

Effects of Vitamin C Supplementation to Bovine Peripheral Blood Mononuclear Cells on Antioxidant and Inflammatory Biomarker Status and Cell Viability with or without Lipopolysaccharide Stimulation

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Abstract

This study aimed to determine the effects of vitamin C supplementation on antioxidant and inflammatory status and cell viability of bovine peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from eighteen clinically healthy Japanese Black calves in this study. PBMCs were cultured with vitamin C (vitamin C group) and without vitamin C (control group) and stimulated with or without lipopolysaccharide (LPS). As a result, the total antioxidant capacities, which are the reducing power of components from Fe³⁺ to Fe²⁺, in the cell culture supernatant with or without LPS stimulation were significantly higher in the vitamin C group than those in the control group ($P < 0.05$, $P < 0.01$, respectively). Tumor necrosis factor- α in the cell culture supernatant with LPS stimulation was significantly higher in the control group than that in the vitamin C group ($P < 0.05$). The viability of cells cultured with LPS stimulation was significantly higher in the vitamin C group than that in the control group after 72 h of culture ($P < 0.05$). These results suggested that vitamin C is related to the antioxidant and anti-inflammatory properties and cell viability of PBMCs obtained from calves.

Discipline: Animal Science

Additional key words: cattle, immunity, *in vitro*

Introduction

Vitamin C is a water-soluble vitamin and a fundamental nutrient for sustaining physiology (Mousavi et al. 2019). Vitamin C has also been reported to have antioxidant and anti-inflammatory properties, which protect against reactive oxygen species and suppress inflammatory responses caused by proinflammatory substances (Mousavi et al. 2019, Niki 1991, Oudemans-van Straaten et al. 2014).

In vivo studies in humans have reported that vitamin C supplementation reduced infectious respiratory diseases (Pauling 1971), oxidative stresses, and inflammatory biomarkers, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in the blood (Du et al. 2003, Mikirova et al. 2012, Yang et al.

2006.). In cattle, vitamin C supplementation has promoted antibody production after *Histophilus somni* vaccination (Otomaru et al. 2020) and reduced the incidence of diarrhea (Hemingway 1991).

Studies reported that *in vitro* vitamin C supplementation to human blood leukocytes reduced the production of TNF- α and IL-6 (Hartel et al. 2004), and vitamin C supplementation to human blood plasma or porcine kidney cells increased antioxidant capacity (Bleilevens et al. 2019, Hong et al. 2002). There have been reports about *in vitro* vitamin E or anti-inflammatory agent supplementation to peripheral blood mononuclear cells (PBMCs) of cattle (Maeda et al. 2011, Otomaru et al. 2022). However, only few *in vitro* studies have investigated vitamin C supplementation to bovine tissues or cells.

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Clarifying the effect of vitamin C supplementation on a cellular level for the health management of cattle is important. Therefore, this study aimed to investigate the effects of vitamin C supplementation to bovine PBMCs on antioxidant and inflammatory biomarker status and cell viability.

Materials and methods

1. Animals, cell isolation, and culture

Eighteen clinically healthy Japanese Black calves kept on a farm in Kagoshima Prefecture, Japan, were used in this study. The calves on the farm were allowed to remain with their dams for 1 week after birth; thereafter, they were separated and housed in individual hutches until 2 weeks of age. After 3 weeks of age, the calves were housed in groups of 18 per paddock and fed with milk replacer until 12 weeks of age. At 13 weeks, calves were weaned and housed in paddocks for 6 calves each. The amount and nutrient composition of feed are shown in Table 1. All calves were managed in the same manner and fed to meet their nutritional requirements according to the Japanese beef cattle feeding standard (MAFF 2008).

