Oxidative stress in calves with acute or chronic bronchopneumonia

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SUMMARY
The oxidant and antioxidant status were investigated in calves with acute and chronic bronchopneumonia (n = 15 and n = 12, respectively) as well as in healthy animals (n = 22). Blood concentrations of thiobarbituric acid reactive substances (TBARS), lipid peroxides (LPO), reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) superoxide dismutase (SOD) catalase (CAT) activities were measured in all subjects. In parallel, some haematological (erythrocyte and leukocyte counts, haemoglobinemia) and biochemical parameters (blood fibrinogen and total protein concentrations) were also determined. Fibrinogen concentrations were dramatically increased in diseased calves, but, in chronically affected animals, the elevation of fibrinogen concentrations was lower than in calves with the acute disease and was associated with reduction of erythrocyte counts, leucopenia and hypoproteinemia. The markers of oxidative stress (TBARS and LPO) were significantly elevated in diseased calves, mainly in those with chronic bronchopneumonia. Among antioxidants, the SOD activity and the GSH concentrations gradually declined according to the disease duration whereas variations of CAT and GSH-Px activities greatly differed in function of the disease grade: the 2 enzyme activities were enhanced during the acute form and they were depressed during chronic bronchopneumonia. These results demonstrated the occurrence of the oxidative stress in bronchopneumonia, particularly intense and coupled to a strong inflammatory reaction in the chronic form.

Keywords: Calves, Bronchopneumonia, Chronic form, Oxidative stress, Inflammation, Antioxidant status.

Introduction
Bronchopneumonia in calves is a multi-aetiological disease caused by numerous combinations of infectious agents (viral, bacterial, and mycoplasma) compromised host defences, and environmental conditions [8, 14].

The prevalence of calf bronchopneumonia has been investigated in many countries, ranged from 10 - 70% and many calves died [20, 31]. The disease increases as herd size increases. It is more prevalent during the growing period, especially in 1-3 months old animals [2]. Calves being raised and confined in small areas, have more environmentally related respiratory problems, crowding in poorly ventilated barns and accumulation of manure and urine, leading to heavy ammonia concentration in barn air, causing severe irritation to the mucous membrane of the upper respiratory tract, pre-disposing to its inflammation [24]. Inadequate intake or poor quality colostrum affect the calves’ defence against respiratory agents and make them more susceptible to infection [30]. In early spring and autumn, because of the unstable weather and sharp changes between day and night temperatures calves become predispose to the respiratory diseases. One to three months old calves are the most susceptible to upper respiratory tract infection [3].

Bronchopneumonia causes greater economic losses than any other diseases in calves. Without effective and fast help big losses are expected including loss of growth rate, premature culling and death of sick calves, and in addition, the treatment is expensive [2, 33]. Calves affected by bronchopneumonia, exhibit signs of respiratory insufficiency, including fever, cough, nasal discharges and dyspnoea. Pulmonary crackles can be heard easily in advance stages. Fibrinous pneumonia is the primary lesion that observed at necropsy.
Several studies have reported that there is imbalance between lipid peroxides and antioxidants in pneumonia and suggested that this factor may contributes to the damage of pulmonary endothelium [11]. Poorly perfusion in pulmonary tissues may induce free radical processes and the inception of free radical peroxidation [7]. Production of oxidizing agents has been suggested by many workers to occur in many disease problems of cattle such as in milk fever [28], mastitis [27] and retained placenta [12], the antioxidant system become impaired with acceleration in the process of lipid peroxidation. To overcome the toxic effects of different forms of reactive oxygen species, the body is equipped with a variety of antioxidants. Superoxide anions generated during metabolic processes are reduced to hydrogen peroxide in the presence of superoxide dismutase. Both catalase and glutathione peroxidase are responsible for the degradation of hydrogen peroxidase [21].

