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Concentration of Products of Nitric Oxide Oxidation and Some Vitamins in Sheep with Naturally Acquired Babesiosis

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Abstract: The aim of the present study was to determine serum concentrations of the products of nitric oxide oxidation (nitrate and nitrite) and some vitamins (retinol acetate, α- and δ-tocopherol, and vitamin D_3) in sheep naturally infected with *Babesia ovis*. The investigation included 30 infected and 10 control sheep. Serum α-tocopherol levels were significantly lower ($P < 0.05$), and nitrate and nitrite concentrations were significantly higher ($P < 0.05$) in infected animals than in controls. It is thought that the elevated nitrate and nitrite levels of the sheep infected with babesiosis were due to the result of damage caused by Babesia. On the other hand, a significant decrease was observed in α-tocopherol levels in sheep with babesiosis because of damage and pathophysiological changes to erythrocytes.

Key Words: Babesiosis, nitric oxide, sheep, vitamin

Babesiosizli Koyunlarda Nitrik Oksit Oksidasyon Ürünleri ve Bazı Vitaminlerin Düzeyleri

Özet: Sunulan çalışmanın amacı doğal olarak *Babesia ovis* ile enfekte olmuş koyunlarda nitrik oksitin (NO) oksidasyon ürünleri (nitrat ve nitrit) ve bazı vitaminlerin (retinol asetat , α- ve δ-tokoferol ve vitamin D_3) serum düzeylerini saptamaktır. Araştırma 30 enfekte ve 10 sağlıklı koyunda yapıldı. Enfekte hayvanlarda kontrollere göre α-tokoferol düzeyi istatistiksel olarak önemli düzeyde azalırken ($P < 0,05$), nitrat ve nitrit miktarları önemli düzeyde artmıştır ($P < 0,05$). Nitrat ve nitrit düzeylerindeki artışın Babaesia tarafından yapılan hasar neticesinde oluştuğu, diğer taraftan babesiosizli koyunlardaki α-tokoferol düzeyinin eritrositlerdeki hasar ve fizyopatolojik değişiklikler sebebiyle azaldığı düşünülmektedir.

Anahtar Sözcükler: Babesiosis, nitrik oksit, koyun, vitamin

Introduction

The genus Babesia is composed of intra-erythrocytic protozoan parasites of domestic and wild animals that cause anemia and hemoglobinuria. Ovine babesiosis is the most important disease of small ruminants caused by *Babesia ovis*, *Babesia motasi*, and *Babesia crassa*. Among those, *Babesia ovis* is the most pathogenic (1).

Nitric oxide (NO) is produced by a number of different cell types in response to cytokine stimulation, and is reported to play a role in immunologically mediated protection against a growing list of protozoan and helminthic parasites, both in vitro and in animal models (2). There is also evidence that NO exerts an important selective pressure on parasites (3). NO results from the oxidative deimination of L-arginine to L-citrulline via NO synthase (NOS) (4). Due to the very short half-life of NO in aqueous solutions (5) it is generally measured indirectly via its metabolites–nitrate and nitrite–collectively referred to as reactive nitrogen intermediates (RNIs).

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Vitamins are essential to health and must be supplied by food. Worldwide, vitamin deficiency still results in death, either directly or by reducing resistance to illnesses. Anti-oxidant vitamins such as E and A protect cells from the damage caused by the free oxygen radicals generated by parasites (6).

The aim of the present study was to measure serum concentrations of the products of NO oxidation and some vitamins (retinol acetate, α- and δ-tocopherol, and cholecalciferol [vitamin D_3]) in sheep with naturally acquired babesiosis.

Materials and Methods

Animals

The study included 40 Akkaraman sheep (weight: 25-30 kg; age 4-5 years old). The sheep were obtained from villages in the region of Van, Turkey between June and August 2005. All the animals were field grazed. Thirty of the sheep were naturally infected with *Babesia ovis* and 10 control animals were clinically healthy. All the sheep were submitted to clinical and parasitological examinations.

