Associations between Inflammatory Factors, Lipid Peroxidation and Antioxidant Capacity in Bovine Seminal Plasma

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Abstract

Oxidative stress and inflammation are cooperative events involved in male reproductive dysfunction. In the present study, we assessed the associations between the spermatozoa motility, inflammatory factors (C-reactive protein and Interleukin-6), total antioxidant status (TAS) and lipid peroxidation expressed as malondialdehyde (MDA) concentration in the seminal plasma of breeding bulls. 17 semen samples were included in the study. Computer-aided sperm analysis (CASA) system was used to assess the spermatozoa motion characteristics, and seminal plasma was collected for further analyses. Interleukin-6 (IL-6) was quantified using ELISA, while C-reactive protein (CRP) and markers of the oxidative balance were evaluated by UV/VIS spectrophotometry. The correlation analysis revealed significantly positive associations between the sperm motility and TAS (P<0.05), while both parameters were in significantly negative correlations with CRP (P<0.05), IL-6 (P<0.05) and MDA (P<0.01). At the same time, the samples were divided according to the motility characteristics into groups of Excellent (Ex) and Moderate (Mo) quality. CRP, IL-6 as well as MDA concentrations were significantly (P<0.05) higher in the Mo group, while the Ex group exhibited a significantly higher antioxidant capacity (P<0.05). The relationships between the oxidative balance and inflammatory markers detected in our study suggest their intricate involvement in the resulting semen quality.

Keywords: antioxidants, inflammation, male reproduction, oxidative stress, spermatozoa

1. Introduction

Mammalian semen contains spermatozoa as well as other populations of nonspermatozoal cells primarily represented by leukocytes and immature germ cells [1, 2]. Relationships between the cell-mediated immunity and male reproductive function have been reported in a variety of studies [3-6]. Activation of seminal leukocytes during genital tract inflammation or reactions against microbial agents may trigger the release of a variety of molecular products such as proteolytic enzymes, cytokines, and reactive oxygen species (ROS).

Negative associations between excessive ROS production and male subfertility have been demonstrated in numerous reports in animals as well as humans [7-9]. At the same time, negative effects of an excessive leukocyte activation on spermatozoa performance has been well documented [10-12], and it is feasible that such negative impact may be caused either by inflammatory factors or ROS produced by leukocytes. It is known that physiological levels of ROS are crucial for sperm maturation, capacitation and acrosome reaction [13]. Nevertheless, it is still not completely clear if or how the presence of leukocytes has an impact on the sperm function. At the same time, it has been repeatedly speculated that inflammatory molecules could...
have a close relationship with sperm dysfunction [6, 14-16]. Proinflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and acute-phase proteins, such as C-reactive protein (CRP) have been questioned as the inflammation process is associated with their presence, and as a consequence, may alter the antioxidant properties of mammalian semen [11, 12]. Therefore, the aim of this study was to evaluate the relationships among selected inflammatory factors, antioxidant capacity and lipid peroxidation of bovine seminal plasma collected from healthy bulls and to evaluate if these characteristics have any impact on the resulting spermatozoa activity.

2. Materials and methods

Semen samples were collected from 17 Holstein Friesian bulls kept in the Breeding Centre of the Slovak Biological Services, Nitra, Slovakia. The animals were 4-6 years old and fed a standard diet consisting of green and cereal fodder, berseem, straw and commercial mixtures for beef cattle. Water was provided regularly.

The samples were acquired on a regular collection schedule using an artificial vagina and immediately transferred to the laboratory. Basic semen assessment was performed in each sample, including volume (mL), pH and spermatozoa concentration (x10⁶/mL).

Spermatozoa motility (MOT; percentage of motile spermatozoa; motility > 5 μm/s; %) was determined using the Computer Assisted Semen Analysis (CASA) system based on the SpermVision™ program (Minitube, Tiefenbach, Germany) and Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan). The samples were placed into the Makler Counting Chamber (depth 10 μm, 37°C; Sefi Medical Instruments, Haifa, Israel) and assessed. At least 1000 cells were evaluated in each sample [17].

The samples were centrifuged (15 min, 10 090 x g, 4°C), seminal plasma was transferred into 1.5 mL tubes and kept frozen (−80 °C) until further analysis [18].

The C-reactive protein (CRP) was quantified using immunoturbidimetry and the DiaSys commercial kit (DiaSys Diagnostic Systems, Holzheim, Germany). The principle of this assay is based on an endpoint determination of the concentration of CRP by photometric measurement of the antigen-antibody reaction of antibodies to mammalian CRP with CRP present in the sample. CRP concentration was measured on a 96-well plate at 340 nm using the Elisa reader (Multiscan FC microplate photometer; Thermo Scientific; Waltham, MA, USA) expressed as ng CRP/g protein.

