Original Article

In vitro Modulatory Effects of Quercetin on Bovine Neutrophil Effector Functions

Phongsakorn Chuammitri* Chiradet Amphaiphan Panupong Nojit

Abstract

Bovine neutrophils perform numerous effector functions to overcome microbial invasions by utilizing innately-equipped intracellular and extracellular killing mechanisms. In this study, we explored the modulatory effects of quercetin hydrate (QH), an herbal flavonoid compound, on bovine neutrophil functional activities. Isolated neutrophils were incubated with various concentrations of QH (0-100 μM). Neutrophil viability results showed no signs of cytotoxicity ($p = 0.33$). In other assays, neutrophils were stimulated with a pre-defined concentration of QH (50 μM). Results showed promising effects of QH on enhancing intracellular ROS generation ($p < 0.0001$) compared to control. Phagocytic activity of fluorescently labeled *E.coli* was also augmented by the effects of this compound ($p = 0.05$). However, intracellular killing of live *E.coli* quantified by MTT assay demonstrated a slight decrease in activity ($p = 0.304$). Additionally, extracellular killing driven by NET-DNA release was remarkably higher in QH-stimulated cells ($p = 0.0004$). Supplementation of QH to LPS stimulated neutrophils resulted in significantly decreased pro-inflammatory cytokines, IL-1β ($p < 0.0001$) and TNF-α gene expression ($p = 0.004$). Taken together, quercetin could exert its noteworthy actions, both at cellular and molecular levels, at a certain physiological concentration.

Keywords: bovine, effector functions, neutrophil, quercetin

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Introduction

Neutrophils are privileged members of innate immune cells. As the first line, non-specific immune response, neutrophils are utmost important cells whose activities are to act against invading microorganisms. These cells sense and signal the presence of microbes which will increase the likelihood of infection by using various pathogen recognition receptors (PRRs) that are readily equipped on the cell surface or even in cytoplasmic parts (Hayashi et al., 2003; Mantovani et al., 2011). When a pathogen has been detected, neutrophils have strategies to properly manage those non-self intruders by performing a variety of processes, including phagocytosis, respiratory burst (reactive oxygen species, ROS), and degranulation, which are considered an intracellular killing mechanism (Bedard and Krause, 2007; Lambeth, 2004; Mantovani et al., 2011). In addition to internal effector function, neutrophils release web-like extracellular DNA, histones, and granule proteins to capture and to restrain bacteria, fungi, protozoa, and viruses in a process called "neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Grinberg et al., 2008; Lippolis et al., 2006). For more details in neutrophil biology and function, please refer to a collection of review articles, previously published elsewhere (Mantovani et al., 2011; Nathan, 2006; Segal, 2005; Witko-Sarsat et al., 2000).

The generation of ROS provokes many subsequent activities governing in neutrophils. To address some, phagocytosis along with the degranulation of acidic content worked in concert with ROS molecules, specifically H_2O_2, in phagolysosome (Segal, 2005). Another example is about the process in NET formations that required stimulation of NADPH oxidase (NOX) protein subunits to drive the release of those structures (Ermert et al., 2009). The unexpected creation of oxidative radicals, granule exocytosis, and NETs released by neutrophils in order to combat microbes were unintentionally detrimental to a surrounding host tissue (Diplock et al., 1998; Mantovani et al., 2011). Subtly, ROS may act like a switch to turn on pro-inflammatory genes through some transcription factors as reviewed by Boots (Boots et al., 2008).

Application of dietary antioxidants from natural plants such as quercetin has recently gained more popularity. Quercetin is naturally available phenolic compounds ubiquitously present in many fruits, vegetables, herbs, nuts, seeds, stems, flowers, and plant-derived beverages such as tea and wine (Formica and Regelson, 1995; Wadsworth and Koop, 1999). *In vitro* and *in vivo* studies revealed that quercetin exerted its effects on antiviral activity, anticancer activity, anti-inflammation, anti-oxidant, antimicrobial activity, cardiovascular protective effects, and vice versa (Alzoreky and Nakahara, 2003; Arima et al., 2002; Formica and Regelson, 1995). The modulatory activities of quercetin and its derivatives may depend on the transformation of original structures and solubility (Loke et al., 2008; Moreira et al., 2007). When studied at a molecular level, quercetin greatly reduced gene expression, especially targeted genes that were involved in early immune responses or pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α, MIP-1α, MIP-2, IL-8, IL-6, iNOS, which is vital to host defense mechanism (Cho et al., 2003; Das et al., 2012; Geraets et al., 2009; Kwon et al., 2005; Liu et al., 2005).

Based on current data of quercetin in neutrophils, macrophages, and other cell types, the knowledge of quercetin pertaining to bovine neutrophils is inadequately established. This prompted us to question how this substance would exert its immunomodulatory effects in bovine neutrophils. The aim of the present study was to explore the effects of quercetin on both intracellular and extracellular functions of bovine neutrophils. Moreover, we investigated the anti-inflammatory effect of this substance in controlling gene expressions in neutrophils, specifically pro-inflammatory cytokines, under quercetin supplementation conditions.

