Lee *et al*. in 2013 observed quercetin effectivity as a ROS scavenger using H2DCFDA staining in

fibrosarcoma culture in vitro. The study revealed that quercetin treatment was able to scavenge ROS level at doses of 5, 10, or 50 50 µg/ml (Lee DE, et al. 2013). In this study, the researchers found no significant difference among three different doses (31,25, 62,5 and 125 µM) of quercetin groups. This indicated that all doses employed in this study have similar effectivity as antioxidant, where the minimum dose of 31.25µM showed similar effectivity to those of higher doses (62.5 and 125 µM). Quercetin dose of 250µM showed significant decrease of ROS levels compared to other groups. Although this dose has the lowest level of ROS among the other quercetin doses, but it also has significantly lower level than negative control.

ROS level that lower than normal condition is not a good condition. Although ROS can make intracellular damage, but ROS can act as a signal transduction mediator. ROS can mediate signals in immune systems and cell proliferation thus it maintains body homeostasis (Holmström, Kira M and Toren F. 2014).

Appropriate quercetin dose to lowering ROS level in adipocyte culture that exposed to *T. gondii* profilin were between 31.25 – 125 µM because at those doses, they have no signifanct difference with negative control. This indicates those doses range can lower ROS level at normal level. The GSH data were considered in order to find the effective dose of quercetin.

The results of this research showed that there are significantly decrease of GSH level in positive control compare with negative control. It indicates that the exposure of profilin *T. gondii* stimulates excessive ROS that induces the production of intracellular GSH. This production used to scavange H2O2 assisted with catalase and glutathione peroksidase, in which GSH then switched into Glutathione disulfide (GSSG). Those phenomena reduced GSH level in positive control. It was consistent with Li et al in 2016 that showed the decreasing level of GSH in endotel of aorta was followed by increasing of GSSG in early 3 hours of treatment.

From the results in previous paragraph, there are significant differences between quercetin dose 62.5 µM and positive control. This means quercetin is able to increase the endogenous antioxidant level (Ebrahimzadeh, et. al. 2008). Furthermore, quercetin can scavenge free radical directly thus it helps endogenous antioxidants works. It caused the increasing level of GSH compared with positive control.

ANOVA test showed the GSH level in negative control has no significant differences with quercetin groups between dose 62.5 – 250 µM. It indicates that at those range, quercatin can increase GSH level equal with normal condition. On the other hand, appropriate dose of quercetin in order to lower ROS level were between 31.25 – 125 µM. Therefore the minimum dose that can increase GSH level and decrease ROS level in normal condition was 62.5 µM. These results indicated that quercetin might be a promising candidate for the development of antioxidant treatment interventions to prevent toxoplasmosis-mediated adipocytopathy.

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CONFLICT OF INTEREST

The authors have no conflict of interest with any third parties.

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