トリパンブルー染色、フルオレセインージアセテート（FDA）発光およびMTTアッセイにより測定分析したシバヤギ好中球の生存能力に対するケルセチンの影響

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<th>誌名</th>
<th>明治大学農学部研究報告 = Bulletin of the Faculty of Agriculture, Meiji University</th>
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<tbody>
<tr>
<td>ISSN</td>
<td>04656083</td>
</tr>
<tr>
<td>巻/号</td>
<td>602</td>
</tr>
<tr>
<td>掲載ページ</td>
<td>p. 31-39</td>
</tr>
<tr>
<td>発行年月</td>
<td>2010年12月</td>
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Effects of Quercetin on the Physiological Viability of Shiba-goat Neutrophils Assayed by Trypan-blue Staining, Fluorescein Diacetate (FDA) Radiation and MTT Assay

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(2010年7月26日受理)

ABSTRACT

Effects of quercetin on the physiological viability of Shiba-goat neutrophils were examined in vitro. Neutrophils are the most numerous type of leucocyte which migrate to the mammary gland and attack microbes at a mastitis outbreak in goats and cows. Quercetin was intended to be used for the medical treatment of subclinical mastitis. The viability of neutrophils isolated from Shiba-goat blood was assayed by Trypan-blue staining, Fluorescein diacetate (FDA) radiation and MTT assay. In Trypan-blue staining, the viability of neutrophils was reduced with over 40 μM quercetin during 48 h cultivation. In FDA radiation, the viability was injured with over 80 μM quercetin during 48 h. In MTT assay, MTT was assumed to be reduced not only by mitochondrial enzyme in the living cells but also by the quercetin added. However, the fall of the viability was observed during the incubation, and a higher concentration (160 μM) of quercetin was more noxious to the cell viability. It was shown conclusively that, a low concentration (20 μM) of quercetin was assumed to be effective for sustaining the neutrophil viability in the early stage (24 h) of leucocyte migration.

Key words: quercetin, neutrophil, trypan-blue, FDA staining, MTT assay.

Introduction

Quercetin is much contained in onions, apples, grape and so on and belongs to flavonoid which acts as free radical scavenger (Lee et al. 2008, Somerset and Johannot, 2008, Wiczkowski et al. 2008). It also acts as antioxidant (Azuma et al. 2007), antimicrobe agent (Alverez et al. 2006, Bertelli et al. 2008, Gonzales-Segovia et al. 2008, Sugiyama et al. 2008), antiallergy agent (Min et al. 2007, Shaik et al. 2006), anticancer agent (Singhal et al. 1995, Yeh et al. 1995, Yoshida et al. 1992), and anti-inflammation agent (Jackson et al. 2006, Li and Xu, 2008, Rogerio et al. 2007). A utilization of quercetin for medical treatment is under research and development (Ossala et al. 2009, Pareek et al. 2005, Potenza et al. 2008). In this study, quercetin was intended to be used for the treatment of subclinical mastitis in dairy cows (Boutet et al. 2004, Lauzon et al. 2006, Lauzon et al. 2005) and she-goats. The mastitis in dairy cows is one of important diseases which bring about a high loss in dairy farming (Food Safety and Consumer Bureau, Japanese Ministry of Agriculture, Forestry & Fisheries, 2007). In the ordinary cases of mastitis outbreak, it is treated with antibiotics permitted for animal use, however, some fears of antibiotics translation into milk and meat are considered. The medical prevention and treatment of clinical and subclinical mastitis is desirable without using antibiotics.
In the early stage of immune response, pathogenic microbes are attacked by the phagocytic action of polymorphonuclear leucocytes, monocytes and macrophages. Neutrophils are most numerous in the leucocytes which migrate to the mammary gland and attack microbes at the mastitis outbreak. Antimicrobial substances such as flavonoids originated from natural food have a possibility to assist the leucocyte function. Quercetin is found in onions, red wine, grapefruit, apples, black tea, and so on, and can be obtained at low price. Quercetin was focused and intended to be used for the medical treatment of clinical or subclinical mastitis by injecting through udder teat. In a practical use, quercetin will exhibit two functions, one is a direct attack against pathogenic microbes in the mammary gland, and another is a reinforcement of leucocytes and the immune system. An efficacy and an application possibility of flavonoids including quercetin in the medical therapy have been numerously reported (Boutet et al. 2004, Kapiszewska et al. 2007, Losa 2003; Suri et al. 2008), however, researches concerning the efficacy of quercetin toward phagocytotic cells in the immune response are scarce.