Blood Sampling

Blood samples from the jugular vein and from ear vessels were collected for the analysis of NO oxidation products and vitamins, and for the preparation of thin blood smears. Serum samples were obtained after centrifugation at 1700 × g for 15 min at room temperature and were stored at -20 °C until used. Thin blood smears were fixed in methanol and stained with Giemsa for microscopic detection of Babesia and the assessment of parasitemia.

Measurement of Serum Vitamins, Nitrate, and Nitrite Concentrations

Quantitative analysis of serum vitamin levels was performed by high performance liquid chromatography (HPLC, Agilent-1100 series, Germany), according to a modified procedure based on the literature (7-9). For the measurement of vitamins A, D, and E, 100 µl of ethanol was added to 100 µl of serum and vortexed. Then, 700 µl of hexane was added and the mixture was vortexed for 2 min. After centrifugation the lipophilic hexane phase was collected and dried under N_2 gas. Then, it was redissolved in 200 µl of ethanol, and 20-µl samples were injected into an HPLC system fitted with a 5-µm C_{18} reverse-phase column (C_{18}, 250 × 4.6 mm, Ace, Scotland). As the mobile phase, methanol-water (98:2) was used at 1.5 ml/min. For the quantitative measurement of vitamins (A, D, and E), a DAD (diode-array detector) was employed at 325, 265, and 290 nm wavelengths, respectively. All chemical reagents were of analytical grade and obtained from Merck (Germany). Double distilled water was used throughout the study.

Concentrations of serum nitrate and nitrite were measured using a coupling reagent (10).

Statistical Analysis

Results are expressed as means ± standard deviation, Duncan's test was used for statistical analysis, and statistical significance was set at $P < 0.05$.

Results

Biochemical parameters of the infected and control groups are shown in the Table. Blood smears prepared from the 30 infected animals showed the presence of piroplasm of *Babesia ovis* in the red blood cells with different parasitemias. On the other hand, no piroplasm was detected in the control animals.

Table. Biochemical parameters of the infected and control groups.

Parameters	Control (n = 10) X ± SD	Infected Group (n = 30) X ± SD
Retinol Acetate (µg/ml)	0.0756 ± 0.0133	0.0717 ± 0.0182
α-Tocopherol (µg/ml)	0.7878 ± 0.1597	0.6122 ± 0.1675*
δ-Tocopherol (µg/ml)	0.6900 ± 0.1271	0.5744 ± 0.1719
Vitamin D_3 (µg/ml)	0.1256 ± 0.0371	0.1217 ± 0.0420
Nitrate (µg/ml)	4.6025 ± 2.2759	9.0147 ± 5.5896*
Nitrite (µg/ml)	1.5413 ± 1.3663	2.6275 ± 1.6673*

*P < 0.05

Serum α-tocopherol values of the infected group were significantly lower, and the nitrate and nitrite concentrations of the infected group were significantly higher than those in the control group ($P < 0.05$).

No statistically significant differences were noted in the concentrations of retinol acetate, vitamin D_3, and δ-tocopherol between the infected and control groups ($P > 0.05$).