Materials and Methods

Reagents: Citrate-dextrose solution (ACD), Hank's balanced salts (HBSS) without calcium, magnesium, and phenol red, Quercetin hydrate, Lipopolysaccharides from *Escherichia coli* O111:B4, Thiazolyl Blue Tetrazolium Bromide (MTT), Poly-L-lysine solution, Trypan Blue solution, and Ethidium bromide were purchased from Sigma-Aldrich, St. Louis, MO, USA. Fetal Bovine Serum (FBS), RPMI-1640, Hoechst 33342 solution, H_2DCFDA, and ProLong Gold with DAPI were obtained from Life Technologies, Carlsbad, CA, USA.

Animals and isolation of bovine neutrophils: Healthy, non-pregnant adult Holstein cows were used as blood donors. A total number of 15 cows in 3 independent experiments, 5 cows each, were recruited in the study. All cows were housed at Mae Hia Agricultural Research, Demonstration and Training Center, Chiang Mai University. The experimental protocol was reviewed and approved by the Faculty of Veterinary Medicine Animal Care and Use Committee, Chiang Mai University, Thailand. Forty (40) mL of whole blood was collected by jugular venipuncture into a sterile syringe containing 10 mL of 1x ACD solution for immediate neutrophils isolation. Neutrophil isolation protocol was previously described by Roth and Kaeberle (1981) with modifications. In short, blood was centrifuged at 1,000 x*g* for 20 min (Allegra X-15R Centrifuge, Beckman Coulter, Brea, CA, USA) to collect only packed RBCs. The remaining RBCs were lysed by flash hypotonic solution followed by a hypertonic phosphate buffered saline (PBS) solution in order to return its isotonicity. The sample was centrifuged at 600 x*g* for 10 min. Cell pellet was washed twice with HBSS and resuspended in cold RPMI-1640 medium supplemented with 1% heat inactivated FBS. Cell purity was greater than 90 % as visualized by DipQuick-stained cytospin preparation (RVL Supply, Bangkok, Thailand) and cell viability was > 95% as

determined by trypan blue exclusion. Finally, cell density was adjusted to approximately one million (1 x 10^6) cells per mL.

Quercetin: Quercetin hydrate (QH) with purity of ≥ 95% by HPLC and containing ≥ 2.9 % of water as determined by Karl Fischer was used. Stock solution (5 mM) was prepared by dissolving dry quercetin powder in 95% ethanol and filter sterile. Working solution at 50 µM concentration made from stock solution was wrapped with foil to protect from light. All solutions were freshly prepared on the day of experiment and stored at room temperature until use.

Bacteria and fluorescent staining of bacteria: *Eschericia coli* (*E.coli*) was isolated from clinical mastitis cow (kindly provided by Assist. Prof. Dr. Sukolrat Boonyayatra, Department of Food Animal Clinics, Faculty of Veterinary Medicine, Chiang Mai University). Fluorescent *E.coli* was prepared by growing *E.coli* to log phase in Luria-Bertani Broth (LB broth) in shaking incubator (120 rpm, 37°C, overnight), heat killed (60°C, 90 min) and centrifuged (12,000 rpm, 5 min) (Chuammitri et al., 2011). Bacterial pellet (10^8 CFU/mL) was stained with Hoechst 33342 at a final concentration of 5 µg/mL in HBSS, and incubated at room temperature in the dark for 20 min. Hoechst labeled *E.coli* was resuspended, adjusted to 1 × 10^7 CFU/mL with PBS, and stored at 4°C until use.

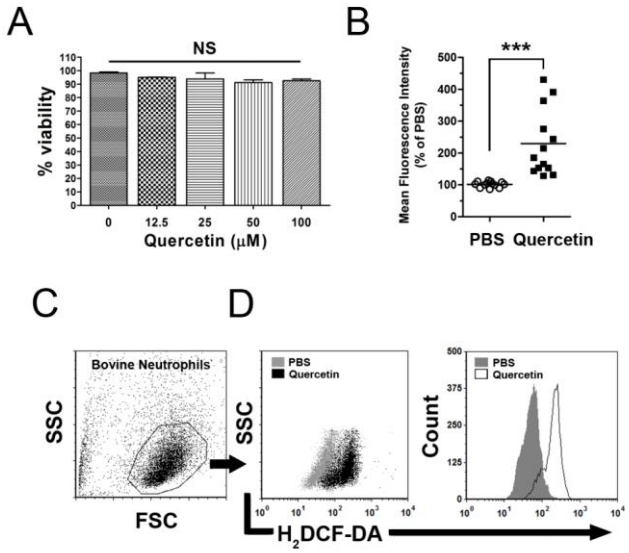

Figure 1 **Cytotoxicity assay and ROS production**
(A) Cytotoxicity test by MTT assay after incubating cell with various concentrations of QH for 60 min, n = 10, NS = no significance by one-way ANOVA. (B-D) Representative flow cytometric analysis of ROS in bovine neutrophils. (B) Each data point in the scatter plot represents an individual animal (n = 13). Horizontal bars indicate median values of three independent experiments. (C-D) Dot plots and histograms show positive staining of H_2DCF-DA for ROS molecules in PBS and 50 µM quercetin-stimulated neutrophils. ***p < 0.001 versus PBS (Mann-Whitney test).