In this study, the neutrophil reinforcement by means of quercetin was focused. The neutrophil viability was assayed by using three methods; Trypan-blue staining, Fluorescein diacetate (FDA) radiation and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. In Trypan-blue staining, the living cells exclude the coloring matter at the membrane by active transport, however, the dead cells cannot exclude and become dyed blue. In FDA staining, Fluorescein diacetate, one of fluorescent pigment, is taken into all the cells, however, it is hydrolyzed in the living cells and radiates green color. In MTT assay, the membrane-transmitting MTT is taken into all the cells, however, it is reduced to dark-blue formazan by the enzyme in mitochondria. The reduction enzyme in mitochondria in the dead cells does not work. For-mazan is solubilized in acidic 2-propanol.

When neutrophils are cultured with quercetin for a physiologically long time, a viability estimation of the neutrophils will differ following the assay methods. In this study, physiologically accurate effects of quercetin on the neutrophil viability were analyzed in vitro.

Materials and Methods

Neutrophil isolation

Fresh blood was taken out from jugular vein of a female goat (6 years old) using a vacuum syringe with sodium heparin solution, gently mixed and cooled. In room temperature, a 15 ml test-tube was poured with 3 ml of Lymphocyte separation medium 1077 for human (LSM), and 3 ml of the fresh blood was added over. The tube was centrifuged (800 x g, 20 min), and the upper layer containing monocytes, plasma and separation solution, was removed. The residual pellet of neutrophils and erythrocytes was suspended in phosphate buffered saline (PBS) and washed (150 x g, 10 min). After hemolysis and washing with PBS, the isolated neutrophils were suspended in the medium RPMI1640 (Lonza) containing glutamine.

The neutrophils were stained with Tuerk solution and observed under Thoma counting plate. The neutrophil density was adjusted to become 4 x 10^6 ml^-1. It became 2 x 10^6 ml^-1 when the neutrophil suspension was added with quercetin solution of the equal quantity in microplate wells.

Quercetin preparation in DMSO

Because of the non-hydrophilic property of quercetin dehydrate (Sigma), the quercetin solution was prepared in dimethyl sulfoxide (DMSO). In the first step, the effect of DMSO on the viability of neutrophils was examined. As the result, over 1% of DMSO in the neutrophil suspension was inhibitable against the neutrophil viability. In the second step, the solubility of quercetin in RPMI1640 medium was examined. The
80 mM quercetin solution was used. When the concentration of DMSO mixed was higher, the quercetin solubility was higher. As the result, the 1 ml diluted solution of 160 μM quercetin consisted of 2 μl of 80 mM quercetin, 8 μl of DMSO, and 990 μl of RPMI1640. The solution was heated at 37°C in advance.

**Trypan-blue exclusion**

The living cells do not take in Trypan-blue pigment, while the dead cells take in the pigment because of the injury in membrane and are stained blue. Trypan-blue solution was diluted with PBS to become 3 mg ml⁻¹. The equal quantities of the neutrophil suspension and Trypan-blue were gently mixed by pipetting. Soon after the pipetting, the living and the dead cells were countered using Thoma counting plate under microscope. The rate of the living cells in the total cells was calculated. The counts were performed on six sample plates and the mean and standard deviations were calculated.

**FDA radiation**

FDA, one of fluorescent pigment, is taken in by all the cells, however, the pigment is hydrolyzed by enzymes in the living cells and radiates green fluorescence. FDA was solubilized in acetone at 5 mg ml⁻¹, and stored at -20°C until use. The FDA solution was diluted with 49 fold volume of PBS. The diluted FDA and the neutrophil suspension were gently mixed at the volume ratio of 1 : 9 by pipetting. Soon after the pipetting, the living cells were countered using Thoma counting plate under microscope. The counts were performed on six sample plates and the mean and standard deviations were calculated.

**MTT assay**

MTT, one of tetrazolium bromide, passes through cell membrane. In the living cells, MTT is reduced by mitochondria enzyme and becomes formazan. Solubilized in organic solvent, formazan presents blue color. In this study, MTT was diluted with PBS at 5 mg ml⁻¹. Each well of the microplate was poured with 50 μl of the neutrophil suspension and 50 μl of the quercetin solution. It was added with 10 μl the diluted MTT solution. After incubation (37°C, 5% CO₂) for 4 h, 100 μl of propanol in 0.04 N HCl was added. After gentle and enough pipetting, blue color of formazan formed was measured using Microplate Reader Model 1680 (Bio Rad) at wavelength 570 nm (reference 620 nm). The measurements were performed on six wells of microplate and the mean and standard deviations were calculated.