Blood from the jugular vein was sampled using vacutainer heparin tubes from 24–32 weeks of age. PBMC isolation and cell suspension were conducted according to a previous report (Maeda et al. 2011). Briefly, 10 mL of heparinized blood diluted twofold in phosphate-buffered saline (PBS) were placed above separation medium solution (Lymphocyte Separation Medium 1077, Immuno-Biological Laboratories, Japan; specific gravity 1.077) and centrifuged at $400 \times g$ for 60 min at room temperature. Mononuclear cells at the interface were collected and washed twice with PBS by centrifugation at $400 \times g$ for 5 min. The isolated PBMCs were treated with 0.83% ammonium chloride and rocked gently for 5 min to remove any residual red blood cells. Subsequently, PBMCs were washed with PBS and suspended in RPMI1640 (Invitrogen, Tokyo, Japan) medium containing 100-U/mL penicillin G (Meiji Seika, Tokyo, Japan). The isolated PBMCs were dispensed into 48-well plates (Greiner Bio-One, Tokyo, Japan) at a density of 5×10^6 cells/well.

The cells were cultured at a final volume of 1 mL/well in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Cansera International Inc., Rexdale, Canada) with vitamin C (L-Ascorbic acid, FUJIFILM Wako, Japan) (vitamin C group) or without vitamin C (control group). Vitamin C was dissolved in distilled water, and the concentration of vitamin C was set to 200 µg/mL with reference to the

previous reports (Bergman et al. 2004, Hong et al. 2002, Perez-Cruz et al. 2003).

In addition, PBMCs were treated with or without lipopolysaccharide (LPS) (*Escherichia coli* O111, Sigma Aldrich, USA). LPS was dissolved in PBS, and the concentration of LPS was set to 1.0 µg/mL following previous reports (Lyu & Park 2005, Uddin et al. 2012). Accordingly, dissolved vitamin C was added to the culture plate for the vitamin C group and distilled water was added to the culture plate for the control. FCS was added to both groups. In the case of LPS stimulation, LPS was added to the culture plate. Eventually, the above reagent-treated cells were added to the culture plate. Cells were not treated with vitamin C before this procedure. The number of samples in the control, as well as the vitamin C group with and without LPS stimulation, was 18. Plates were incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 h. After centrifugation, the supernatants were collected and stored at –30°C until analysis.

Examination of cell viability in culture was conducted following previous reports (de Abreu Costa et al. 2017, Yang et al. 1995). The isolated PBMCs were dispensed into 48-well plates at a density of 5×10^5 cells/well, with a final volume of 1 mL/well in RPMI 1640 medium. The order of adding and the concentration of each reagent was the same as described above. The number of samples in the control and the vitamin C group with and without LPS stimulation was 18 for each. PBMCs were incubated at 37°C in a humidified 5% CO₂ atmosphere. The protocol of these experiments was reviewed and approved by the Kagoshima University Laboratory Animal Committee, Japan (Approval No. VM19046).

2. Sample analysis

The total antioxidant capacity in the cell culture supernatant was measured using a commercially available reagent and assayed with a free radical analyzer (FREE Carrio Duo, Wismerll, Italy) (Abuelo et al. 2013, Otomaru et al. 2018). The principle of this test is the reduction from Fe³⁺ to Fe²⁺ by components in a sample. The optical density was measured at 505 nm using a spectrophotometer. TNF-α was measured using specific quantikine enzyme-linked immunosorbent assay kit systems (Fujifilm, Japan); PBMCs were stained with trypan blue (0.5% trypan blue stain, Nacalai Tesque, Japan) after 24 h, 48 h, and 72 h of culture; each cell was classified into live or dead and quantified.