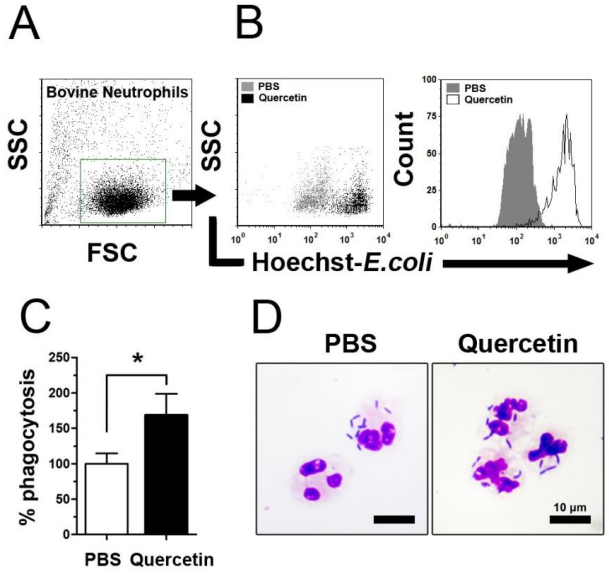

Figure 2 **Representative flow cytometry results of phagocytosis in bovine neutrophils**
(A-B) Dot plots and histograms show phagocytosis of fluorescently labeled *E.coli* in PBS and 50 µM quercetin-stimulated neutrophils. (C) Bar graphs show percentage of phagocytosis relative to control (PBS). Data are mean ± SEM of three independent experiments (n = 4-5 animals per experiment). *p < 0.05 versus PBS by unpaired Students t test. (D) Representative images from cytospin preparation slides depict neutrophils with *E.coli* detained in phagosomes. Scale bars = 10 µm, 40x objective.

In vitro cytotoxicity assay of quercetin on bovine neutrophils: Cytotoxicity of bovine neutrophils was determined as published protocol (Das et al., 2012) with modifications. In brief, 1 x 10^5 neutrophils were seeded into a 96-well flat bottom plate in duplicate. Quercetin treatments were infused at final concentrations of 0, 12.5, 25, 50, and 100 µM. The cell culture plate was incubated at 37°C with 5% CO_2 for 60 min. Cell viability determination was conducted by reduction of MTT to formazan (2 µg/mL in HBSS). Optical density (OD) of colored formazan was measured at OD_{570} using an automated microplate reader (Anthos Labtec Instruments, Wals, Austria).

Reactive Oxygen Species (ROS) assay: Neutrophils (1 x 10^5 cells) were seeded into a 96-well flat plate in duplicate. The cells were stimulated with 50 μM quercetin (optimal concentration from preliminary studies) or PBS, served as control, for 30 min in an incubator set at 37°C with 5% CO_2. The plate was centrifuged at 1,200 rpm for 3 min (LMC-3000, BioSan, Riga, Latvia) then supernatant was discarded. The cells were activated to produce ROS with 100 nM phorbol myristate acetate (PMA, Millipore, Billerica, MA, USA) for 15 min in the dark. Subsequently, H_2DCFDA (20 μM/mL final conc.) were added (He et al., 2003), and incubated for 15 min at 37°C, 5% CO_2. The plate was centrifuged (1,200 rpm, 5 min) and washed with iced-cold PBS. Sample acquisitions (10,000 events) were performed by CyAn ADP High-Performance Flow Cytometer (Beckman Coulter). Data were analyzed by using the FCS Express 4 software package (De Novo Software, Los Angeles, CA, USA). The region of background fluorescence was set at 10 units for cut-off value. The ROS generation was reported as mean fluorescence intensity (MFI).

Phagocytosis: Phagocytic activity of Hoechst labeled *E.coli* was assessed via flow cytometry (Yan et al., 2012). In brief, neutrophil suspension (1 x 10^5 cells) was incubated, in duplicate wells, with 50 μM quercetin or PBS for 30 min at 37°C, 5% CO_2. Fluorescent *E.coli* (1 x 10^6 bacteria from no.3) was opsonized with 10% heat-inactivated normal bovine serum for 20 min at 37°C. Opsonized bacteria (MOI of 10) were then added to the cells, centrifuged (1,200 rpm, 5 min), and incubated (37°C, 5% CO_2) for 60 min. After incubation, the plates were washed twice with cold PBS and centrifuged at 1,200 rpm for 5 min. Cell pellet was resuspended in 1% PFA and samples were submitted to flow cytometry. Data acquisition and analysis were performed as stated in the ROS assay.