**Finding of apoptosis and necrosis**

Based on the results of Trypan-blue exclusion, a necrosis induction of neutrophils submitted to quercetin exposure was judged (Kusaba et al. 1998). Apoptosis is defined as a cell death which is self-induced by DNA programming and characterized by cell shrinking. Necrosis is defined as a cell death which is injured by exogenous factors and characterized by breakdown of cell membrane and organelle. In this study, therefore, the sum of living and dead cells in Trypan-blue exclusion test at the start of cultivation was evaluated 1.0.

The sum of the cells remained after 24 h cultivation was compared with that at the start of cultivation. When the rate of the sums (after 24 h at the start) was significantly lower than that without quercetin (0 μM), the physiological action with quercetin was estimated as neutrophil necrosis. The same fall of the rate after 72 h was fundamentally estimated as neutrophil apoptosis.

**Statistical analysis**

Data are presented as mean±SD. Statistical significances between experimental treatments were determined using the Student's *t*-test at *p*<0.05 and *P*<
Results

Neutrophil viability estimated by Trypan-blue exclusion

The rates of living cells clearly decreased during 72 h cultivation, especially after 48 h of cultivation (Fig. 1). The inhibiting effect of higher quercetin concentrations from 10 μM to 180 μM on the cell viability was also recognized. The inhibiting effect of 40 μM and more was significantly recognized already after 24 h of cultivation. It was noticeable when the cultivation time was prolonged from 24 h to 48 h and 72 h.

Neutrophil viability estimated by FDA radiation

The rates of the cells radiating green fluorescence at each observation time compared with those at the start of cultivation also clearly decreased to below 0.2 of the relative value during cultivation of 72 h, while an increase of the ratio was recognized with 10 μM of quercetin after 24 h (Fig. 2). The significant rate increase with quercetin specifically differed from other values, however, it suggested the effectiveness of quercetin in low concentration to the neutrophil viability. The inhibiting effect of 40 μM and more was also noticeable when the cultivation time was prolonged to 48 and 72 h.

Neutrophil viability estimated by MTT assay

The relative values of optical density (570 nm, reference 620 nm) in MTT assay increased during 24 h, especially more increased with 80 μM quercetin (Fig. 3). The values a little decreased after 48 and 72 h. The larger declines were recognized with higher concentrations of quercetin, however, the relative values with all the concentrations of quercetin at 48 and 72 h did not fall so largely as recognized in Trypan-blue exclusion test and FDA staining.

Figure 1. The rates of living neutrophils judged by Trypan-blue staining test. The living cells remained not stained with Trypan-blue. Asterisks represent significant differences between the value concerned and that with 0 μM quercetin at the same cultivation time (*P < 0.05, **P < 0.01).
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Figure 2. The relative values of living neutrophils judged by FDA radiation test. The living cells radiated green colour from the hydrolyzed FDA. Asterisks represent significant differences between the value concerned and that with 0 µM quercetin at the same cultivation time (*P < 0.05, **P < 0.01).

Figure 3. The relative values of living neutrophils judged by MTT assay. The living cells presented dark blue colour of the formazan formed. The optical densities were converted compared with those (1.00) at the start of cultivation. Asterisks represent significant differences between the value concerned and that with 0 µM quercetin at the same cultivation time (*P < 0.05, **P < 0.01).
Neutrophil necrosis with quercetin

The concentration-dependent neutrophil necrosis with quercetin was judged based on Trypan-blue exclusion test (Kusaba et al. 1998). The sums of living and dead cells in Trypan-blue exclusion a little decreased with or without quercetin after 24 h cultivation compared with those (relative values = 1.0) at the start of cultivation, although the fall was larger with 160 μM of quercetin (data not shown). After 72 h, the sum of living and dead cells with 0 μM of quercetin moreover decreased, and the other sums with 20 to 160 μM of quercetin furthermore decreased significantly (Fig. 4). From the fall of the sums with 20 to 160 μM of quercetin after 72 h, a slow outbreak of neutrophil necrosis with high concentrations of quercetin was judged together with apoptosis.

Discussion

Although the clear and large decline in the neutrophil viability was recognized during 72 h cultivation in Trypan-blue exclusion test and FDA radiation, it was not recognized in MTT assay. In MTT assay, MTT reagent introduced into cells is reduced by mitochondria enzyme and becomes formazan. However, quercetin will also act as a reductant. It is feared that MTT is reduced not only by mitochondria enzyme in the living cells but also by quercetin added. In the supplementary test, MTT reduction only with quercetin was analyzed. As the result, concentration-dependent MTT reductions with quercetin was confirmed (Fig. 5). The reductions were higher in the cultivations with higher concentrations of quercetin. The MTT reduction increased in longer cultivation times, and, the reduction augmented with higher concentrations of quercetin. Formazan reoxidation with other elements in the reaction solution was guessed in longer cultivation time.