Table 1. Amount and nutrient composition of feed

Item		Age (weeks)								
		1	4	8	12	16	20	24	28	32
Amount (dry matter)										
Milk replacer	(kg)	0.54	0.72	0.92						
Concentrate A	(kg)		0.18	0.45	0.90					
Concentrate B	(kg)			0.45	0.90	2.52	1.80	1.80	1.80	1.80
Concentrate C	(kg)						1.80	1.80	1.80	1.80
Hay (oats)	(kg)	0.02	0.09	0.13	0.34	0.85	3.40	3.40	4.42	4.42
Composition (dry basis)										
Total digestible nutrients	(%)	103.4	102.4	100.2	95.8	90.3	77.5	77.5	77.5	77.5
Crude protein	(%)	27.4	27.1	26.3	24.9	23.1	18.8	18.8	18.8	18.8
Crude fat	(%)	15.5	15.1	14.0	11.8	9.0	2.2	2.2	2.2	2.2
Calcium	(%)	1.34	1.31	1.25	1.13	0.97	0.61	0.61	0.61	0.61
Phosphorus	(%)	0.72	0.71	0.69	0.66	0.62	0.52	0.52	0.52	0.52
Magnesium	(%)	0.11	0.11	0.12	0.14	0.16	0.21	0.21	0.21	0.21

3. Statistical analyses

Data were expressed as mean $\pm$ SD. Statistical analysis was conducted to determine the differences between the two groups within the same stimulation and incubation time using Student's *t* test with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 2.13.0). *P* values of <0.05 were considered statistically significant.

Results

Chelate quantification revealed that total antioxidant capacity in the vitamin C group with or without LPS stimulation was significantly higher than that in the control group ($P < 0.01$ and $P < 0.05$, respectively), and this antioxidant capacity tended to be higher with LPS stimulation than without LPS stimulation (Fig. 1). The TNF- α without LPS stimulation did not show a significant difference between the groups (Fig. 2). However, with LPS stimulation, the TNF- α in the control group was significantly higher than that in the vitamin C group ($P < 0.05$). Cell viability without LPS stimulation gradually decreased in both groups, and there was no significant difference between the groups (Fig. 3 a)); in contrast, cell viability with LPS stimulation decreased sharply after 24 h of culture in both groups, and there was a significant difference between the groups after 72 h of culture ($P < 0.05$) (Fig. 3 b)).

Discussion

This study evaluated the effects of vitamin C supplementation to bovine PBMCs on antioxidant and inflammatory biomarker status and cell viability. As a result, the total antioxidant capacity in the vitamin C group with or without LPS stimulation was significantly higher than that in the control group. The TNF- α in the control group with LPS stimulation was significantly higher than that in the vitamin C group. Cell viability in the vitamin C group with LPS stimulation after 72 h of culture was significantly higher than that in the control group.

TNF- α , an inflammatory cytokine whose secretion is induced by LPS, is mainly produced by monocytes and T-cells (Caims et al. 2018). Vitamin C is essential for the biosynthesis of collagen and a cofactor in the biosynthesis of catecholamines and some peptide hormones (Mousavi et al. 2019). Further, it has been shown to capture free radicals, remove active oxygen species, and possess anti-inflammatory properties (Block et al. 2009, Oudemans-van Straaten et al. 2014, Pauling 1971, Yang et al. 2006). Vitamin C has also been reported to increase nuclear factor-erythroid 2-related factor 2 (Nrf2) activity in mouse macrophages and human hepatocytes (Kim et al. 2015, Li et al. 2019). Nrf2 is a transcription factor whose activity induces antioxidant and anti-inflammatory properties in cells (Bao et al. 2021, Mo et al. 2014, Nadeem et al. 2020). Thus, vitamin C supplementation to bovine PBMCs might enhance antioxidant capacity and suppress TNF- α production by increasing Nrf2 activity.