Bacterial killing assay: Bovine neutrophil bacterial killing was evaluated using a formazan metabolic conversion in the MTT colorimetric assay as previously described with modifications (Chuammitri et al., 2011; Das et al., 2012). *Escherichia coli* were freshly prepared for each assay by colony picking method and propagation in LB broth until OD_{600} of 0.5 or greater was reached. Live bacteria were opsonized and diluted to a final concentration (1 x 10^6 CFU/mL) to be used in the assay. Neutrophils (1 x 10^5 cells) were loaded into duplicate wells of a 96-well plate, subsequently added 50 μM quercetin or PBS, and incubated (37°C, 5% CO_2) for 30 min. Opsonized *E. coli* were seeded with stimulated neutrophils in 1:10 ratio. The plate was centrifuged and placed in an incubator for 45 min. After incubation, the plate was again centrifuged to remove non-ingested bacteria. Hypotonic solution (diH_2O) was used for releasing internalized bacteria from lysed neutrophils. After lysing, LB broth was supplemented to all wells. Incubation took place for a total of 90 min at 37°C. The plate was taken out and 2 μg/mL MTT was added in LB broth (working solution) to develop colored formasan for 15-30 min. The plate was centrifuged and the supernatant was transferred to a new plate. Absorbance (OD_{570}) was measured after adding dimethyl sulfoxide (DMSO) to dissolve the purple formazan crystals using an automated microplate reader. In each experiment, OD from control well (MTT solution only) was included to indicate no live bacteria were present. The MTT assay was also validated by counting colony forming units. Percentage of killing was calculated by substituting measured OD values into the following formula:

$$\% \text{ of killing} = 100 - [(OD_{sample} - OD_{MTT}) \times 100]$$

Cytospin slides were prepared from an aliquot of neutrophils before lysis process. For details, 0.001% poly-L-lysine coated circular coverlips (15 mm diameter) were placed into a 24-well plate. Cells were seeded and subsequently centrifuged at 1,200 rpm for 5 min. Slides were fixed with absolute ethanol, stained with DipQuick, and examined with a Zeiss Axio Scope A1 (Carl Zeiss, Thornwood, NY, USA).

Bovine neutrophil extracellular trap (NET) formation: Circular 0.001% poly-L-lysine coated coverslips (15 mm diameter) were placed into 24-well tissue culture plates (Grinberg et al., 2008). Neutrophils (2.5 x 10^4 cells in RPMI-1640) were seeded on coverslips, followed by stimulating with 50 μM quercetin or PBS for 30 min in a 37°C, 5% CO_2 incubator. For NETs release, solutions from previous process were aspirated and the cells were subsequently stimulated with PMA (100 nM) (Remijsen et al., 2010) or PBS for 90 min to allow NET formation (optimal concentration and incubation time were determined in preliminary experiments). The plates were centrifuged (1,200 rpm, 5 min), supernatant was removed, and cells were fixed with 4% PFA for 25 min at room temperature. After washing with cold PBS, NET structures were fluorescently stained and mounted with ProLong Gold with DAPI for 10 min in the dark. NET releases were captured by using 10x objective equipped with a Zeiss Axio Scope A1 fluorescence microscope with the Axio Vision Image System. DNA-staining NETs were manually counted from 5 random fields at 10x objective by three observers in a blinded technique (Yan et al., 2012).

Semi-quantitative reverse transcription PCR (RT-PCR): Semi-quantitative reverse transcription PCR (RT-PCR) was performed to investigate the effect of quercetin on pro-inflammatory cytokine gene expressions in bovine neutrophils. One million neutrophils (1 x 10^6 cells in RPMI-1640) were treated with 100 ng/mL *Escherichia coli* LPS 0111:B4 (Liu et al., 2005) coupled with 50 μM quercetin or PBS in a 24-well culture plate (duplicate wells) for 60 min at 37°C with 5% CO_2 incubator. After stimulation, total RNA was extracted and purified by NucleoSpin RNA II (MACHEREY-NAGEL, Bethlehem, PA, USA) according to the manufacturer's recommendations. The RNA concentration was quantified using a DU 730 nanoVette UV/Vis spectrophotometer (Beckman Coulter) at 260 nm. Complementary DNA (cDNA) was reverse-transcribed from a starting amount of 2 μg of total RNA by Tetro cDNA Synthesis Kit (Bioline, Taunton, MA, USA). Briefly, 20 μL reaction mixture contained 4 μL 5x RT Buffer, 1 μL 10mM dNTP mix (2.5 mM each), 1 μL Oligo$(dT)_{18}$ (10 μM/μL), 4 μL Random Hexamer (10 μM/μL), 1 μL RNase Inhibitor (10 U/μL),

1 μL Tetro Reverse Transcriptase (200U/μL), 2 μg Total RNA, and DEPC-treated H_2O. The cDNA synthesis was carried out by G-STORM GS1 thermocycler (Somerton Biotechnology, Somerset, UK) by incubating the samples at 25°C for 10 min, 45°C for 30 min, followed by heating at 85°C for 5 min, chilled on ice, and stored reaction at -20°C until further use.

Primer sequences of bovine IL-1β and TNF-α were described previously (Tao et al., 2004). The primer pair of GAPDH was designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The sequences of oligonucleo- tides used in this study were as follows: GAPDH (NM_00103403), forward, 5′-AGTTCAA CGGCACAGTCAAG-3′, reverse, 5′-TCACGC CCATCACAAACATG-3′; IL-1β (M35589), forward, 5′-CGTACCTGAACCCAT CAACGAAAT-3′, reverse, 5′-GGCGTATCACCTTTTTTCACACAA-3′; TNF-α (Z14137), forward, 5′-TCTCAAGCCTCAAGT AACAAGCCG-3′, reverse, 5′-CAGGTAGTCCGGCAG GTTG AT CTC-3′. PCR primers were synthesized by Macrogen, Seoul, Korea.

