Respiratory tract infection induced experimentally by *Ornithobacterium rhinotracheale* in quails: effects on heat shock proteins and apoptosis


**Department of Pathology, Faculty of Veterinary Medicine, Atatürk University, 25240 Erzurum, TURKEY;
**Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University, 36100 Kars, TURKEY;
**Department of Anatomy, Faculty of Medicine, Atatürk University, 25240 Erzurum, TURKEY;
**Department of Pathology, Faculty of Veterinary Medicine, Atatürk University, 25240 Erzurum, TURKEY.

*Corresponding author: kbraterim@gmail.com

SUMMARY

*Ornithobacterium rhinotracheale* (ORT), a Gram-negative pathogen microorganism, causes respiratory tract diseases (RTD) in poultry. The objective of this experiment was to determine how RTD caused by ORT was associated with heat shock protein 70 (HSP-70) and apoptosis in quails. A total of 60 male quails, 10-week old, were equally divided into 2 groups: control (non-infected) and ORT (infected with 1 mL of a pure culture of ORT strain B3263/91, serotype A, 3.8 x10^6 CFU/ml by aerosol route). Then, 10 quails in both groups were sacrificed on days 10, 21, and 42 post-inoculation and organs (nasal sinuses, larynx, trachea, lungs and air sacs) were collected for bacteriological, serological and histopathological (conventional histology, HSP 70 immunohistochemistry and apoptotic index determined with the TUNEL method) evaluations. No ORT was isolated from the control and infected birds. However, 46.7% of infected birds were seropositive. The ORT infected birds exhibited inappetence, sneezing and nasal discharge a week after inoculation. The protein HSP-70 showed immunoreactivity in epithelial cells of the secondary and tertiary bronchi and air vesicles of the lungs and the epithelium of the air sacs, trachea, larynx, and sinuses but significantly declined when histopathological lesions and the apoptotic index increased since the 10th day. On day 42, inflammatory lesions and apoptosis were maximal whereas the HSP-70 expression was markedly depressed in lungs, air sacs and in trachea. As a conclusion, HSP-70 production was inversely correlated with apoptotic cells in ORT infected quails.

Keywords: quails, *Ornithobacterium rhinotracheale*, respiratory tract infection, histopathology, apoptosis, heat shock proteins, hsp-70

Introduction

Respiratory tract diseases (RTD) are commonly encountered health problems in the poultry operations and causes great economical loss. Many biological agents (*i.e.* bacteria, virus, fungus and parasites) cause RTD either alone or together. Non-infectious factors such as seasonal conditions, ventilation, management, and nutrition may contribute to the development of RTD, as well [25]. *Ornithobacterium rhinotracheale* (ORT) is a Gram-negative pathogen microorganism and causes RTD in chickens and turkeys, associated with retarded growth, decreased egg production and quality, increased mortality, and inevitably economic losses in poultry [12, 25]. It has also been isolated...
from other birds including ostriches, geese, ducks, guinea fowl, partridges, pheasants, quails, pigeons and rooks, and also from guinea pigs [6, 18]. The rapid spread of the disease across the world drew the attention of researchers [25] and the bacterium has been isolated from infected animals in many countries, including the United States [6], Germany [14], Turkey [23], and France [18]. A multitude of studies has been conducted in recent years on Ornithobacterium rhinotracheale (ORT) infection, which has gained importance over time due to being one of major causes of RTD [25].

Heat shock proteins (HSP) are a group of highly conserved proteins that are constitutively expressed in most cells under normal physiological conditions in every organism from bacteria to man [1]. They make up approximately 5-10% of the total protein content of cells under conditions of normal, healthy growth [22] and are expressed or increased in response to various biological stressors, including high temperatures [24, 37] viral infections, inflammation [21, 34, 35], malignant tumours [28], irritant substances [30], alcohol, toxic chemicals, and radiation [1]. They are also involved in both intracellular and extracellular immune functions [22] and apoptosis [11, 16]. Among HSPs [31], HSP 70 plays an important role in apoptosis. It is a decisive negative regulator of the mitochondrial pathway of apoptosis that can block apoptosis at different levels: at a pre-mitochondrial stage by inhibiting stress-inducing signalling, at the mitochondrial stage by preventing mitochondrial membrane permeabilisation through the blockage of Bax translocation, and finally, at the post-mitochondrial level by interacting with AIF and Apaf-1 [16].

