EFFECT OF THE BVD-MD VIRUS ON COAGULATION AND FIBRINOLYTIC SYSTEMS IN DAIRY COWS

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Abstract

The experiment was performed on 30 cows, including 10 animals infected with the BVD-MD virus. The following parameters were determined: prothrombin time, thrombin time, activated partial thromboplastin time, fibrinogen and D-dimer concentrations, antithrombin III activity, and blood platelet counts. The obtained results indicated that the BVD-MD virus lowered blood platelet counts and led to dysfunctions in plasma coagulation and fibrinolytic systems. The observed changes in the examined haemostatic indicators suggest that BVD-MD infections could lead to the development of disseminated intravascular coagulopathy that has adverse consequences for the animals' health.

Key words: cattle, BVD/MD, coagulation system, fibrinolysis, coagulopathy.

Haemostatic processes in animals and humans maintain the liquid form of blood circulating in the vascular bed and prevent blood loss in the event of vessel wall rupture. General systemic homeostasis requires a balance between coagulation and fibrinolytic systems, and a disruption of that balance could lead to haemorrhagic diathesis and thrombotic complications. Procoagulation and anticoagulation processes take place with the involvement of blood vessels, blood platelets, coagulation and fibrinolytic system proteins, their inhibitors and activators (36, 38). The general body of processes taking place during haemostatic reactions can be divided into three stages. The first is primary haemostasis, which involves vessel walls and blood platelets and continues until a platelet plug is formed.

The second stage involves secondary haemostasis, which activates a cascade of plasma coagulation factors and converts soluble fibrinogen into fibrin, to stabilise the platelet plug. The last stage comprises fibrinolysis during which the resulting clot is dissolved and eliminated. Fibrinogen is converted to an insoluble fibrin fiber mesh as a result of two pathways that activate the coagulation process. The first, referred to as the extrinsic pathway of coagulation, is determined by the tissue factor (TF), while the second, known as the intrinsic pathway of coagulation, is dependent on contact factors. For many years, the intrinsic pathway was believed to be the key determinant of the coagulation process, but recent findings have demonstrated that both systems are closely interrelated and equally significant in the physiology of blood coagulation. Blood coagulation processes are initiated by the TF-activated factor VII in the extrinsic pathway of coagulation. In physiological states, the activated enzymatic cascade is inhibited by the anticoagulation system, which prevents the formation of clots in healthy organisms. Antithrombin III (AT III) is one of the key elements of the anticoagulation system. When coagulation processes cease, the fibrinolytic system removes clots from blood vessels. Plasmin affects fibrin and fibrinogen, leading to the formation of fibrin and fibrinogen degradation products (FDP). Enmeshed fibrin is decomposed more slowly than non-enmeshed fibrin, which leads to the production of differently sized fragments. The smallest are D-dimer fragments, which are covalently bonded with fragment E (12, 17, 36, 38).

Tests evaluating intrinsic and extrinsic pathways of coagulation are performed to investigate and interpret dysfunctions in coagulation and fibrinolytic systems. The key parameters for diagnosing a haemostatic imbalance are: prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT), fibrinogen and D-dimer concentrations, and antithrombin III (AT III) activity. PT values are used to assess the extrinsic coagulation pathway. TT selectively evaluates the common pathway of coagulation, and is a measure of fibrinogen conversion to fibrin. Referred to as kaolin-kefalin time in older nomenclature, APTT examines the intrinsic pathway of coagulation. The results of the measurement of PT, TT, and APTT are dependent from the concentration of suitable plasmatic factors being a party to each pathways of coagulation. Fibrinogen levels are usually determined using a modified method of TT measurement, as
described by Claus in 1957. AT III activity and D-dimer concentrations are determined to illustrate the degree of activation of the anticoagulation and fibrinolytic systems. The use of the above tests in the diagnosis of haemostatic disturbances explains the causes of haemorrhaging or clotting in the disease process. An analysis of changes in coagulologic factors confirms the presence of disseminated intravascular coagulopathy (DIC) in the body (3, 8, 12, 36).