Interleukin-6 was quantified using Enzyme-Linked Immuno Sorbent Assay (ELISA). The Thermo Scientific (Waltham, MA, USA) IL-6 ELISA kit contains a 96-well microtiter plate precoated with monoclonal antibody to the cytokine. A measured volume of the samples and standards were added to each test well and incubated. The wells were washed, and a biotin-labeled polyclonal antibody to the tested cytokine was added to bind the captured IL-6. The wells were washed again and a peroxidase-labeled avidin reagent was added to attach the biotin on the plate. After incubation the wells were washed and a peroxidase-labeled immunoglobulin G was added to attach the polyclonal antibody (in the immune complex) on the plate. After a third wash, a substrate buffer (peroxide) and chromogen were added to the wells. The reaction was stopped by the addition of sulfuric acid. The intensity of the colorimetric reactions was in a direct proportion to the amount of tested cytokine present in the studied sample or standard. The absorbance was read with Elisa reader (Multiscan FC microplate photometer; Thermo Scientific; Waltham, MA, USA) at 450 nm, and a standard curve was constructed to quantitate IL-6 concentrations. The final concentrations are expressed as ng IL-6/g protein.

The assessment of TAS originates from the ability of all antioxidants in the sample to neutralize a prooxidant compound. The TAS Randox (Randox Laboratories, Crumlin, Great Britain) assay follows an incubation of ABTS (2,2’-Azino-di-[3-ethylbenzthiazoline sulphonate]) with a peroxidase (metmyoglobin) and H2O2 to produce the ABTS+ radical. This has a relatively stable blue-green color, which may be measured at 600
nm. Antioxidants present in the sample supress this color production to a degree, which is proportional to their concentration. TAS was assessed using the Randox Monza spectrophotometer (Randox Laboratories, Crumlin, Great Britain) and is expressed as µmol/g protein. Lipid peroxidation (LPO) expressed through malondialdehyde (MDA) production was assessed with the help of the TBARS assay, modified for a 96-well plate and ELISA reader. Each sample was treated with 5% sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, MO, USA), and subjected to 0.53% thiobarbituric acid (TBA; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 20% acetic acid adjusted with NaOH (Centralchem, Bratislava, Slovak Republic) to pH 3.5, and subsequently boiled at 90-100°C for 1h. Following boiling, the samples were placed on ice for 10 min and centrifuged at 1750 x g for 10 min. Supernatant was used to measure the end-product resulting from the reaction of MDA and TBA under high temperature and acidic conditions at 530–540 nm with the help of the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) [18]. MDA concentration is expressed as µmol/g protein.

Protein concentration was quantified using the DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit, based on the Biuret method: copper sulphate reacts with proteins to create a violet blue color complex in alkaline solution, whose intensity is directly proportional to the protein content at 540 nm using the Randox Monza spectrophotometer (Randox Laboratories, Crumlin, Great Britain).

All data were subjected to statistical analysis using the GraphPad Prism program (version 3.02 for Windows, GraphPad Software incorporated, San Diego, California, USA, http://www.graphpad.com/). Results are quoted as arithmetic mean ± standard error (SEM). Pearson product-moment correlation coefficient analysis for paired samples was used to assess correlations between all examined parameters. Additionally, the samples were categorized in two quality groups according to their motility rates. Comparative analysis of selected parameters in the seminal plasma as well as in the quality groups was carried out by one-way ANOVA with the Bonferroni multiple comparison test. The level of significance for the comparative as well as correlation analysis was set at *** (P<0.001); ** (P<0.01); * (P<0.05).

3. Results and discussion

Results from the seminal examination are shown in Table 1. Animal donors presented with no signs of disease or pathology. At the same time, the semen characteristics met the criteria established for the Holstein Friesian bovine breed, which is why a possible health impact on the outcomes collected from further examinations was ruled out.

Table 1. Semen characteristics of the samples (n=17)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>6.47±0.07</td>
</tr>
<tr>
<td>pH</td>
<td>6.44±0.33</td>
</tr>
<tr>
<td>Concentration [x10^6 cells/mL]</td>
<td>2999±40.90</td>
</tr>
<tr>
<td>MOT [%]</td>
<td>87.79±1.27</td>
</tr>
<tr>
<td>CRP [ng/g prot]</td>
<td>25.30±46.00</td>
</tr>
<tr>
<td>IL-6 [ng/g prot]</td>
<td>1.46±0.38</td>
</tr>
<tr>
<td>TAS [µmol/g prot]</td>
<td>32.13±2.47</td>
</tr>
<tr>
<td>MDA [µmol/g prot]</td>
<td>0.31±0.04</td>
</tr>
</tbody>
</table>

Mean±SEM. MOT: motility; CRP: C-reactive protein, IL-6: interleukin-6; TAS: total antioxidant status; MDA: malondialdehyde.