Apoptosis, or programmed cell death, is a physiological, genetically controlled, cellular response to external and internal stimuli whose purpose is to eliminate unwanted cells, including infected cells, while preventing damage to surrounding cells or tissue. Apoptosis can be stimulated or inhibited by different signals or cytokines. Some of these regulators are pro-apoptotic (Bax, Bik, Bak, Bad, Bcl-Xs, TNF/TNFR1, Fas/FasL, caspases family, NFkB, ATP, H2O2) and some are anti-apoptotic (Bcl-2 family of genes, HSP, proteins-inhibitor of apoptosis) [7]. The balance between pro-apoptotic and anti-apoptotic factors influences the resolution of inflammatory process or development of disease [26]. In addition to this anti-apoptotic (cytoprotective) effect of HSP-70, its anti-inflammatory effect was recently revealed and is thought to be important for its protective role against various diseases [34].

Recent studies have focused on the release of HSPs also known as stress proteins, in response to bacterial infections [31]. However, the production of HSPs and the occurrence of apoptosis in ORT infection are limited. The present study was conducted to determine production of HSP-70 protein and presence of apoptotic cells in quails infected experimentally with ORT using immunohistochemical staining methods.

Materials and Methods

PREPARATION OF BACTERIAL INOCULATE

The bacterial inoculate was prepared from ORT strain B3263/91 (serotype A). Bacteria were grown on blood agar plates with 7% sheep blood and they were incubated in a 5-10% CO2 atmosphere at 37°C for at least 48 hours. Bacterial suspension was adjusted to yield 3.8 x 10^6 viable colony forming units per millilitre (CFU/mL).

ANIMALS AND EXPERIMENTATION

Ten-week old 60 male quails were obtained from the Quail Breeding Unit of Kafkas University and then divided into two equal groups: non-infected control (CONT) and infected with Ornithobacterium rhinotracheale (ORT). The animals in all groups were housed on the floor in wire-separated pens, and a commercial grower diet was provided ad libitum throughout the experiment. Control and treatment groups were reared in separate rooms.

Birds in the ORT group were infected with a pure culture of ORT strain B3263/91 (serotype A) (3.8 x 10^6 CFU/mL, 1 mL) by aerosol route, a fine spray to the quails in an isolator using a commercial paint sprayer. The developed mist was maintained for at least 10 minutes without air circulation. After inoculation, birds were clinically examined every day and weighed weekly. Ten quails in both groups were killed by cervical dislocation on days 10, 21, and 42 post-inoculation. Nasal sinuses, larynx, trachea, lungs, and air sacs were collected immediately for bacteriological, serological, and histopathological evaluations.

LABORATORY ANALYSES

Bacteriology

Swabs from the lungs and trachea were aseptically inoculated on blood Agar supplemented with 7% sheep blood and 10 μg/mL of gentamicin. The plates were incubated in a 5-10% CO2 atmosphere at 37°C for at least 48 hours. Bacterial species were then identified by the colonial morphology and Gram staining results.

Serology

Blood samples were collected prior to slaughtering by antemortemally cardiac puncture into sterile microtubes without anticoagulant. After overnight clotting at 4°C, the samples were centrifuged for 20 min at 4000xg. The sera were carefully harvested and stored at -20°C until assessed.

All sera were tested for antibodies against ORT using an ELISA kit (IDEXX Laboratories, Westbrook, ME, USA). Briefly, samples were diluted in a 1/500 ratio and the OD was measured on an ELISA plate reader (Anthus 2020, Anthos labtec instruments, Salzburg, Austria) at 650 nm. Results
were determined by calculating the sample to positive (S/P) ratio. Samples with S/P ratios of 0.4 or less were considered as negative, and samples having S/P values higher than 0.4 were considered as positive.

**Histopathology**

The tissues were removed and immediately half of them were fixed in 10% neutral buffered formalin. After dehydration in a graded ethanol series and clearing with xylene, the sample material was embedded in paraffin and 4-μm-thick sections were stained with Haematoxylin-Eosin for observation under the light microscope.

**HSP 70 protein immunostaining**

Four µm sections from all the tissue samples were cut and processed for immunohistochemical examination by a standard avidin-biotin-peroxidase method as described by the producer. Mouse monoclonal antibodies that react with human HSP-70 (Clone: BRM-22, Sigma, St. Louis, MO, USA) were used at dilution of 1:5000 for 60 minutes. Negative control tissue sections were incubated with normal mouse serum.

**Detection of apoptotic cells**

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) stain using a commercial ready-to-use kit (In Situ Cell Death Detection Kit, POD, Roche, Mannheim, Germany). Briefly, paraffin sections (4 µm) were mounted on silanized slides. After deparaffinization and rehydration, sections were digested with proteinase K (20 µg/mL, 30 minutes) and quenched with 3% hydrogen peroxide in methanol. Sections were incubated in a humidified chamber in 200 μL of TUNEL (TdT and label solution) at 37°C for 60 minutes and with anti-fluorescein antibody conjugated Peroxidase (POD) converter at 37°C for 30 minutes. The sections were then treated with 3, 3’-diaminobenzidine (DAP) for 5 minutes, washed with phosphate buffered saline (PBS) and counterstained with Mayer’s haematoxylin.