Oxidative stress is thought to play an important role in the pathogenesis of a number of lung diseases, not only through direct injurious effects, but by involvement in the molecular mechanisms that control lung inflammation [18]. In respiratory tract infections, neutrophils are recruited to the pulmonary tissues to remove the invading micro-organisms by their phagocytic activity [17]. A number of tissue damaging products secreted by neutrophils such as reactive nitrogen intermediates and nitric oxides modulate both acute and chronic inflammatory reactions. Nitric oxide is a mediator of capillary dysfunction and macromolecular leakage. Reaction between superoxide radical and nitric oxide produce the non-radical oxidant peroxynitrite causing severe damage to the pulmonary tissues [32]. When phagocytes are exposed to appropriate stimuli, they form large quantities of superoxide radical, an important precursor of other more reactive species that contribute to pulmonary damage. Moreover, administration of antioxidants has been reported to decrease lung lipid peroxidation after endotoxin infusion [10]. Superoxide radical was found to be ten times higher in calves with bronchopneumonia in its acute and chronic phases by measuring the thiobarbituric acid reactive substances (TBARS), the final products of lipid peroxidation after endotoxin infusion [10]. Superoxide radical and nitric oxide produce the non-radical oxidant peroxynitrite causing severe damage to the pulmonary tissues [32]. When phagocytes are exposed to appropriate stimuli, they form large quantities of superoxide radical, an important precursor of other more reactive species that contribute to pulmonary damage. Moreover, administration of antioxidants has been reported to decrease lung lipid peroxidation after endotoxin infusion [10].

The experiment was conducted on 49 Holstein, 2-3 months old calves during December 2006 to March 2007, belongs to three heard of cattle sized 100-150 head per heard, and situated in Ajloun area, a northern province of Jordan. The animals were housed in stalls that had a concrete floor and were provided green fodder and water ad libitum. Calves of two herds suffer from respiratory distress and bronchopneumonia.

Calves were classified into three groups: the first group comprised clinically healthy calves (n = 22) whereas animals with acute bronchopneumonia (n = 15) and those with chronic bronchopneumonia (n = 12) were included in the second and third groups respectively. The clinical signs used as criteria for acute bronchopneumonia includes high hyperthermia (40°C), anorexia, depression with respiratory grunt, tachycardia and reluctance to lie down, crackles and harsh breath sounds are abnormal lung sounds heard over the thoracic cage during auscultation. For the chronic form of bronchopneumonia a history of pneumonia and bronchitis was observed for at least two weeks. Calves have a rough hair coat and gaunt appearance, respiratory and heart rate are above normal, body temperature usually normal, in some cases a moderate persistent fever seen. A copious bilateral muco-purulent nasal discharges and chronic moist productive cough are common, crackles and loud breath sounds are usually audible over the ventral part of the lung. Calves with chronic bronchopneumonia exhibited characteristic clinical signs for more than 14 days. Healthy calves were monitored twice a week and sick animals were daily examined. General situation of the animals and physical examination including temperature, pulse and respiration, the mucous membrane, appetite of the animals, urination and defecation for each calf were monitored. Auscultation of the heart and lungs were also performed.

Blood samples were collected from the external jugular vein aseptically, while restraining the animal in a standing position, using disposable needles blood was drawn and transferred into a two sealed vacutainer tubes, the first tube containing acid citrate dextrose anticoagulant for the measurements of glutathione peroxidase (GSH-Px) superoxide dismutase (SOD) catalase (CAT) and reduced glutathione (GSH), the second tube containing lithium heparin anticoagulant for the measurements of thiobarbituric acid reactive substances (TBARS) and lipid peroxides (LPO). All samples were placed on ice after collection and transferred to the laboratory without delay for analysis to avoid the in vitro oxidation. Plasma was immediately separated by centrifugation at 3000 g for 10 min at 4°C. Samples were transferred in to vials and stored in deep freezer at -20°C until analysed. Erythrocytes were washed four times with 3 ml of 0.9% NaCl solution for 10 min at 2200 g at 4°C, and then red blood cells were lysed by hypotonic shock using 2.0 ml of cold ultra pure water. The haemolysate was analysed to determine GSH-Px, SOD, catalase and reduced glutathione.

**ANALYTICAL METHODS**

Plasma levels of TBARS were analyzed spectrophotometrically after extraction with n-butanol according to the optimized method of YAGI [34]. Lipid peroxides in plasma were estimated with a test kit (K-Assay, LPO-CC; Kamia Biomedical Company, Seattle, WA, USA) in which lipid per-
oxides are colorimetrically quantified by measuring methylene blue (at 675 nm) produced by cleavage of the methylcarbamoyldimethylamin phenothiazine chromogen during oxidation reactions.

Glutathione peroxidase (GSH – Px) activities were measured by the oxidation of glutathione using tert-butyl hydroperoxide, the oxidized glutathione being later converted to the reduced form in the presence of glutathione reductase and NADPH, leading to the NADPH oxidation into NADP+ and to the reduction of NADPH absorbance at 340 nm. The absorbance change per min and the molar extinction coefficient of NADPH were used to calculate the glutathione peroxidase activity expressed in U/L [23].