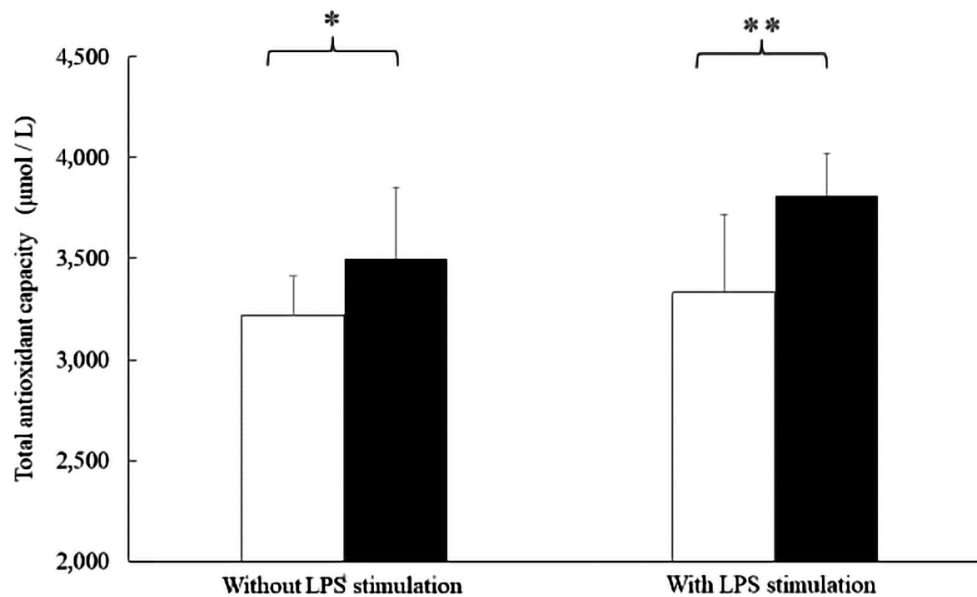


Fig. 1. Effects of vitamin C on total antioxidant capacity in the cell culture supernatant
Control group (empty square, n = 18), vitamin C group (dark square, n = 18)
Data are shown as mean ± SD.
Asterisks indicate significant difference between groups (*: $P < 0.05$, **: $P < 0.01$).

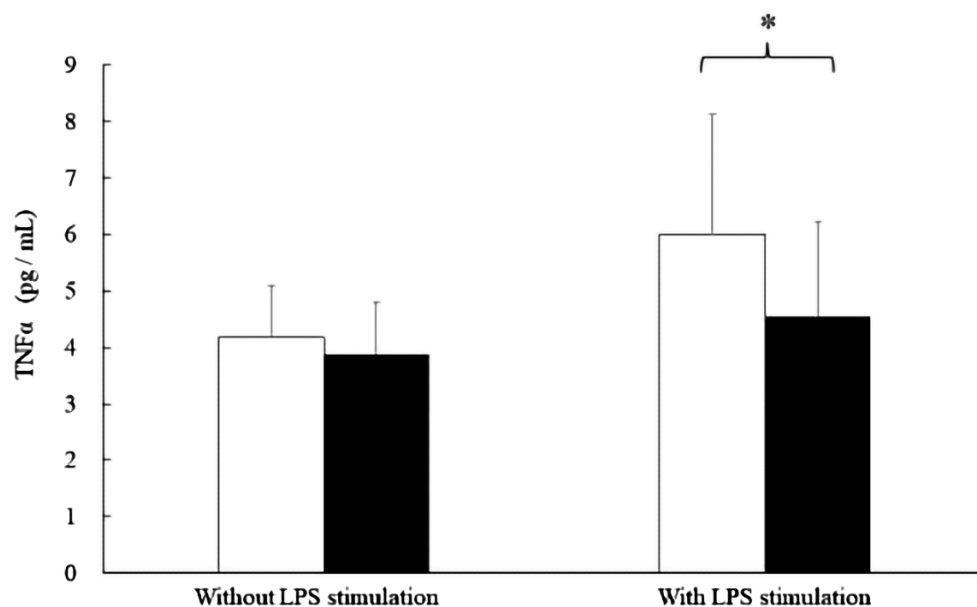


Fig. 2. Effects of vitamin C on TNFα in the cell culture supernatant
Control group (empty square, n = 18), vitamin C group (dark square, n = 18)
Data are shown as mean ± SD.
Asterisks indicate significant difference between groups (*: $P < 0.05$).