Viral infections may be an indirect cause of haemostatic disturbances in cattle. In 2002, DeMaula et al. (9) noted increased levels of blood coagulation in cattle affected by the bluetongue disease. The clinical symptoms and pathological changes observed in cattle attacked by the BVD virus may be the effect, as well as the cause of haemostatic imbalance, including primary as well as secondary haemostasis. The BVD-MD virus is commonly found in dairy and beef cattle around the world and the resulting infections cause massive financial losses (16). The BVD-MD virus is a member of the family Flaviviridae, the genus Pestivirus, and, owing to its specific properties, has been divided into two genotypes – I and II. Every genotype has various biotypes: cytopathic (cp) and non-cytopathic (n-cp) (27, 33). The analysed virus has a tendency to infect various organs, therefore, the clinical picture of the disease may vary significantly. During BVD-MD infections, cows display a transient rise in body temperature, weaker appetite, general weakness, loose diarrhoea, leukopenia, mucous and purulent discharge from nasal openings and conjunctival sacs, as well as cough (2). When individuals permanently infected with the n-cp biotype are additionally attacked by the cp biotype of the BVD-MD virus with an identical antigen, the result may be mucosal disease (MD), a lethal form of BVD-MD. In such cases, erosions of various size and intensity are found in the mucosa of the oral cavity, oesophagus, rumen, abomasums, and intestines. The intensity of changes may vary in different organs. In the intestine, they may be observed only in the area of Peyer’s patches, ranging from fiber deposits and blood clots to an ulcerative inflammation of the jejunum, ileum, and colon (21-23). According to Lieber-Tenorio et al. (21), the intensity of tissue changes is determined by the spread of the virus throughout the body. Tissue damage is usually much more extensive in locations where the BVD-MD antigen is found. Acute BVD-MD may also damage the wall structure of vessels in the submucosa of the small and large intestines (19) and arteries of the infected lymph nodes (22). The BVD-MD virus was also found in the smooth muscles in the inner layers of arterioles (10). The resulting damage to blood vessels and mucosal membranes could activate the coagulation and fibrinolysis cascade following TF release. When the haemostatic balance is disrupted by the BVD-MD virus, the immune system could also affect blood coagulation and fibrinolysis, in particular cytokines produced in a state of infection, such as interleukin-12 (IL-12) (26).

The resulting disturbances in blood coagulation pathways, manifested by extravasations or haemorrhaging from mucous membranes, could deteriorate the functioning of internal organs as DIC progresses. DIC’s adverse effect on bovine health has also been observed during left abomasal displacement, metritis, and mastitis (5).

The objective of this study was to determine the effect of the BVD-MD virus on coagulation and fibrinolytic systems in dairy cows.

**Material and Methods**

Experimental blood samples were collected from 20 Holstein-Friesian cows, which were not vaccinated and free of BVD-MD virus infection (group I, control) and 10 Holstein-Friesian cows infected by the BVD-MD virus with clinical symptoms of the disease (group II). All the cows were between 2 and 6 years of age. The infection of group II cows with the BVD-MD virus was confirmed by serological and virological tests (ELISA, RT-PCR). Blood samples for the determination of coagulologic and haematochemical parameters were taken from the external jugular vein on three occasions at 48 h intervals. The samples were collected into test tubes containing sodium citrate and K$_2$EDTA. Coagulation and fibrinolysis tests were carried out using the Coag-Chrom 3003 device and reagents (Bio-Ksel, Poland). PT, APTT, TT, fibrinogen concentrations, D-dimer concentrations, and AT III activity were determined. Thrombocyte counts (PLT) were determined in a Vet ABC haematology analyser (ABX Diagnostics).

The results of laboratory tests were presented in SI units and processed statistically by an analysis of variance (ANOVA) using the LSD test.

**Results**

The selected haematochemical and coagulologic parameters of peripheral blood samples from groups I and II are presented in Table 1.

