Natural humoral immunity in turkey breeders and broilers, healthy and with hereditary muscular dystrophy, reared under comfortable or stressful microclimatic conditions

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SUMMARY

The aim of the present study was to investigate the co-influence of hereditary muscular dystrophy (HMD) and microclimatic stress factors on blood serum lysozyme concentrations and alternative pathway of complement activation (APCA) in turkey breeders and turkey broilers. The HMD prevalences in Lightweight laying (LL) and Heavyweight bred-for-meat (HM) 180 day old turkey breeders were 5.0% and 7.1% respectively. The lysozyme concentrations and APCA were measured in 20 healthy controls (10 LL and 10 HM) and in all diseased breeders. These 2 parameters were strongly increased in diseased LL and HM breeders compared to the corresponding healthy controls and they were significantly lower in the HM line than in the LL one (P < 0.01). In 52 day old turkey breeders, HMD has been identified in 11.7% of birds reared under comfortable housing conditions and in 13.3% of birds reared under stressful conditions (high temperatures and ammoniac concentrations). The 2 non specific immune parameters were determined in groups of 10 breeders each with or without muscular dystrophy and reared under favourable or unfavourable microclimatic conditions. Lysozyme concentrations and APCA values were dramatically increased in diseased breeders compared to the respective controls and they were significantly depressed in birds reared under an ambient stress compared to broilers reared under comfortable conditions (P < 0.01 for healthy controls and P < 0.05 for diseased animals). These results clearly indicate that the hereditary muscular dystrophy activated non-specific defence systems and that both high ambient temperatures and ammoniac concentrations had a negative impact on the non-specific immunity.

Keywords: Hereditary muscular dystrophy, turkeys, breeder, turkey broiler, lysozyme, complement, microclimatic stress, ambient temperature, ammoniac.

RÉSUMÉ

Immunité humorale naturelle chez des dindons reproducteurs et des dindonneaux, sains et atteints de dystrophie musculaire héréditaire, élevés dans des conditions microclimatiques convenables ou stressantes.

Le but de cette étude a été de rechercher les effets conjoints de la dystrophie musculaire héréditaire (DMH) et des facteurs microclimatiques de stress sur les concentrations sérico-lysosomiques de lysozyme et l’activation du complément par la voie alterne (ACVA) chez les dindons reproducteurs et les dindonneaux. Chez les reproducteurs âgés de 180 jours appartenant à la lignée PL (pondreuse légère) et HPV (Haute production en viande), les prévalences de la DMH ont été respectivement de 5,0 % et de 7,1 %. Les concentrations de lysozyme et l’activation du complément ont été mesurées sur 20 contrôles sains (10 PL et 10 HPV) et sur tous les reproducteurs malades. Ces 2 paramètres ont été fortement augmentés chez les oiseaux malades de types PL et HPV. Le rapport aux contrôles sains correspondants et ils sont apparus significativement plus faibles chez les reproducteurs HPV que chez les PL (P < 0,01). Chez les dindonneaux de 52 jours, la DMH a été constatée chez 11,7 % des oiseaux élevés dans des conditions d’ambiance favorables et chez 13,3 % des oiseaux soumis à une ambiance stressante (fortes températures et fortes concentrations d’ammoniac). Les 2 paramètres de l’immunité non spécifique ont été mesurés sur des groupes de 10 animaux sains ou atteints de DMH, élevés dans des conditions d’ambiance correctes ou stressantes. Les concentrations en lysozyme et les valeurs de l’ACVA ont été très fortement augmentées chez les oiseaux malades par rapport aux contrôles sains correspondants et elles ont été significativement diminuées chez les dindonneaux soumis à un stress ambiant (P < 0,01 pour les sujets sains et P < 0,05 pour les malades). Ces résultats montrent clairement que la dystrophie musculaire héréditaire active les principaux composants de l’immunité non spécifique (lysozyme et complément) et que de fortes températures combinées à des concentrations élevées en ammoniac, ont des effets négatifs sur ces systèmes.

Mots clés : Dystrophie musculaire héréditaire, dindes, reproducteurs, dindonneaux, lysozyme, complément, stress microclimatique, température ambiante, ammoniac.

Introduction

The systemic resistance is determined by the natural humoral immunity, whose main factors are the complement system, represented by the two pathways of activation - classical and alternative, and lysozyme. Phagocytosis cells are a source of lysozyme and when they are activated, lysozyme activity increases. The complement as a non-specific protection factor was mainly studied in pigs, rabbits, in poultry and some other species [20, 28, 38, 41, 48, 49, 51, 54, 56]. FAINARI et al. [9] reported lysozyme antigenic differences in chickens, quails and turkeys. NATH et al. [32] utilised four chicken synthetic lines (CSML, WSML, CSFL and NNL), selected on the basis of high serum lysozyme activities and
by crossing them obtained a significant heterosis effect with regard to lysozyme and therefore, they achieved a high natural resistance of chickens against pathogens.