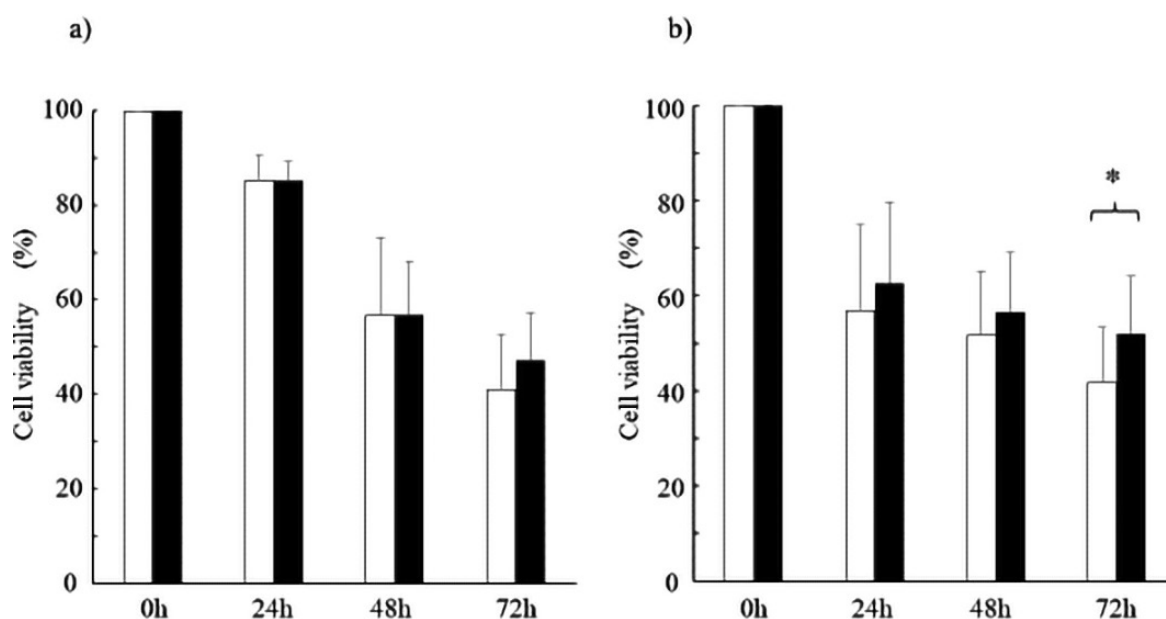


Fig. 3. Effects of vitamin C on cell viability cultured without and with LPS stimulation

a) without LPS stimulation, b) with LPS stimulation

Control group (empty square, n = 18), vitamin C group (dark square, n = 18)

Data are shown as mean $\pm$ SD.

Asterisks indicate significant difference between the groups at same incubation time (*: $P < 0.05$).

Reports showed that vitamin C supplementation to human blood plasma and lymphocytes increased antioxidant capacity (Bouamama et al. 2017, Hong et al. 2004), and vitamin C supplementation to human blood monocytes and lymphocytes or human alveolar macrophages reduced TNF- α production (Chen et al. 2014, Hartel et al. 2004).

Apoptosis is induced by many factors and has complex pathways (Elmore 2007), such as the TNF- α -related pathway (Mendez et al. 1995, Obeng 2021). When TNF- α binds to the TNF receptor on the cell surface, the TNF receptor-related death domain induces the death-inducing signaling complex, which induces cell apoptosis. It has been reported that vitamin C suppresses TNF- α production (Chen et al. 2014, Hartel et al. 2004) and caspases, which are enzymes involved in the apoptosis cascade (Gong et al. 2022, Perez-Cruz et al. 2003). Thus, vitamin C supplementation to bovine PBMCs might suppress cells apoptosis by either decreasing TNF- α production or attenuating the apoptosis cascade. Reports also showed that vitamin C supplementation to human blood monocytes, human hepatocytes, and mouse lymph node cells decreased cells apoptosis (Campbell et al. 1999, Li et al. 2019, Perez-Cruz et al. 2003).

These results suggested that vitamin C supplementation to bovine PBMCs with LPS stimulation

improves antioxidant property, anti-inflammatory effects, and cell viability. However, further studies are needed to clarify the effect of vitamin C on bovine cells, such as activity against viral and parasitic infections, for effective health management of cattle.