PLT counts in animals of group I were within the normal physiological range, although a statistically significant difference (P<0.05) was noted between the first and third test. PLT counts in group II showed highly significant differences (P<0.01) in comparison with group I. A decrease in PLT counts was also noted in the successive analyses of blood samples from this group from 192.11 x 10$^3$/L to 110.90 x 10$^3$/L (P<0.05) on average, reaching values below the physiological norm. The analysis of blood coagulation and fibrinolysis parameters in cows infected and not infected with the BVD-MD virus also demonstrated significant differences between the examined groups. Changes extending beyond the reference range for this animal species were not found in the coagulologic results for group I cows. In group II, PT was extended by 30.99 s in the first test to 39.62 in the third test, producing a statistically significant difference (P<0.05). APTT values in successive blood samples showed highly significant differences (P<0.01) in the range of 54.92 to 68.85 s.
Table 1
Average values of selected blood haematological and coagulologic parameters in cows

<table>
<thead>
<tr>
<th>Test</th>
<th>PLT (10^3/L)</th>
<th>PT (s)</th>
<th>APTT (s)</th>
<th>TT (s)</th>
<th>Fibrinogen (g/L)</th>
<th>AT III (%)</th>
<th>D-dimers (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td></td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>I</td>
<td>II</td>
<td>I</td>
<td>II</td>
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</tr>
<tr>
<td>I</td>
<td>547.65±</td>
<td>192.11±</td>
<td>25.86</td>
<td>30.39±</td>
<td>43.72</td>
<td>54.92±</td>
<td>15.85±</td>
</tr>
<tr>
<td></td>
<td>6.65±</td>
<td>5.12±</td>
<td>119.05±</td>
<td>103.9±</td>
<td>158.60±</td>
<td>1012.9±</td>
<td>±3.08</td>
</tr>
<tr>
<td>SD</td>
<td>±3.08</td>
<td>±70.9</td>
<td>±9.0</td>
<td>±7.5</td>
<td>±5.6</td>
<td>±8.0</td>
<td>±3.3</td>
</tr>
<tr>
<td>2</td>
<td>646.85±</td>
<td>136.60±</td>
<td>25.86</td>
<td>34.89±</td>
<td>42.74</td>
<td>59.44</td>
<td>15.90±</td>
</tr>
<tr>
<td></td>
<td>6.94±</td>
<td>4.61±</td>
<td>119.10±</td>
<td>74.0±</td>
<td>159.051</td>
<td>1205.8±</td>
<td>±4.2</td>
</tr>
<tr>
<td>SD</td>
<td>±88.2</td>
<td>±4.2</td>
<td>±5.0</td>
<td>±6.7</td>
<td>±46.0</td>
<td>±97.0</td>
<td>±34.0</td>
</tr>
<tr>
<td>3</td>
<td>390.35±</td>
<td>110.90±</td>
<td>25.87</td>
<td>39.62±</td>
<td>42.93±</td>
<td>68.85±</td>
<td>15.99±</td>
</tr>
<tr>
<td></td>
<td>6.76±</td>
<td>3.51±</td>
<td>119.30±</td>
<td>69.5±</td>
<td>160.15±</td>
<td>1322.7±</td>
<td>±4.82</td>
</tr>
<tr>
<td>SD</td>
<td>±59.2</td>
<td>±4.82</td>
<td>±5.0</td>
<td>±38.0</td>
<td>±56.0</td>
<td>±5.0</td>
<td>±24.0</td>
</tr>
<tr>
<td>Mean group</td>
<td>528.28±</td>
<td>146.54±</td>
<td>35.17±</td>
<td>43.13±</td>
<td>61.07±</td>
<td>32.39±</td>
<td>6.78±</td>
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<td></td>
<td>1180.47±</td>
<td>1322.7±</td>
<td>1205.8±</td>
<td>1012.9±</td>
<td></td>
<td></td>
<td>159.27±</td>
</tr>
<tr>
<td>SD</td>
<td>±79.2</td>
<td>±77.2</td>
<td>±95.0</td>
<td>±38.0</td>
<td>±36.0</td>
<td>±97.0</td>
<td>±35.0</td>
</tr>
</tbody>
</table>

Average APPT values in group II (61.07 s) were significantly higher than in group I. TT of infected animals was extended in successive tests from 28.72 s to 36.65 s, demonstrating highly significant differences (P≤0.01) in comparison with control. Fibrinogen concentrations in the blood samples of group II animals reached 4.41 g/L on average (P≤0.05) and were lower than in non-infected cows, but the obtained results were within the physiological norm. A highly significant drop in AT III activity was also noted in comparison with group I. D-dimer concentrations increased gradually (P≤0.05) to 1,322.7 µg/L in successive tests in the group of BVD-MD infected animals, and average D-dimer levels in this group differed significantly (P≤0.01) from the values of healthy animals.