PCR condition was set up in a reaction volume of 25 μL. The reaction mixture contained 12.5 μL 2x MyTaq HS Red Mix (Bioline), 1 μL forward and reverse primers (10 μM), 100 ng cDNA template, and molecular biology grade H_2O. The cycling conditions for IL-1β and GAPDH were set as follows: an initial denaturation of 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 59°C for 15 sec, extension at 72°C for 15 sec, and a final extension at 72°C for 2 min. The annealing temperature for TNF-α set at 57°C for 15 sec and the rest of the conditions were identical to other genes. The amplified PCR products were electrophoresed in 1.5% agarose gel at 100V for 30 min (Mini-Run Gel GE100, Hangzhou Bioer Technology, Hangzhou, China). The gels were stained with ethidium bromide (0.5 μg/mL), documented using the Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA). Amplicon sizes at 243, 564, and 414 bp were considered specific products of GAPDH, IL-1β, and TNF-α, respectively. Gel band intensities were quantified by Gel Analyzer Options in ImageJ version 1.46r (http://rsb.info.nih.gov/ij/index.html) (Das et al., 2012). Expression levels are represented relative to GAPDH expression.

Data analysis: Results obtained from 3 independent experiments were tested for differences between treatments. Prior to statistical analyses, outliers were removed from the data set using the Grubbs' test or extreme outlier studentized deviate (ESD) method. Most analyses were done with GraphPad Prism version 5 (GraphPad software, San Diego, CA, USA) using either the unpaired two-tailed Student *t* test or Mann-Whitney test. Cytotoxicity data were analyzed using one-way ANOVA. Results are considered significant when *p*-value was ≤ 0.05. Information obtained from the statistical analyses are represented as graphs generated by GraphPad.

Results

Cytotoxicity test of Quercetin on bovine neutrophils: The *in vitro* cytotoxicity of quercetin on bovine neutrophils, as determined by the MTT cytotoxicity assay, showed no cytotoxicity (Fig 1A, $p = 0.33$). Since the conversion of formasan from MTT was almost identical in every concentration tested (0, 12.5, 25, 50, and 100 μM), this could be interpreted that the cells were not affected by quercetin. We also confirmed the cytotoxicity results from MTT assay with trypan blue exclusion under light microscope. The percentages of cell viability from trypan blue were consistent with MTT assay (data not shown). The percentages of cell viability from both assays indicated no cytotoxicity at any tested concentrations, thus we primarily chose only one concentration (50 μM) to be applied to the other cellular function assays. Considering 50 μM quercetin as an optimal concentration under the condition we used, we critically compared our results with published works of the quercetin effects on either neutrophils or PBMCs (Liu et al., 2005; Nair et al., 2006).

Reactive Oxygen Species (ROS) generation: To assess the effects of quercetin on the production of intracellular reactive oxygen species (ROS), specifically H_2O_2, in bovine neutrophils, we stained the cells with fluorescent dye (H_2DCF-DA). Flow cytometry data showed that the cells treated with 50 μM quercetin markedly increased the amount of ROS (MFI = 229.80 ± 29.08) compared with the PBS treated cells (MFI = 101.90 ± 2.41; $p < 0.0001$, Fig 1B-D).

Phagocytosis assay: We next investigated the phagocytic property of bovine neutrophils to phagocytose fluorescently labeled *E.coli* after stimulating with quercetin or PBS. Results verified that the increase in phagocytosis of neutrophils after being induced by quercetin was found (Fig 2). The exposure of quercetin resulted in enhancing phagocytosis in stimulated neutrophils (169.00 ± 29.66 %) versus PBS control (100.00 ± 14.56 %; $p = 0.05$, Fig 2C).

Bactericidal assay: The killing of live bacteria in bovine neutrophils is a complex process requiring the generation of ROS, degranulation, cytoskeletal rearrangement, and phagosome-lysosome fusion. In our assay setting, we examined the neutrophil bactericidal ability by the MTT assay. Results demonstrated that the control cells (PBS) had the ability to destroy 92.73 ± 0.50 % of the *E. coli* they harbored in phagosomes (Fig 3A-B). Like the control neutrophils, the quercetin-stimulated cells were able to equally kill phagosome-bearing *E. coli* (92.06 ± 0.35%; $p = 0.30$, Fig 3A-B). The dead/live bacteria inside the bovine neutrophils were further visualized by cytospin preparation slides after DipQuick staining. Some bacteria inside phagosomes underwent cell lysis (Fig 3B), whereas the live ones were apparently encircled in the confinements. Moreover, some neutrophils casted neutrophil extracellular traps (NETs) with different length to destroy bacteria outside the cells, as depicted in Figure 3D.