References

- Abuelo, A. et al. (2013) Oxidative stress index (Osi) as a new tool to assess redox status in dairy cattle during the transition period. *Animal*, **7**, 1374-1378.
- Apetrei, N. S. et al. (2010) The effects of some Curdian derivatives on Dectin-1 expression and cytokine production in human peripheral blood mononuclear cells. *Roum. Arch. Microbiol. Immunol.*, **69**, 61-66.
- Bao, H. et al. (2021) Nrf2 exerts anti-inflammatory effects in LPS-induced gEECs by inhibiting the activation of the NF- κ B. *Pol. J. Vet. Sci.*, **16**, 443-451.
- Bergman, M. et al. (2004) In vitro immune response of human peripheral blood cells to vitamins C and E. *J. Nutr. Biochem.*, **15**, 45-50.
- Bleilevens, C. et al. (2019) Influence of vitamin C on antioxidant capacity of in vitro perfused porcine kidneys. *Nutrients*, **11**, 774.
- Block, G. et al. (2009) Vitamin C treatment reduces elevated C-reactive protein. *Free Radic. Biol. Med.*, **46**, 70-77.
- Bouamama, S. et al. (2017) Effects of exogenous vitamins A, C, and E and NADH supplementation on proliferation, cytokines release, and cell redox status of lymphocytes from healthy aged subjects. *Appl. Physiol. Nutr. Metab.*, **42**, 579-587.