Discussion

PLT counts and the results of blood coagulation and fibrinolysis tests varied significantly throughout the experiment, both during successive blood sample collections in group II and between the analysed groups. The PLT counts of infected cows with mucous extravasations and haemorrhaging changed between tests, exceeding the normal physiological range (42) in the third test. A drop in PLT counts caused by BVD-MD in adult cattle was observed by Rebhun et al. (32). According to the authors, thrombocyte counts in animals affected by dysenteric diarrhoea and mucous extravasations ranged from 2,000/µl to 33,000/µl (2 x 10^3/L to 33 x 10^3/L). Such intense clinical symptoms were not noted in this study, but PLT counts were visibly lower. In the 1980s in North America, a new form of BVD-MD caused by a highly fatal genotype II of the BVD-MD virus appeared. Infections caused by this genotype are marked by mortality rates over 30%, and they affect cattle in all age groups. Clinical symptoms include persistent fever, diarrhoea and haemorrhaging, e.g. from the nasal cavity or places of injection, while thrombocytopenia is commonly diagnosed in laboratory tests. Owing to its characteristic clinical symptoms, this form of BVD-MD is termed as the haemorrhagic syndrome (HS) (7, 25, 34). The manner in which the BVD-MD virus affects thromboocytes remains a topic of debate. The most probable mechanism of action includes the virus direct negative effect on cells, or an indirect influence on immune response produced during the disease (41). In a study of young elk, Corapi et al. (6) noted the presence of the BVD-MD virus in blood platelets 11 d after the animals had been infected with the virus, but the presence of immunoglobulins in the process of platelet destruction and sequestration was not confirmed. A drop in PLT counts is a characteristic symptom of DIC during which, according to Hughes-Jones and Wickramasinghe (17), blood platelets are trapped in fibrin deposits, thus contributing to thrombocytopenia.