Bovine Neutrophil Extracellular Traps (NETs) Formation: Neutrophil Extracellular Traps (NETs) in bovine neutrophils has been previously reported by Grinberg et al. (2008) and Lippolis et al. (2006). To elucidate the effects of quercetin on accommodating the release of NET structures in the current study, we pretreated the neutrophils with saline (PBS) or 50 μM quercetin and restimulated cells with the known potent stimulant, PMA. As we expected, the neutrophils released NET structures in response to the PMA activation. The fluorescently labeled DNA as a major component of bovine NETs was evident under fluorescence microscope. The DNA stained structures were protruded and lengthened away from the quercetin-stimulated cells (Fig 3F). An average number of 6.08 ± 0.77 NETs/field was enumerated from the neutrophil incubated with quercetin, whereas the neutrophils receiving PBS had approximately three times less in the number of NETs (2.20 ± 0.32 NETs/field) as presented in Figure 3C (p = 0.0004).

Semi-quantitative Reverse Transcription PCR (RT-PCR): The fact that quercetin controlled certain molecular mechanisms in neutrophils have been proposed. Quercetin possessed the suppressive action for pro-inflammatory cytokine gene expression, for example IL-1β, IL-6, and TNF-α were firmly established (Cho et al., 2003; Liu et al., 2005). We examined whether the use of quercetin at 50 μM, which were the earlier enhanced bovine neutrophil functions, could suppress pro-inflammatory cytokine genes. In this study, the neutrophils from quercetin-treated group subtly altered IL-1β and TNF-α gene expressions. Semi-quantitative analysis of the band intensities of IL-1β PCR products illustrated many differences between the control and quercetin-treated neutrophils (Fig 4A). The profound effect of quercetin implemented in alteration of IL-1β fold changed relatively to the housekeeping gene (GAPDH). The untreated cells (PBS) had 1.00 ± 0.04 relative fold change compared with the quercetin group (0.69 ± 0.03 fold change; $p < 0.0001$, Fig 4B). A similar pattern was also depicted from the TNF-α PCR products, but with less extent than what was found in the IL-1β gene expression. For TNF-α gene expression, the quercetin-treated group showed a decrease in fold change (0.85 ± 0.03) when compared to the PBS-treated group (1.00 ± 0.04; p = 0.0044, Fig 4C).

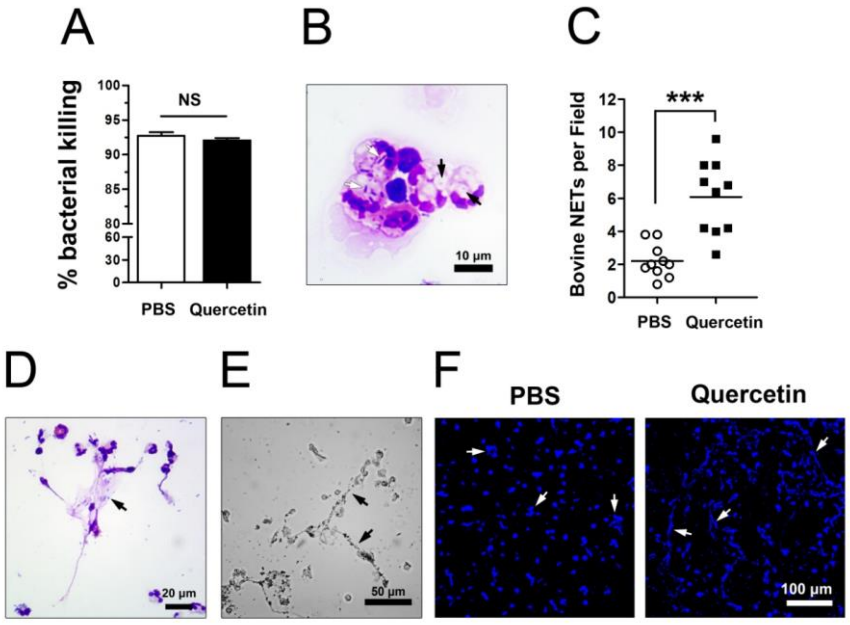

Figure 3 MTT bacterial killing and bovine NET formation
(A) Bar graphs show percentage of killing of live *E.coli* in neutrophils treated with PBS or 50μM quercetin. Data are mean ± SEM of two independent experiments (n = 5 animals per experiment). NS = no significance by unpaired Students t test. (B) Representative image from cytospin slides prepared before MTT assay shows dead (black arrows)/live (white arrows) bacteria in quercetin-stimulated neutrophils. Scale bar = 10 μm, 40x objective. (C-F) Bovine neutrophil extracellular traps (NETs) formation. (C) Bar graphs show quantity of NETs in control (PBS) or quercetin- stimulated cells. Each data point in the plot represents an individual animal (n = 10). Horizontal bars indicate median values of two independent experiments. ***$p < 0.001$ versus control by Mann-Whitney test. (D-F) Representative images of NET structures (arrows) as visualized by (D) DipQuick-stained, 20x (E) Bright field, 20x (F) Hoechst 33342-stained structures (5x), scale bars as indicated on each image.