The superoxide dismutase (SOD) activity was measured in the haemolysate by the method of SUN et al. [26] in which a xanthine-xanthine oxidase complex produces superoxide radicals which react with nitro blue tetrazolium (NBT) to form the formazan compound. The SOD activity is measured at 560 nm by detecting the inhibition degree of this reaction. One unit of SOD activity is defined as the activity that causes 50% inhibition of (NBTH2) reduction rate and the SOD activity was expressed in U/L.

The catalase (CAT) activity in haemolysate was measured by the method described by AEBI [1], where one unit of catalase activity was defined as the amount of enzyme that produces one mmol hydrogen peroxide per minute at an initial concentration of 10 mmol/L at pH 7.4 and 37°C.

The reduced glutathione concentration (GSH) was determined by the titration with 0.1 mmol/L dithiobis (2-nitrobenzoic acid) in a 0.1 mol/L disodium phosphate buffer solution. The formation of the reduced product, thionitrobenzene, was measured spectrophoto-metrically at 412 nm. The GSH content was expressed as g/L of haemolysate [4]. The fibrinogen concentration was measured by the heat precipitation method according to SCHALM [25].

STATISTICAL ANALYSIS

The collected data were initially assessed for their normal distribution before doing further statistical analysis. The parameters from the 3 different calve groups were compared using a one-way ANOVA followed by a stepwise analysis (Bonferroni correction). All statistical analysis was performed using SPSS v 13® software. Differences were considered as significant when p values were less than 0.05.

Results

The 3 groups of calves were similar in age and sex (Table I) whereas significant differences in weight (p ≤ 0.01) were observed between healthy calves and diseased calves: calves suffering from acute or chronic forms of bronchopneumonia exhibited lower body weights than healthy calves, the mean difference of body weights being 11.3% between calves with acute form and healthy animals and reaching 28.4% in chronically diseased calves and controls.

Whereas no significant difference for erythrocyte count, haemoglobin concentration and hematocrit values was noticed between healthy controls and calves with acute bronchopneumonia (Table II), erythrocyte and leukocyte counts, hematocrit values and total protein concentrations were markedly decreased in calves with chronic disease compared to the 2 other groups (p < 0.01). On the other hand, diseased calves (groups 2 and 3) exhibited higher plasma fibrinogen concentrations than healthy controls (p < 0.01) and the increase of this biochemical parameter was particularly important in calves with acute bronchopneumonia (group 2) (group 2 vs. group 3: p < 0.01).

The variations of lipid peroxidation indicators, i.e. plasma TBARS and LPO concentrations, of antioxidant enzyme (SOD, CAT and GSH-Px) activities and of reduced glutathione concentrations according to groups were shown in figure 1. The 2 markers of oxidative stress were significantly increased in diseased calves compared to the healthy controls (p < 0.01). Furthermore, calves with chronic bronchopneumonia exhibited maximal values (chronic vs. acute form: p < 0.01). On the contrary, antioxidant SOD activity and GSH concentrations gradually declined among the 3 groups (p < 0.01), diseased calves exhibiting lower enzyme activities and reduced glutathione concentrations than controls, particularly those with chronic bronchopneumonia. Whereas CAT and GSH-Px activities were also markedly depressed in chronically affected calves (p < 0.01), these 2 enzyme activities were significantly increased in calves with the acute form compared to controls (p < 0.01).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy (n = 22)</td>
<td>Acute BP (n = 15)</td>
</tr>
<tr>
<td>Age (months)</td>
<td>2.73 ± 0.44</td>
<td>2.76 ± 0.57</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Females</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>87.23 ± 5.70a</td>
<td>77.40 ± 5.40ab</td>
</tr>
</tbody>
</table>

Different superscripts in the same row indicate significant differences. NS: not significant.