- Cairns, C. B. et al. (2018) Bench to bedside tumor necrosis factor- α : from inflammation to resuscitation. *Acad. Emerg. Med.*, **7**, 930-941.
- Campbell, J. D. et al. (1999) Ascorbic acid is a potent inhibitor of various forms of T cell apoptosis. *Cell Immunol.*, **194**, 1-5.
- Chen, Y. et al. (2014) Vitamin C mitigates oxidative stress and tumor necrosis factor- α in severe community-acquired pneumonia and LPS-induced macrophages. *Mediators. Inflamm. Med. Sci. Monit.*, **22**, 687-696.
- De Abreu Costa, L. et al. (2017) Dimethyl sulfoxide (DMSO) decreases cell proliferation and TNF- α , IFN- γ , and IL-2 cytokines production in cultures of peripheral blood lymphocytes. *Molecules*, **22**, 1789.
- Du, W. D. et al. (2003) Therapeutic efficacy of high-dose vitamin C on acute pancreatitis and its potential mechanisms. *World J. Gastroenterol.*, **9**, 2565-2569.
- Elmore, S. (2007) Apoptosis: a review of programmed cell death. *Toxicol. Pathol.*, **35**, 495-516.
- Gong, L. et al. (2022) Protective effects of vitamin C against Neomycin-induced apoptosis in HEI-OC1 auditory cell. *Neural Plast.*, **2022**, 1298692.
- Hartel, C. et al. (2004) Effects of vitamin C on intracytoplasmic cytokine production in human whole blood monocytes and lymphocytes. *Cytokine*, **27**, 101-106.
- He, L. et al. (2017) Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *Cell Physiol. Biochem.*, **44**, 532-553.
- Hemingway, D. C. (1991) Vitamin C in the prevention of neonatal calf diarrhea. *Can. Vet. J.*, **32**, 184.
- Hong, S. Y. et al. (2002) Effect of vitamin C on plasma total antioxidant status in patients with paraquat intoxication. *Toxicol. Lett.*, **126**, 51-59.
- Janský, L. et al. (2003) Dynamics of cytokine production in human peripheral blood mononuclear cells stimulated by LPS or infected by *Borrelia*. *Physiol. Res.*, **52**, 593-598.
- Li, Y. et al. (2019) Identification of lead-produced lipid hydroperoxides in human HepG2 cells and protection using rosmarinic and ascorbic acids with a reference to their regulatory roles on Nrf2-Keap1 antioxidant pathway. *Chem. Biol. Interact.*, **314**, 108847.
- Lyu, S. Y. & Park, W. B. (2005) Production of cytokine and NO by RAW 264.7 macrophages and PBMC in vitro incubation with flavonoids. *Arch. Pharm. Res.*, **28**, 573-581.
- Maeda, Y. et al. (2011) Comparison of the Immunosuppressive effects of dexamethasone, flunixin meglumine and meloxicam on the in vitro response of calf peripheral blood mononuclear cells. *J. Vet. Med. Sci.*, **73**, 957-960.
- Mendez, C. et al. (1995) Antioxidants attenuate endotoxin-induced activation of alveolar macrophages. *Surgery*, **118**, 412-420.
- Ministry of Agriculture, Factory and Fisheries Research Council Secretariat (MAFF) (2008) Japanese feeding standard for beef cattle. Japan Livestock Industry Association, Tokyo [In Japanese].
- Mo, C. et al. (2014) The crosstalk between Nrf2 and AMPK signal pathways is important for the anti-inflammatory effect of berberine in LPS-stimulated macrophages and endotoxin-shocked mice. *Antioxid. Redox Signal.*, **20**, 574-588.
- Mousavi, S. et al. (2019) Immunomodulatory and antimicrobial effects of vitamin C. *Eur. J. Microbiol. Immunol.*, **9**, 73-79.
- Nadeem, A. et al. (2020) Differential regulation of Nrf2 is linked to elevated inflammation and nitrative stress in monocytes of children with autism. *Psychoneuroendocrinology*, **113**, 104554.
- Niki, E. (1991) Action of ascorbic acid as a scavenger of active and stable oxygen radicals. *Am. J. Clin. Nutr.*, **54**, 1119S-1124S.
- Obeng, E. (2021) Apoptosis (programmed cell death) and its signals - A review. *Braz. J. Biol.*, **81**, 1133-1143.
- Otomaru, K. et al. (2018) Effect of beta-carotene supplementation on the serum oxidative stress biomarker and antibody titer against live bovine respiratory syncytial virus vaccination in Japanese Black calves. *Vet. Sci.*, **5**, 102.
- Otomaru, K. et al. (2020) Effects of vitamin C supplementation on the blood oxidative stress and antibody titre against *Histophilus somni* vaccination in calves. *J. Vet. Res.*, **65**, 73-78.
- Otomaru, K. et al. (2022) Effects of vitamin E supplementation on antioxidant, inflammatory biomarker, and cell viability of peripheral blood mononuclear cells from Japanese black calves with or without lipopolysaccharide stimulation. *J. Nutr. Sci. Vitaminol.*, **68**, 470-474.
- Oudemans-van Straaten, H. M. et al. (2014) Vitamin C revisited. *Crit. Care.*, **18**, 460.
- Pauling, L. (1971) The significance of the evidence about ascorbic acid and the common cold. *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 2678-2681.
- Perez-Cruz, I. et al. (2003) Vitamin C inhibits FAS-induced apoptosis in monocytes and U937 cells. *Blood*, **102**, 336-343.
- Saha, S. et al. (2020) An overview of Nrf2 signaling pathway and its role in inflammation. *Molecules*, **25**, 5474.
- Thimmulappa, R. K. et al. (2007) Preclinical evaluation of targeting the Nrf2 pathway by triterpenoids (CDDO-Im and CDDO-Me) for protection from LPS-induced inflammatory response and reactive oxygen species in human peripheral blood mononuclear cells and neutrophils. *Antioxid. Redox Signal.*, **9**, 1963-1970.
- Uddin, M. J. (2012) Expression dynamics of Toll-like receptors mRNA and cytokines in porcine peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. *Vet. Immunol. Immunopathol.*, **147**, 211-222.
- Yang, C. C. et al. (2006) Effects of vitamin C infusion and vitamin E-coated membrane on hemodialysis-induced oxidative stress. *Kidney Int.*, **69**, 706-714.
- Yang, Y. et al. (1995) Retinoic acid inhibition of ex vivo human immunodeficiency virus-associated apoptosis of peripheral blood cells. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 3051-3015.