Discussion

In the current study, our goals were to explore the response of the innate immune system, bovine neutrophils in particular, once they have been stimulated with quercetin hydrate. Our finding is the first to witness the initiation of extracellular NET formation from quercetin-stimulated bovine neutrophils after encountering live bacteria or PMA *in vitro*.

The cytotoxicity test revealed that quercetin was safe to be utitilized for bovine neutrophils. This result is consistent with previous reports in human-, rabbit neutrophils and macrophage cell line (Boesch-

Saadatmandi et al., 2011; Kaneider et al., 2004; Moreira et al., 2007; Tang et al., 2009). The importance of reactive oxygen species (ROS) in neutrophil biology has long been addressed. Quercetin acting as antioxidants or the scavenger of ROS in neutrophils was currently known to date (Loke et al., 2008). From our observation, the *in vitro* stimulation of bovine neutrophils with 50 μm quercetin resulted in the elevation of the ROS level within the cells. The generation of ROS molecules is fully regulated by NADPH oxidase (NOX) enzyme (Lambeth, 2004). In resting, unstimulated neutrophils, NOX subunits (e.g. $p22^{phox}$ and $p47^{phox}$) are inactive. A logical reason for our finding is that quercetin influenced, in part or in whole, the NOX subunits, which then become active. As a consequence, active NOX proteins interact with each other to form a viable NADPH oxidase and to be able to execute ROS production (Lambeth, 2004). Another possible explanation is that quercetin is involved in ROS signaling pathway by inducing through protein kinase C (PKC)/Akt pathway (Piccoli et al., 2007). However, further investigations need to fulfill this specific information.

Phagocytosis and destruction of invading pathogens are some of the major effector functions equipped within mammalian neutrophils. This particular process of ingesting bacteria initiates when cell surface receptors, for example, FcγR, complement receptors (CR), scavenger receptor (SR), and Toll-like receptors (TLRs), are activated. Cells will recognize pathogens by specifying receptor, send a signal downstream, secrete some cytokines, and provoke the immune response toward phagocytosis. Upon activation by quercetin, bovine neutrophils were biologically active and efficiently phagocytose opsonized *E.coli*. The result here is in line with the experiment by Moreira et al. (2007) in which quercetin promoted phagocytosis in rabbit neutrophils. It was previously reported that increasing ROS, especially H_2O_2, will enhance FcR phagocytosis (Pricop et al., 1999).

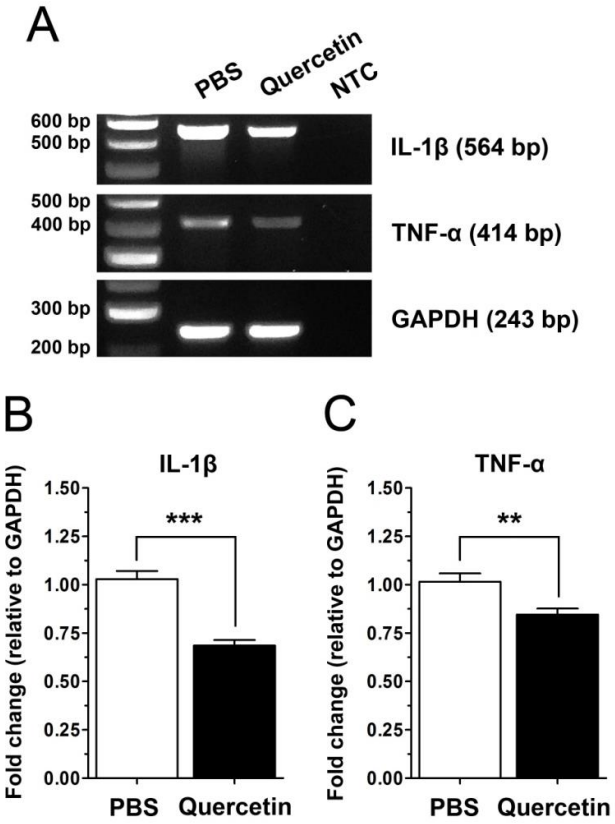

Figure 4 **Effects of quercetin on IL-1β and TNF-α mRNA expressions in bovine neutrophils by RT-PCR**
(A) Quercetin down regulated the expression of both IL-1β and TNF-α genes as demonstrated by representative band intensities of PCR amplicons. GAPDH was used as an internal control. NTC = no template control. (B) Densitometric analysis of IL-1β mRNA expression level compared to GAPDH. (C) TNF-α mRNA expression level compared to GAPDH. Data are expressed as mean ± SEM of two independent experiments (n = 5 animals per experiment). $^{**}p < 0.01$, $^{***}p < 0.001$ versus PBS by unpaired Students t test.

It is apparent that bovine neutrophils utilize extraordinary structure (NETs) to entrap and to kill extracellular bacteria alongside with other effector functions (Grinberg et al., 2008). Hakkim et al. (2011) reported that the signaling pathway of NET formation involved the Raf-MEK-ERK pathway. As signals went downward, the mediators of oxidative radicals, specifically NADPH oxidase, increased and extended the creation of NETs (Brinkmann and Zychlinsky, 2007; Giambelluca and Gende, 2008; Hakkim et al., 2011; von Köckritz-Blickwede and Nizet, 2009). In our study, we did show the formation of bovine NETs after receiving quercetin treatment. Ermert et al. (2009) and Röhm et al. (2014) suggested that ROS was required for NET production. Our results in ROS assay also showed the increase in ROS molecules in the quercetin-primed cells. Altogether, the increase in ROS level may somehow assist in more bovine NET formation.