The complement system is also important for the natural resistance and antibody response [3, 18, 62]. It is known that the immune factors do not independently operate. Conclusive evidence with this respect is provided by ADI- NOLFI et al. [1], GLYNN [11] and HEDDLE et al. [16], indicating that lysozyme (from chicken eggs) couldn’t lyse E. coli alone. The complement, activated by classic pathway, kills bacteria in the presence of antibodies within a longer time period, but the lytic reaction is enhanced in the presence of lysozyme. PARMENTIER et al. [36] established linear variations in the complement activity in hens. Later, the same authors [37] examined the relationship between the activity of the alternative pathway of complement activation (APCA) and the chicken major histocompatibility complex (B) in chickens and found out that poultry with blood group antigens B2 and B31 had a statistically significant higher activity and were more resistant to the Marek’s disease virus whereas the holders of antigens B14 and B15 had a lower activity. Perhaps, the higher APCA activity was one of the reasons for the higher resistance to this virus.

On the other hand, communications for the stressors’ impact on the performance of non-specific immune defence are scarce. The results of LECHOWSKI et al. [22] from the exposure of 15 pigs to different types of stress showed that immobilization highly increased the serum lysozyme activities. The authors explained this with the mobilization of natural systemic defence. According to GUDEV et al. [13], the higher complement activity after stress had a positive impact on the lysozyme microbicidal effect. DEMERS and BAYNE [6] obtained similar results by removing a group of rainbow trouts from the water stress and then determined the concentrations of lysozyme, cortisol and adrenaline. The authors observed increases of all these indicators. In their opinion, the acute short-time stress had a beneficial effect on cellular and humoral components of natural immunity. Moreover, LIU et al. [26] found out that lysozyme had a protective effect against free radicals and oxidative stress. Unlike the short-term effect of stress factors, prolonged exposure to stress suppressed the animal immune system [17, 29, 42, 59, 63]. The high ambient temperature and ammoniac concentrations in poultry reared on litter are such stress factors [14, 19, 24, 25, 39, 40, 53]. Prolonged stress had a negative effect on the immune responsiveness of poultry, exacerbated the course of various diseases and made difficult the treatment of already existing illnesses [4, 61]. In our previous experiments [56], we examined the influence of dietary induced muscular dystrophy (experimentally reproduced by deficiency of Se, vitamin E and sulphur-containing α-amino-acids in the diet), in turkey broilers reared under environmental stress conditions, on parameters of natural defence (lysozyme and complement), and reported a decrease in these parameters. The hereditary muscular dystrophy (HMD) in turkey breeders and broilers was differentiated from the dietary induced muscular dystrophy by a significantly lower blood creatine kinase activity [21, 27, 33, 56, 58] and by a smaller number and more volatile creatine kinase SH-groups in birds with HMD [34, 46].

As there is no report on the impact of hereditary muscular dystrophy, combined with environmental stress factors on lysozyme and complement activity in poultry, the aim of the present study was to investigate the co-influence of hereditary muscular dystrophy and microclimatic stress factors on blood lysozyme and complement activities in turkey breeders [Light weight laying (LL) and Heavy weight bred-for-meat (HM)] and turkey broilers.

**Materials and Methods**

**ANIMALS AND PROTOCOL DESIGN**

The experiments were carried out at the base of the Agricultural Institute, Stara Zagora, and the poultry farm in Krushovitza, Vratsa, Bulgaria.

**Turkey breeders**

The turkey breeders (700 Lightweight layers (LL) and 700 Heavyweight bred-for-meat (HM)) were reared in conditions corresponding to the zoohygienic requirements for this category of poultry [2] for the whole experimental period, i.e. under comfort microclimatic conditions on litter at a density of 1.43/m² (normal density rate: 1.7/m²). Until the 180th day of age, the number of LL turkey breeders with HMD (hereditary muscular dystrophy) was 35 (5.0%), while the number of diseased turkey breeders was 50 (7.14%). On this basis, the following groups of turkey breeders were formed: group I: healthy LL controls (DLL, n = 10) and group II: healthy HM controls (CHM, n = 10), group III: HMD affected LL breeders (DLL, n = 35) and group IV: HMD affected HM breeders (DHM, n = 50).