When estimated by MTT assay, the neutrophil viabilities during cultivation were maintained in higher levels or near levels to those at the start of cultivation. However, the results would have been affected by the reductant ability of quercetin.

The viability and definite life time of neutrophils will be difficult to be declared, because the somatic cells migrate to the inflammation regions according to infections. Human neutrophils cultured with medium alone for 24 h exhibited an apoptotic change with positive staining for annexin at the rate of 51% and a necrotic change with positive staining for PI at the rate of 8% (Liu et al, 2005). In peak lactating goats, deter-
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Figure 5. Formazan formation in the solution of quercetin and RPMI1640. In MTT assay, 50 μl of the medium RPMI1640 was poured into well instead of the neutrophil suspension. It was added with 50 μl of quercetin solution and 10 μl of the diluted MTT solution. After incubation and propanol (in HCl) addition, the optical densities (570 nm, 620 nm) were measured. A part of MTT reagent in the culture solution was reduced to become formazan which was augmented with higher concentrations of quercetin. The concentration-dependent MTT reduction with quercetin was represented.

mined by dual labeling with annexin-V and PI, ca. 8% of blood neutrophils are undergoing cell death, either necrosis or apoptosis, in contrast to 30% of milk neutrophils (Tian et al, 2005).

When a life time of goat neutrophil is supposed to be 8 days, 1/8 (12.5%) of the whole neutrophils submitted to the experiment will die in the first 24 h of cultivation, and 3/8 (37.5%) will die in the first 72 h. In this study, the rate of the living cells in the culture without quercetin (0 μM) decreased to 0.77 compared to that at the initiation during 24 h cultivation (not shown), while that after 72 h cultivation decreased to 0.64 (Fig. 4). The rate 0.64 without quercetin (36% reduction) closely resembled the above calculated value (37.5%). The rates of the living cells in the cultures with 20, 40, 60 and 80 μM quercetin were significantly lower and below 0.40. Therefore, it will be judged that the neutrophil necrosis was caused with 20 ~80 μM of quercetin accompanied by apoptosis.

In longer cultivation time, a damage of neutrophils with quercetin will be under the apprehension. In practical treatment, lower concentrations (20 μM and below) of quercetin is recommended, and it will work for the viability retention of neutrophils.

When the extent of mastitis in dairy cows is ranked by somatic cell count (SCC) in fresh milk, the milk with 3~5×10^6 cells ml^-1 will be judged as milk of subclinical mastitis (Kawada, 1987). In these cases of subclinical mastitis, flavonoids, such as quercetin, catechin, anthocyanin, will be effective by injecting and impregnating into mammary gland through teat canal together with frequent, physical massage by hands.

In this study, basic functions of quercetin to phagocytotic neutrophils was examined in vitro. Other flavonoids which have high reductive values are left for in vitro examination. A practical application of flavonoids to mastitis in dairy cow and goats is also left as a serious theme.

References


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トリパンブルー染色、フルオレセイン-ジアセテート（FDA）発光およびMTT アッセイにより測定分析したシバヤギ好中球の生存能力に対するケルセチンの影響

三浦奈都美・小西 晶也・平田 雄也・小林 茂樹

要約　シバヤギ好中球の生存能力に対するケルセチンの影響を in vitro で測定分析した。好中球は乳牛およびヤギにおける乳房炎発生時に乳頭部に遊走する白血球の中で最も多く存在し、病原菌を攻撃する。本研究は、潜在性乳房炎を薬物治療するときケルセチンを使用可能かを調べるために、実施した。シバヤギ血液から分離された好中球の生存能力をトリパンブルー染色、フルオレセイン-ジアセテート（FDA）発光およびMTT アッセイにより測定分析した。トリパンブルー染色による判定では、好中球の生存能力は48時間の培養中に40 μM 以上のケルセチンで阻害された。FDA 発光による判定では、同生存能力は48時間の培養中に80 μM 以上のケルセチンで阻害された。MTT アッセイによる判定では、MTT が好中球内ミトコンドリア酵素によって還元されるだけでなく、添加されたケルセチンによっても還元されることが、推定された。しかし、好中球生存能力低減は培養中に観察され、160 μM 以上の高濃度のケルセチンが好中球生存能力に対しより有効であった。結論として、低濃度（20 μM）のケルセチンが白血球遊走の初期段階（24 h）で好中球生存能力の保持に有効であることが推定された。

Key words: quercetin, neutrophil, trypan-blue, FDA staining, MTT assay.