It is well recognized that quercetin remarkably modulates inflammation at a molecular level (Cho et al., 2003; Liu et al., 2005; Nair et al., 2006; Tang et al., 2009). In this report, we observed the reduction in proinflammatory cytokine gene expressions, IL-1β and TNF-α in bovine neutrophil by the action of quercetin after stimulated with LPS. The results were in agreement with reports elsewhere (Cho

et al., 2003; Tang et al., 2009). The macrophage cell line (RAW 264.7) treated with quercetin was found to have the decrease in IL-1β and TNF-α mRNA expressions under a time-dependent manner. Quercetin also inhibited the expression of TNF-α in human PBMC with increasing doses (Nair et al., 2006). Furthermore, there was an evidence that quercetin abrogated IL-6 production in isolated human neutrophils (Liu et al., 2005).

It is, therefore, not surprising that quercetin is involved in the multi-process of stimulating the generation of ROS, thus sustaining other effector functions that follow. Quercetin helped to enhance phagocytosis, and moreover released NETs. From our point of view, ROS, phagocytosis, microbial killing and degranulation, and NETs act in concert to immediately combat the pathogen invasion. These functions can be nicely organized and induced by the effects of quercetin. Our current understanding regarding the effects of quercetin on bovine innate immune cells, neutrophils, could be concluded here. Under the conditions used, we have obtained a logical evidence that quercetin, at a physiological concentration (50 μM) can manipulate major effector bovine neutrophil functions, namely respiratory burst, phagocytosis, and NETs. In order to fully exploit the benefits of quercetin, the understanding of exact molecular mechanisms behind the immunomodulatory effects of quercetin, is one of our future particular interests.

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บทคัดย่อ

ผลของสารเคอร์ซิตินต่อการทำหน้าที่ของเซลล์นิวโทรฟิลของโคในหลอดทดลอง

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นิวโทรฟิลในโคเป็นเซลล์ในระบบภูมิคุ้มกันที่มีมาแต่กำเนิดซึ่งมีหน้าที่ในการต่อต้านและทำลายเชื้อโรคที่บุกรุกเข้าสู่ร่างกายโดยอาศัยการทำงานทั้งแบบการทำลายภายในเซลล์และภายนอกเซลล์ ในการศึกษานี้ ผู้วิจัยได้ศึกษาผลของสารเคอร์ซิตินไฮเดรต (QH) ซึ่งเป็นสารพฤกษเคมีในกลุ่มฟลาโวนอยด์ที่ส่งผลต่อการทำหน้าที่ของเซลล์นิวโทรฟิลในโค เซลล์นิวโทรฟิลที่แยกได้จากเลือดนำมากระตุ้นด้วยสาร QH ที่ความเข้มข้นตั้งแต่ 0 ถึง 100 ไมโครโมล (μM) เพื่อตรวจสอบความเป็นพิษที่มีต่อเซลล์ การทดลองไม่พบว่าเกิดความเป็นพิษในทุกความเข้มข้นที่ทดสอบ ($p = 0.33$) และเมื่อใช้สาร QH ที่ความเข้มข้น 50 μM มากระตุ้นเซลล์พบว่าความสามารถในการผลิต Reactive Oxygen Species (ROS) ภายในเซลล์เพิ่มขึ้น ($p < 0.0001$) เปรียบเทียบกับเซลล์ในกลุ่มควบคุม ความสามารถในการเก็บกินเชื้ออีโคไล (*E.coli*) ที่ย้อมสีฟลูอออเรสเซนส์เพิ่มขึ้น ($p = 0.05$) แต่การทำลายเชื้ออีโคไลมีชีวิตภายในเซลล์ลดลงเล็กน้อย ($p = 0.304$) นอกจากนี้ยังพบว่าความสามารถในการผลิตโครงสร้าง Neutrophil Extracellular Trap (NET) มีปริมาณที่เพิ่มขึ้น ($p = 0.0004$) ในเซลล์กลุ่มที่ถูกกระตุ้นด้วย QH เมื่อตรวจสอบการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการอักเสบในนิวโทรฟิลที่ได้รับการเสริมสาร QH ก่อนการกระตุ้นเซลล์ด้วย lipopolysaccharide (LPS) พบว่าระดับการแสดงออกของยีน IL-1β ลดลง ($p < 0.0001$) และระดับการแสดงออกของยีน TNF-α ลดลงเช่นกัน ($p = 0.004$) โดยสรุป การกระตุ้นนิวโทรฟิลด้วยสารเคอร์ซิตินไฮเดรตที่ความเข้มข้นที่เหมาะสมจะส่งผลต่อการทำงานทั้งในระดับเซลล์และระดับชีวโมเลกุลของเซลล์ชนิดดังกล่าว

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