**Turkey broilers**

A total of 1 200 one day old turkey broilers were reared under different microclimatic conditions. The groups I and III were reared under comfortable microclimatic conditions corresponding to the zoohygienic requirements for this category of poultry for the whole experimental period, whereas from the 11th to the 70th day turkey broilers from the groups II and IV were reared under conditions of microclimatic stress, i.e. at a higher temperature than the recommended one, and at the maximal allowed ammoniac concentration (Table I). Microclimatic parameters were determined by routine methods. The temperature and the relative humidity were determined by a thermohygrograph, the ventilation by a catathermometre, the intensity of light by a luxmetre and the ammoniac concentration (ppm) was determined by indicator tubes. Until the 52 days of age, the number of HMD affected turkey broilers reared under comfort microclimatic conditions was 70 (11.7%), whereas under stress microclimatic conditions, the number of diseased birds was 80 (13.3%). The following groups (10 broilers in each group) were constituted: group I: healthy controls reared under normal
microclimatic conditions (normal temperature and ammoniac concentration) (C-NTNH₃); group II: healthy controls reared under ambient stress conditions (high ambient temperature and ammoniac concentrations) (C-HTNH₃); group III: diseased turkey broilers reared under normal microclimatic conditions (D-NTNH₃) and group IV: diseased turkey broilers reared under ambient stress (D-HTNH₃).

**BLOOD SAMPLING AND ANALYSES**

Blood for analyses (5 mL) was sampled into sterile tubes without anticoagulant at the 180th day from the v. subcutaneus ulnaris from the turkey breeders and at the 52nd day from broilers. The blood was allowed to clot for one hour at room temperature (25°C) and the samples were centrifuged at 2 000g for 10 min at room temperature.

**Serum lysozyme concentrations** were determined by the method of LIE et al. [23]. Briefly, 20 mL of 2% agarose (ICN, UK, Lot 2050) dissolved in phosphate buffer (70 mM Na₂HPO₄ and NaH₂PO₄, pH 6.2) were mixed with 20 mL of a suspension of Micrococcus lysodeicticus-24 hours culture at 67°C. This mixture was poured out in Petri’s dish (14 cm diameter). After solidifying at room temperature 32 wells (5 mm diameter) were made. Fifty microliters of undiluted sera were poured out in each well. Eight standard dilutions (from 0.025 to 3.125 mg/l) of lysozyme (Veterinary Research Institute, Veliko Tirnovo) were used in the same quantity as well. The samples were incubated for 20 hours at 37°C and lytic diameters were measured.

The alternative pathway of complement activation (APCA) was studied by the method of SOTIROV [47]. Each serum sample was first diluted by mixing 100 µL serum with 300 µL veronal – veronal Na buffer (composed by 146 mM NaCl; 1.8 mM 5,5-diethylbarbituric acid sodium salt; 3.12 mM 5,5-diethylbarbituric acid; 1 mM EGTA and 0.8 mM MgCl₂ (these chemicals having to be diluted in 2 L distilled water, pH 7.5, in a stable maternal form and diluted 1/5 with distilled water before use). In U bottomed microplates (Flow Laboratories, UK) 7 other dilutions from each diluted serum were again prepared in veronal-veronal Na buffer: 70 µL diluted serum + 30 µL buffer, 60 µL diluted serum + 40 µL buffer, 50 µL diluted serum + 50 µL buffer, 40 µL diluted serum + 60 µL buffer, 30 µL diluted serum + 70 µL buffer, 20 µL diluted serum + 80 µL buffer and 10 µL diluted serum + 90 µL buffer. The final serum dilutions were respectively 7/40, 6/40, 5/40, 4/40, 3/40, 2/40 and 1/40. Then, 50 µL buffer and 100 µL of 1% rabbit erythrocyte suspension to each dilution were added to each well. After incubation at 37°C for 1 hour, the optical density were measured by “SumaPE2” ELISA reader (Karl Zeiss, Germany) at 540 nm. The final APCA activity was calculated using special computer program developed in the Trakia University, and expressed as CH50 units (a CH50 unit corresponds to 50% of complement-induced haemolysis of applied erythrocytes).

**STATISTICAL ANALYSIS**

The statistical analysis was performed using the Mann–Whitney U-test and using the chi test for comparing the frequency of the hereditary muscular dystrophy according to the breeder line and to the microclimatic conditions. The results were reported as means ± SEM (standard error of the mean). Differences were considered significant when *P < 0.05*.

### Results

The HMD prevalence tended (*P < 0.10*) to be higher in the 180 days old Heavyweight bred-for-meat (HM) breeder line (5.0%) than in the Lightweight layers (LL) line (7.1%) whereas
the proportions of diseased broilers reared under comfortable or stressful ambient conditions were similar, 11.7% and 13.3% respectively.

The results of the lysozyme concentrations and alternative pathway of complement activation (APCA) activities in turkey breeders are presented in Table II. Lysozyme concentrations and complement activation were significantly increased in breeders with muscular dystrophy compared to the corresponding healthy controls whatever the breed bird (Lightweight layers (LL) or Heavyweight bred-for-meat (HM)) (group III vs. group I and group IV vs. group II: \( P < 0.01 \)). For the lysozyme concentrations, the increase percentages were 61.8% for LL breeders and 67.9% for HM breeders and for the complement activity, they were 9.9% and 13.8% respectively. Moreover, the 2 parameters were significantly higher in healthy or diseased LL breeders than in HM breeders (group I vs. group II and group III vs. group IV: \( P < 0.01 \)). The percentages of variations between values observed in healthy or diseased breeders from the 2 breeds were 21.4% and 17.0% for the lysozyme concentrations and 11.9% and 7.9% for the APCA.