Discussion

NO is produced by inducible nitric oxide synthase (iNOS) in macrophages during acute infection and has been shown to mediate resistance against several pathogenic species (11). Parasite-activated macrophages inhibit parasite growth during acute infection, and contribute to the development of acquired T-cell-mediated and humoral immunity by presenting antigens and directing a type-1 immune response via the production of certain cytokines (12). Cytokines, including interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), produced by macrophages and other antigen-presenting cells are critical for generating and regulating innate and acquired immune responses against many pathogens (2,13). IFN-γ and TNF-α are also thought to enhance NO-mediated parasiticidal activity (2,14). TNF-α enhanced neutrophil-mediated killing of mouse malarial parasites and TNF-α is an important component of immune effector mechanisms involved in the destruction of the malarial parasite (15), and in concert with IFN-γ stimulates the production of NO by murine and bovine macrophages (16). Because IFN-γ activates macrophages, it is hypothesized to be a key cytokine in the protective immune response to Babesia parasites (17). Administration of the iNOS inhibitor aminoguanidine to calves experimentally infected with *B. bovis* resulted in reductions in fever, parasitemia, and the severity of anemia, providing evidence of a role for NO produced in response to acute infection in the pathology of bovine babesiosis (18). Conversely, Hanafusa et al. (13) reported that, while NO production increased in horses experimentally infected with *B. caballi*, inhibition of NO led to an increase in parasitemia and NO may have been a critical effector molecule of immune defense against the parasite. Intact and fractionated *B. bovis* parasites stimulated iNOS and NO production in bovine macrophages in vitro (12,19,20), while NO donors partially inhibited the growth of *B. bovis* in culture (12,20).

In the present study serum concentration of NO products increased significantly in sheep with naturally acquired babesiosis. Nitrate and nitrite concentrations in the infected group were significantly higher than in the control group ($P < 0.05$). The elevated levels of serum nitrate and nitrite in the sheep infected with babesiosis were the result of damage caused by Babesia.

Reactive oxygen species (ROS) substantially contribute to the pathogenesis of parasitic diseases (21). Production of cytokines and free radicals is reported to be partially involved in the pathogenesis of bovine and canine babesiosis (22,23). Antioxidant systems comprised of vitamins have cellular protective action against oxidative stress induced by the parasite, resulting in cell, organ, and tissue damage. ROS could affect host tissues, including unparasitized erythrocytes (RBC), as well as parasitized erythrocytes (PRBC) and their contents. PRBC not only trigger the oxidative burst of macrophages, but also act as target cells in a cytotoxic assay, resulting in intra-erythrocytic death of the parasite (24).

Vitamin E is one of the most potent antioxidants. Bovine erythrocytes infected with *B. bovis* were analyzed and a decrease in the antioxidant vitamin E was observed (25). In the present study serum vitamin E levels were significantly lower in the infected sheep ($P < 0.05$) than in the control group, whereas serum δ-tocopherol levels were not statistically different between the 2 groups ($P > 0.05$).

Vitamin A has antioxidant activity and plays an important role in the body's ability to develop an immune response to infection. Vitamin A-deficient animals have impaired immune responses in the presence of infection (26). Serum retinol was also reported to be significantly lower in children with malarial infection (27). Stoltzfus et al. (28) reported that vitamin A deficiency increases the severity of malarial infection in rats. In the present study no significant differences were observed in the concentrations of retinol acetate between the infected and control groups ($P > 0.05$).

Vitamin D plays an important role in calcium and skeletal homeostasis. In addition, there is increasing evidence that 1,25-dihydroxyvitamin D_3 may serve as a modulator of the immune response. The function of 1,25-dihydroxyvitamin D_3 in the immune system may depend, in part, on its ability to alter cytokine signals (29). 1,25 Dihydroxyvitamin D_3 inhibits production of monocyte/macrophage-derived cytokines, such as interleukin-1α, interleukin-6, and TNF-α, at the post-transcriptional level, most likely by reducing the half-life of specific mRNAs. The proliferation of T-cells and their release of such cytokines as IL-2 and interferon gamma are also suppressed by 1,25-D_3, partly as a result of the reduced production of T-cell-activating cytokines (interleukin-1α, TNF-α) because of a direct effect on T-cells (30); however, in the present study no significant differences were observed in the concentrations of

vitamin D_3 between the infected and control groups ($P > 0.05$).

It is thought that elevated nitrate and nitrite levels of the sheep with babesiosis were the result of damage caused by Babesia. On the other hand, a significant decrease was observed in α-tocopherol levels in the sheep with babesiosis because of damage and pathophysiological changes to erythrocytes.

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