The data collected from the CRP, IL-6, TAS and MDA assessments suggest that all the studied characteristics were within the physiological limits and comparable to the results of other authors [6, 18-20]. Table 2 displays the results of the correlation analysis between the selected motion characteristics, inflammatory, prooxidant and antioxidant parameters quantified in bovine seminal plasma.

MOT as the basic characteristic of spermatozoa vitality was significantly (P<0.05) positively correlated with TAS. Inversely, CRP, IL-6 as well as MDA were significantly negatively correlated with the sperm motility (P<0.05 with respect to CRP and IL-6; P<0.01 in case of MDA) as well as the antioxidant capacity of the samples (P<0.05 in relation to CRP and IL-6; P<0.01 in case of MDA).

To have a better understanding of the results, the samples were categorized in two groups of excellent (Ex; >90% motile; n=10) and moderate (Mo; <80% motile; n=7) quality according to their motility rates (Table 3). Mean value for MOT was significantly different between the
groups (P<0.01). TAS was significantly higher in the Ex group (P<0.05) while CRP, IL-6 and MDA were significantly higher in the Mo group (P<0.05).

**Table 2.** Correlations between motility parameters, prooxidant and antioxidant markers as well as inflammatory factors in bovine seminal plasma evaluated by the Pearson product-moment correlation (n=17)

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>MOT</th>
<th>CRP</th>
<th>IL-6</th>
<th>TAS</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOT</td>
<td>1</td>
<td>-0.419*</td>
<td>-0.471†</td>
<td>0.571</td>
<td>-0.510**</td>
</tr>
<tr>
<td>CRP</td>
<td>-0.419*</td>
<td>1</td>
<td>-0.462*</td>
<td>0.485**</td>
<td>-0.575**</td>
</tr>
<tr>
<td>IL-6</td>
<td>-0.471†</td>
<td>-0.462*</td>
<td>1</td>
<td>0.20±0.03</td>
<td>0.44±0.07</td>
</tr>
<tr>
<td>TAS</td>
<td>0.382*</td>
<td>-0.462*</td>
<td>-0.376*</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>0.510**</td>
<td>0.571</td>
<td>0.20±0.03</td>
<td>0.44±0.07</td>
<td>1</td>
</tr>
</tbody>
</table>

The interpretation of the results was based on the value of the correlation coefficient: ±0.111 - ±0.333: low correlation; ±0.334 - ±0.666: moderate correlation; ±0.667 - ±0.999: high correlation. *P < 0.05; †P < 0.01; **P < 0.001. MOT: spermatozoa motility [%]; CRP: C-reactive protein [ng/g prot]; IL-6: interleukin-6 [ng/g prot]; TAS: total antioxidant status [μmol/g prot]; MDA: malondialdehyde [μmol/g prot].

**Table 3.** Average values of motility parameters, inflammatory, prooxidant and antioxidant markers in the quality groups (Mean ± SEM) and Bonferroni multiple comparison test results

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>Ex Group (n=10)</th>
<th>Mo Group (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOT [%]</td>
<td>91.07±0.97</td>
<td>83.10±1.49*</td>
</tr>
<tr>
<td>CRP [ng/g prot]</td>
<td>118.40±35.63</td>
<td>253.30±48.69*</td>
</tr>
<tr>
<td>IL-6 [ng/g prot]</td>
<td>0.62±125.50</td>
<td>2.64±2.26</td>
</tr>
<tr>
<td>TAS [μmol/g prot]</td>
<td>35.29±3.08</td>
<td>25.22±2.97</td>
</tr>
<tr>
<td>MDA [μmol/g prot]</td>
<td>0.20±0.03</td>
<td>0.44±0.07**</td>
</tr>
</tbody>
</table>

*P < 0.05; †P < 0.01; **P < 0.001. MOT: spermatozoa motility; CRP: C-reactive protein; IL-6: interleukin-6; TAS: total antioxidant status; MDA: malondialdehyde.