As reported in the Table III, HMD affected broilers reared under comfortable or stressful microclimatic conditions. Group I (healthy LL controls (n = 10)); group II (healthy HM controls (n = 10); group III: HMD affected LL breeders (n = 35); group IV: HMD affected HM breeders (n = 50). Results are expressed as means ± standard errors.

**Table II:** Lysozyme concentrations (mg/L) and complement activation by the alternative pathway (APCA, expressed in CH50) in the Lightweight laying (LL) and Heavyweight bred-for-meat (HM) turkey breeders with and without hereditary muscular dystrophy (HMD), reared under comfortable microclimatic conditions. Group I: healthy LL controls (n = 10); group II: healthy HM controls (n = 10); group III: HMD affected LL breeders (n = 35); group IV: HMD affected HM breeders (n = 50). Results are expressed as means ± standard errors.

**Table III:** Lysozyme concentrations (mg/L) and complement activation by the alternative pathway (APCA, expressed in CH50) in diseased turkey breeders and broilers. In addition, the stressful ambient conditions (especially high temperatures and ammonia concentrations) significantly and negatively affected the lysozyme concentrations and the complement activities in turkey breeders with or without HMD.

The activation of the non specific defence systems probably occurs under the influence of dystrophic products of the disease. In our previous studies [55, 56], we found out that blood lysozyme concentrations and complement activities were increased in birds with dietary muscular dystrophy, induced experimentally by artificial deficiency in selenium, vitamin E and sulphur-containing amino acids in poultry feed. Similar increases in blood lysozyme concentrations were reported by SUZUKIA et al. [57], MORGANTE et al. [31], FANG et al. [10] and TSENG et al. [60]. Our data emphasize the important role of complement in the pathogenesis of hereditary muscular dystrophy as shown also by others [5, 8, 15, 44]. Thus, ENGEL and BIESECKER [8] and CORNELIO and DONES [5] observed C3- and C9- components of complement in damaged muscle fibres. At the same time, SEWRY et al. [44] found out C9 and C8 in necrotic fibres and mainly C9 as membrane attack complex. Contrary to all cited investigators, SPULER and ENGEL [52] do not support the opinion about the role of antibody-dependent complement in damaged muscle fibres in many inflammatory muscular diseases. They found no plausible explanation for the involvement of complement in the pathogenesis of various types of muscular dystrophy.
The negative effect of microclimatic stress factors (high temperatures and ammoniac concentrations equal to the maximum allowed) on the lysozyme concentration in turkey broilers could be related to a possible increase in glucocorticoid (cortisone in poultry) concentrations. Many authors believe that the reduction of the lysozyme concentrations under stress results from the increased blood cortisol concentrations [7, 12, 30]. According to PANARELLI et al. [35], glucocorticoid would induce repression of the lysozyme gene transcription, leading to the strong reduction of the circulating lysozyme concentrations, but these authors also outlined that this inhibiting effect was obtained with dexamethasone and cortisol concentrations of at least 1 mmol/L. The effect of microclimatic stress on the decrease in the complement activity in groups reared under different microclimatic conditions could be explained by the results of SHIGO et al. [45] who observed an inhibiting effect of corticosterone upon complement activity in chickens under stress. A similar inhibiting effect of glucocorticoid on complement was also reported by DIMITROV et al. [7] and SEMERDJIEV et al. [43].

Moreover, the blood lysozyme concentrations and ACPA values were significantly lower in the Heavyweight bred-for-meat (HM) control and HMD-affected turkey breeders than in the Lightweight layer breeders, evidencing type differences in these 2 components of the non specific defence system according to turkey lines. In parallel, the frequency of hereditary muscular dystrophy tended to be greater (P < 0.10) in the HM line at the 180th day of age. However, although the stressful housing conditions were coupled to decreased lysozyme concentrations and ACPA values, the proportions of HMD-affected broilers have remained closely related, 11.7% and 13.3% respectively at 52 days of age. These results suggest that the decreases in the non specific defence systems are insufficient for significantly promoting the hereditary muscular dystrophy occurrence in turkey.

As a conclusion, our results were in line with the above-mentioned data and confirmed the important role of complement- and lysozyme activity in the pathogenesis of hereditary muscular dystrophy. At the same time, microclimatic stress factors (higher temperatures and ammoniac concentrations) inhibited both lysozyme concentrations and the complement activation by the alternative pathway in healthy and HMD-affected turkey broilers.

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