**STATISTICAL ANALYSIS**

The microscopic changes in the all tissue were evaluated by scoring each item that ranged from −, none; +, weak; ++, moderate; and ++++, marked. Serology data were subjected to the MEAN procedure. The main effect of time relative to inoculation and staining methods and their interactions on organ histopathology score were determined by two-way ANOVA (SPSS Inc., Version 17.0, Chicago, IL, USA). In another model, time was replaced with staining method to evaluate staining method by organ interaction. Statistical significance was declared at p < 0.05. Data were expressed as median.

**Results**

**BACTERIOLOGICAL AND SEROLOGICAL FINDINGS**

The bacterium was isolated from respiratory organs of neither the control birds nor the infected birds. None of the control birds yielded positive result in the ELISA technique whereas positive ORT serology was achieved in 46.7% of the inoculated quails (Table I). However, the positivity rate decreased with the advancing experimentation.

**MACROSCOPIC FINDINGS**

Within one week post-infection, the animals exhibited clinical signs including inappetence, nasal discharge, sneezing, and in some, swelling of the sinuses. Throughout the trial, mortalities were observed on days 21 and 42. At the necropsy performed on the 10th day post-infection, oedema, congestion and patches of haemorrhages were observed in the mucosa of the trachea, sinuses, and larynx. The lungs were dark due to congestion, haemorrhages, and oedema and displayed patches of consolidated areas. The air sacs, in particular the abdominal air sacs had thickened and displayed an opaque appearance. Necropsy findings on days 21 and 42 post-inoculation were similar to those noted on day 10 post-inoculation. However, differently, two animals on day 21 and three animals on day 42 displayed thickening of the pleura and the lungs, which contained consolidated areas covered with yellowish fibrin fibrils and were adhered to the costae.

**MICROSCOPIC AND IMMUNOHISTOCHEMICAL FINDINGS**

Microscopic examination revealed the presence of necrosis or hyperplasia of the epithelium of the secondary and tertiary bronchi and air vesicles, the presence of heterophil leukocytes in the lumen, desquamated epithelial

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**Table I:** Frequencies of circulating anti-Ornithobacterium rhinotracheale (ORT) antibodies in quails experimentally inoculated by aerosol route with ORT strain B3263/91 (serotype A) (3.8 x10^8 CFU/mL, 1 mL) determined using specific ELISA test.

<table>
<thead>
<tr>
<th>Day relative to inoculation</th>
<th>ELISA</th>
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<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Day 10 (n = 10)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Day 21 (n = 10)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>Day 42 (n = 10)</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>Total (n = 30)</td>
<td>16 (53.3%)</td>
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Revue Méd. Vét., 2013, 164, 3, 132-140
cells, cellular deposits composed of erythrocytes and mucus, and oedema, congestion, haemorrhages, heterophil leukocytes and mononuclear cell infiltration in the interstitial space (figure 1). Furthermore, lymphoid hyperplasia was noticed in the periphery of blood vessels and secondary and tertiary bronchi. Air sacs exhibited epithelial degeneration and patches of hyperplasia associated with the presence of oedema, congestion, haemorrhages and heterophil leukocyte, lymphocyte, and macrophage infiltration in the propria. No histopathological lesion was evidenced in control birds.

The respiratory tract organs such as sinuses, larynx and trachea (figure 2) showed similar histopathological findings. Mucosae displayed slight epithelial degeneration and desquamation. The propria mucosa showed slight oedema, congestion, haemorrhages of varying severity, and inflammatory cell infiltration. The cellular reaction was of intermediate intensity and was induced by heterophil leukocytes, lymphocytes, and macrophages. The propria mucosa also displayed focal lymphoid hyperplasia. The lumen contained an exudate with necrotic epithelial cells and erythrocytes.

In infected quails, the degree of inflammation varied according to the organs and the day relative to post-inoculation (p < 0.0001 for both factors; figure 3). Moreover, organ by time interaction showed that lung was the most affected respiratory organ (p < 0.001).

The protein HSP-70 showed immunoreactivity in epithelial cells of the secondary and tertiary bronchi and air vesicles of the lungs (figure 4a), the epithelium of the trachea (figure 5a) and a lesser extent in the epithelium of larynx and sinuses. Moreover, HSP was secreted in vascular endothelium of lungs (figure 4b). On the other hand, the presence of apoptotic cells in the above mentioned organs was inversely proportional to the presence of the protein HSP-70 (figures 4c and 4d, figure 5b).