Many inflammation factors are involved in the regulation of male reproduction [6, 12, 14]. In agreement with our results, a negative correlation was observed between a higher level of CRP and IL-6 and spermatozoa motility; sperm motion was lower in subjects with elevated levels of CRP or IL-6 in semen as reported by Vera et al. [6], Sanocka et al. [12] and Gruschwitz et al. [21]. It is well known that both molecules are related to the presence of different cell types in semen, including macrophages, leukocytes and endothelial cells. It would be therefore interesting to associate the presence and/or concentration of such cells in bovine ejaculates with higher levels of inflammation factors, as possibly a correlation exists between these parameters. It would be also feasible to search for the exact origin of such molecules in further studies, and to detect specific receptors in male reproductive cells and tissues, in order to establish which could be the targets for CRP or IL-6 in the male reproductive system. Our results have shown that higher levels of inflammation factors present in male genital tract have the ability to change the profile of oxidants and antioxidant activity in seminal plasma. It has been shown in previous studies that proinflammatory molecules, such as IL-1b, IL-6, IL-8, and TNFa seem to have the ability to modulate the activity of prooxidant or antioxidant enzymes, and thus can severely affect basic semen parameters [12].

The presence of inflammation factors in semen is believed to be a clinically significant factor in the etiology of male reproductive dysfunction [5]. Nevertheless, existing discrepancies in literature do not sufficiently emphasize on the role of genetic tract inflammation in male subfertility or infertility. Studies by Sanocka et al. [12, 22], Kurpisz et al. [23] and Miesel et al. [24] however suggest that alterations to the seminal antioxidant system may be an important reason for these conflicting results. Aitken et al [5] postulated that leukocytospermia had no influence on the motility or morphology of human spermatozoa. On the other hand, a significant impact of the immune system on the functional capacity of spermatozoa and sperm–oocyte fusion was observed. What is more, Tomlinson et al. [25] suggested that elevated concentrations of neutrophils or macrophages improved sperm morphology and semen density. Male genital tract inflammation has been repeatedly associated with increased levels of free radicals, and its effects on semen parameters may be dependent on the initial antioxidant capacity [12]. Correspondingly, a comparison of leukocytospermic samples with nonleukocytospermic samples revealed significant differences at the levels of ROS, however, the antioxidant capacity was not precisely
characterized [25]. Inversely, Sanocka et al. [12, 22], Kurpisz et al. [23] and Miesel et al. [24] reported that an inefficient semen antioxidant system correlated with infertility and semen pathology, and was especially associated with asthenozoospermia. Furthermore, and compatible to our results Zalata et al. [11] and Sanocka et al. [22] observed that elevated levels of peroxidated lipids in the cell membranes of spermatozoa hinted at significant generation of ROS. During genital tract inflammation, beside leukocyte contamination, pathological bacterial strains may appear in semen. A substantial number of studies [26-29] indicated that bacterial infections might cause visible alterations to the semen volume, sperm motility, and viability. Immobilization or death of spermatozoa can be a biological response to the action of bacterial toxins. The impact of genital inflammation on fertility was mediated through diminished sperm motility due to the adherence of dialyzable factors in semen samples [26]. Our results may complement the hypothesis risen by Sanocka et al. [12] who suggested that pathological bacterial strains present in semen may cause an increase of cytokine levels that in turn may abolish the activity antioxidant enzymes. These changes are the direct consequence of exposure to ROS, constantly exhausting the antioxidant scavenging capacity and causing a higher degree of lipid peroxidation of the membrane structures of spermatozoa. We believe that the crucial role in perpetuation of the inflammatory process may belong to cytokines, and that these bioactive substances may constitute an important link between inflammation and male infertility. Sikka et al. [30] suggested that combinations of lipopolysaccharides and interferon-g are detrimental to human spermatozoa and may contribute to male infertility in patients with chronic genitourinary inflammation. In the present study, we have strongly indicated that the activity of the antioxidant system is dependent on particular inflammatory molecules (Tables 2 and 3), which can be potent modulating factors of antioxidant semen capacity. A clear relationship between basic semen parameters and oxidative stress was well observed. It has been recently established that ROS may act as intracellular signaling molecules to mediate the biological effects of cytokines. One of the main targets of ROS is transcription factor kB (nuclear factor-kB [NFkB]). NFkB-dependent transcription is inhibited by antioxidants and its activation is induced or potentiated by ROS [31-33]. The precise molecular mechanisms for regulating the antioxidant response to male genital tract inflammation remains unclear, however, results generated so far indicate that cytokines may play an important role during the inflammatory reactions and are connected with oxidative metabolism.

4. Conclusions

In conclusion, our study shows that inflammation factors such as CRP and IL-6 have the ability to modulate the pro- and antioxidant properties of semen. As the oxidative balance may directly affect semen parameters, long-term genital tract inflammations may lead to seminal oxidative stress and subsequently to male subfertility. As such, we suggest that the assessment of male genital tract inflammation in relation to reproductive dysfunction should be based on a joined evaluation of seminal leukocytes, inflammation factors and the antioxidant capacity of male reproductive fluids.

Acknowledgements

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References