Rebhun et al. (32) did not observe any changes in the plasma coagulation system of six cows infected with the BVD-MD virus. This study revealed cumulative changes in the coagulation and fibrinolytic system pointing to the development of DIC. The stimulation and functioning of the coagulation system in animals may be analysed using parameters, which describe different pathways of activation of the coagulation cascade. The effectiveness of the extrinsic pathway of coagulation is assessed based on PT, i.e. the time between the moment of adding TF and calcium chloride to platelet-rich plasma and the formation of the fibrin clot. PT is determined by the content of
prothrombin, factors V, VII, X, and fibrinogen in plasma (31). Prolonged PT values that do not fit in the normal physiological range were observed in cows infected with the BVD-MD virus. A similar increase in PT values was noted by Gokce et al. (13) in traumatic reticuлоperitonitis, and by Irmak et al. (18) and Sobiech et al. (39) in abomasal displacement. In all cases, the disease resulted in the development of DIC. Prolonged PT values in DIC are related to a drop in fibrinogen concentration, a decrease in the content of factors V and X, and the presence of fibrinogen degradation products. To evaluate the functioning of the intrinsic pathway of coagulation, the time between the maximum activation of the pathway dependent on contact factors (in particular factors XI and XII) in citrate plasma, and the formation of the fibrin clot (APTT) is measured with the use of kaolin, kefalin, and calcium chloride. APTT values are affected by factors II, V, VIII, IX, X, XI, II, and fibrinogen (31, 38). The average APTT values in control animals did not differ significantly from the physiological norm (15, 40). In cows infected with BVD-MD virus APTT increased in successive tests from 54.92 s to 61.07 s. The variations in APTT were most probably caused by the plasmin effect on the biodegradation of plasma coagulation factors V, VIII, IX, XI, as well as hypofibrinogenemia and the presence of FDP (3, 38). APTT could have also been influenced by liver malfunction that developed in the course of the BVD-MD infection. Fiore et al. (11) observed an increase in the following enzyme activity in animals infected with the BVD-MD virus: aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl transferase (GGT), which pointed to hepatocyte damage. TT is an indicator of fibrinogen conversion to fibrin, and it is a time measured from the moment of adding a standard quantity of thrombin to citrate plasma until clot formation. The stages of activation of all coagulation factors, except fibrinogen, are thus omitted in the experiment. TT values are determined by fibrinogen concentrations, the presence of defective fibrinogen, correct fibrin polymerisation and stabilisation, as well as the presence and activity of thrombin inhibitors (31, 38). This study revealed prolonged TT values in group II cows in comparison with group I, similarly to the values described by Heuwieser et al. (15) and Sobiech et al. (40). The above change could result from a decrease in fibrinogen concentrations in plasma and an increase in FDP levels (3). The fibrinogen content of plasma is relative to various factors, including stress, inflammatory processes, hormones, and exercise (36). In this experiment, fibrinogen levels in the blood plasma of cows infected with the BVD-MD virus were lower than in animals free of BVD-MD virus infection, nevertheless, they were within the physiological norm described by Gentry (12). A study of humans showed that fibrinogen levels decrease in less than 50% of patients affected by DIC (1). The correct diagnosis of DIC requires a thorough evaluation of the anticoagulation system. The authors of this study relied on the analysis of AT III activity, the most important physiological inhibitor of thrombin (8). Pusterla et al. (30) determined the threshold of AT III activity in healthy cows at 105% to 150%. The above findings were validated by other studies, where average AT III activity was noted at 152.3% (40). In this experiment, AT III activity in three successive sample collections was similar, reaching 119.5% on average in group I and 82.47% in group II. A drop in AT III activity in cows affected by DIC was observed by Pusterla et al. (30). This decrease is attributed to an excessive use of antithrombin and increased permeability of vessel endothelium (35). Clear differences in fibrinogen concentrations between the studied cow groups could point to dysfunctions in fibrinogen polymerisation. The above observation justified the determination of D-dimer content, specific products of fiber degradation (38). D-dimer concentrations in group II were higher than those described by other researchers within the physiological norm (40), and the above most probably resulted from the activation of intravascular fibrinolysis. A set of parameters supporting an intravital diagnosis of DIC has been identified in human medicine. They are: FDP levels (including D-dimers), PLT counts, fibrinogen concentrations, and PT. Based on those parameters and published data (3, 31, 35), the coagulologic indicators of cows infected with the BVD-MD virus point to the progression of DIC, and do not confirm the results reported by Rebhun et al. (32). As noted by Sawicka (35), DIC is an “acquired blood coagulation defect that results from a haemostatic imbalance and excessive thrombin formation”. The occurrence of DIC in BVD-MD should be attributed to several factors. The damage of tissue and vessel walls in the intestines, lymph nodes, and even reproductive organs during the disease prevents TF release from various organs (4, 19, 37), thus activating the coagulation cascade (38). The progressive vessel endothelial dysfunction could decrease anticoagulant synthesis, stimulate the production of anti-clotting agents, and inhibit fibrinolysis. The secretion of cytokine in DIC, including IL-12, whose synthesis is stimulated by the BVD-MD virus, could lead to haemostatic disturbances (14, 28). The IL-12-induced activation of clotting and fibrinolysis was described in humans and chimpanzees, but the exact mechanism of those disturbances has not been explained to date (20, 29). The above proclotting and anticoagulation reactions lead to the formation of numerous microclots in the circulatory system, which could deepen tissue damage, leading to multi-organ system failure that is encountered in humans and animals (eg dogs) (24, 35). In patients affected by BVD-MD, the above processes could cause tissue and organ damage not only as a direct result of virus activity but also in consequence of recurrent DIC in infected cows.

References