Table I: Physical characteristics of healthy (n = 22) and calves with acute (n = 15) or chronic (n = 12) bronchopneumonia (BP). Results are expressed as mean ± standard deviation.
TABLE II: Haematology parameters of healthy (n = 22) and calves with acute (n = 15) or chronic (n = 12) bronchopneumonia (BP).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy (n = 22)</th>
<th>Acute BP (n = 15)</th>
<th>Chronic BP (n = 12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (1012/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.3 ± 1.0a</td>
<td>5.8 ± 0.9a</td>
<td>5.0 ± 0.5b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Range: min - max</td>
<td>4.8 - 8.2</td>
<td>4.5 - 7.3</td>
<td>4.2 - 5.8</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g / L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>9.6 ± 1.6</td>
<td>9.7 ± 1.6</td>
<td>9.1 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Range: min - max</td>
<td>7.4 – 12.0</td>
<td>7.2 – 12.0</td>
<td>7.5 – 11.0</td>
<td></td>
</tr>
<tr>
<td>Hematocrit (L / L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.30 ± 0.04a</td>
<td>0.29 ± 0.03a</td>
<td>0.24 ± 0.03b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Range: min - max</td>
<td>0.25 – 0.38</td>
<td>0.24 – 0.34</td>
<td>0.19 – 0.29</td>
<td></td>
</tr>
<tr>
<td>WBC (109/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>9.7 ± 3.3a</td>
<td>8.2 ± 2.7a</td>
<td>6.8 ± 1.6b</td>
<td>&lt; 0.01</td>
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<tr>
<td>Range: min - max</td>
<td>4.4 – 16</td>
<td>8.2 ± 2.7a</td>
<td>4.8 – 9.5</td>
<td></td>
</tr>
<tr>
<td>TP (g /L)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>58 ± 6a</td>
<td>54 ± 6a</td>
<td>51 ± 4b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Range: min - max</td>
<td>50 – 70</td>
<td>42 – 63</td>
<td>45 – 60</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g /L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>3.58 ± 0.77a</td>
<td>9.33 ± 2.39c</td>
<td>7.27 ± 2.86b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Range: min - max</td>
<td>2.42 – 5.61</td>
<td>6.00 – 13.00</td>
<td>5.00 – 11.60</td>
<td></td>
</tr>
</tbody>
</table>

NS: not significant; SD: standard deviation; RBC: red Blood Cells; WBC: White Blood Cells; TP: Total Protein concentration.
Different superscripts in the same row indicate significant differences.

FIGURE I: Variations of lipid peroxidation indicators, i.e. plasma TBARS and LPO concentrations, of antioxidant enzyme (SOD, CAT and GSH-Px) activities and of reduced glutathione concentrations according to groups: healthy controls (n = 22), calves with acute (n = 15) and chronic (n = 12) bronchopneumonia.
* all mean differences between groups were statistically significant (p < 0.01).
Oxidative stress results from an imbalance between reactive oxygen species production and the antioxidant enzymatic and non-enzymatic systems, leading to cell damage, and some antioxidant deficiencies may contribute to the oxidative stress occurrence [19]. Antioxidants protect cells against oxidative injury by preventing initiation of peroxidation process and production of the final products such as TBARS, which are able to induce severe cellular damage [13]. The major antioxidant enzymes that are responsible for the degradation of reactive oxygen species are superoxide dismutase (which converts superoxide anion radicals to hydrogen peroxide), glutathione peroxidase and catalase (which reduce hydrogen peroxides to alcohols and to water respectively) [13].

Several studies have reported the decline of the enzymes in human patients with pneumonia [9, 29]. LEDWOZYW et al. [16] have reported a significant decrease of SOD activity in calves with bronchopneumonia correlated with high blood concentrations of superoxide radicals. Moreover, marked decreases of blood GSH concentrations have been reported in human patients [22]. In the present study, marked increases of oxidative stress markers (TBARS and LPO) were observed in calves with bronchopneumonia in agreement with the previous studies. Furthermore, antioxidant systems (SOD, CAT and GSH-Px enzymes and GSH concentrations) were notably depressed in animals with chronic pneumonia. The decreases of the antioxidant enzyme activities and the dramatic reduction of blood GSH concentrations would result from a massive and durable production of radical species during bronchopneumonia development and lead to the exacerbation of oxidative damage in lungs. By contrast, calves with the acute form of the disease exhibited significant increases of GSH-Px and CAT activities compared to healthy controls whereas the antioxidant deficiencies may contribute to the oxidative stress occurrence [19].

In conclusion, the results demonstrate the presence of oxidative stress in calves with bronchopneumonia, particularly in chronically affected animals. The identification of molecular mechanisms involved in oxidative injury in lungs should allow the development of antioxidant therapy approaches in future.

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References