The staining scores according to the respiratory organs and to time after ORT inoculation via the aerosol route were summarized in Table II. Histopathological lesions were prominent in lungs and also in trachea and air sacs at a lesser extend whereas they were remained weak in sinuses and larynx and lesions were maximal on day 42. In the same way, the numbers of apoptotic cells in epithelia and in vascular endothelium of respiratory organs increased in parallel according to time and were maximal in lungs, air sacs and trachea on day 42. Strong positive correlations were found between histopathological lesions and TUNEL staining in lungs and air sacs (r = 0.85, p < 0.0001), trachea (r = 0.80, p < 0.0001) and in sinus (r = 0.89, p < 0.0001) (Table III).
By contrast, HSP immunolabelling was maximal on day 0 in all respiratory organs explored and gradually declined according to time, since the 10th day (Table II). The release of HSP 70 from cells decreased in proportion to the severity of the lesion and the number of the apoptotic cells increased in all respiratory organs; negative and significant correlations were obtained between HSP expression and histopathological scores or the apoptotic index in lungs (r = -0.52, p < 0.001 and r = -0.44, p < 0.01, respectively) air sacs (r = -0.51, p < 0.001 and r = -0.45, p < 0.01) and in trachea (r = -0.42, p < 0.01 and r = -0.40, p < 0.01) (Table III).

**Discussion**

*Ornithobacterium rhinotracheale* was first described in 1991 by DU PREEZ in South Africa as an emerging respiratory disease causing 1-2% mortality in 28-day-old broiler chickens [12]. As it is a bacterial agent difficult to
isolate and identify, ORT has long been overlooked in poultry populations as secondary and opportunistic infections, and it was not until 1994 when its presence was first demonstrated [33]. The disease has been described to have an onset characterized with the swelling of the sinuses, followed by nasal discharge and sneezing, and ever-increasing mortality rates and worsening performance parameters until the end of the growth period. The most significant finding observed at the necropsy of infected birds is the presence of a mucoid white coloured foamy exudate particularly in the abdominal air sacs and the observation of signs of unilateral purulent pneumonia in the lungs. Furthermore, infected animals display lesions of the respiratory system such as rhinitis, sinusitis and tracheitis [9, 13, 15, 32] as well as systemic lesions including hepatitis, pericarditis, arthritis, and neuritis [27]. The macroscopic and microscopic findings obtained in the present study are similar to previous reports.

Bacterial isolation can be performed from the trachea and lungs of poultry infected either naturally or experimentally with ORT. However, tissue localizations of the causative agent, other than the respiratory system, remain unknown.
Heat shock proteins appear to be regulated at the transcriptional level by heat shock factors (HSF), transcription factors that interact with heat shock elements in the promoter regions of heat shock genes [22]. During infection, HSPs serve as an early warning signal during their extracellular release, which activates proinflammatory innate immunity [3], as reflected by increased production of nitric oxide, a product with prominent antimicrobial activity [2, 13]. Studies on the release of HSPs in ORT infection are limited. It is shown that these proteins are expressed at high levels when cells are exposed to high or low temperature or other stressors [1]. In cases of stress [20, 21] and infection, such as with ORT [5], infectious bursal disease [17] and E. coli [21], elevated reactive oxygen species and consequent lipid peroxidation were shown to trigger HSP induction. Among them, HSP-70 family is the most conserved one and performs a series of biological functions such as molecular chaperone, assisting immune response, anti-apoptosis, and enhancing tolerance to stressors [1, 22]. In the present experiment, lesions severity was positively correlated with HSP production, especially at early stage of infection, which indicates activation of body response. Indeed, an ameliorative effect of HSP-70 due to its cytoprotective, anti-inflammatory and molecular chaperone properties has been reported for various respiratory diseases, such as acute respiratory distress syndrome [34], pulmonary hypertension [10], bronchiolitis obliterans syndrome after lung transplantation [36], allergic asthma [19], pulmonary fibrosis [29]. It was also observed that the number of apoptotic cells increased inversely proportional to the secretion of the HSP-70 protein, suggesting that HSP-70 could limit the degree of apoptosis and severity of infection, possibly through inhibiting cytochrome c activity and depressing ROS production [21].

As a conclusion, ORT infection caused HSP secretion at early stage whereas apoptosis became significant at later stage. The data suggest that HSP-70 exerts protective role in ORT infection.

Acknowledgement

The authors would like to thank the Ataturk University Scientific Research Foundation in Turkey for the financial support (Project No. BAP-2009/285). Thanks are extended to Dr. HM. Hafez for donating ORT serotypes and Dr. O. Erganiş, Dr. HH. Hadimli, Dr. H. Eröksüz, and Dr. G. Ozbey for helping in providing serum.

References

ORNITHOBACTERIUM RHINOTRACHEALE CAUSES APOPTOSIS AND DECREASES HSP70 EXPRESSION IN QUAILS


