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THE INFLUENCE OF BOVINE SEMINAL PLASMA ON BHV -1, EHV-1, BVD and MORBUS AUJESZKY VIRUS REPLICATION IN VITRO

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The aim of this investigation was to estimate the possible influence of bovine seminal plasma (BSP) on the replication in vitro of some sexually transmitted viruses: BHV - 1 (bovine herpes virus - 1) and BVD (bovine viral diarrhea) significant for bovine reproduction. In addition, we tested the influence of the on EHV-1 (equine herpes virus - 1) and Morbus Aujeszcky virus replication in the same model. Our results showed that BSP has an inhibitory effect on the replication in vitro of all tested viruses and their strains. This activity was lost following dialysis suggesting that the active inhibitory factor is of small molecular veight (less than 12 kDa).Furthermore, inhibition was preserved after repeated freezing and thawing and after inactivation for 30 min at 56 ^0C indicating that the inhibitory factor is heat stable.

Key words: bull, seminal plasma, virus replication

INTRODUCTION

Mammalian seminal plasma (SP) is an extremely complex body fluid whose constituents play an important role in the processes before fertilization. For a long time, it was thought that SP only serves to maintain the viability of the sperm cells by supplying them with a sufficient amount of energy and balancing pH. Stating from the mid-seventies and during the last two decades numerous scientists have discovered other important functions of SP. The constituents of mammalian SP exert inhibitory effects on the functions of cells and molecules of the immune system providing sperm with specific immunological protection within the female genital tract (revieved by James and Hargreave 1984; Witkins 1988; Hunter 1989; James and Skibinski 1994). These inhibitory mechanisms are numerous and different in nature, but it is important to mention here that bull, ram and boar seminal plasma contains an enzyme named AS RNAase that is responsible for the inhibition of lymphocyte proliferation *in vitro* (Souček et al. 1978.) Seminal RNAse also has embryotoxic (Matoušek and Grozdanović 1973) and cancerostatic effects (Souček and Matoušek 1979). The native dimer of AS RNAse has a molecular weight of 45 kDa (Souček at al. 1981).

As early as in 1957 Bhargava discovered that bovine seminal plasma (BSP) contains substances capable of inhibiting RNA and protein synthesis. Twenty years later, Reddy and Bhargava (1979) isolated from BSP an antimicrobial protein called seminalplasmin (MW 19.8 kDa) which acts in *E. coli* by specific inhibition of rRNA synthesis. The authors stated that only a part of the total transcription-inhibiting activity of BSP is due to the seminalplasmin that inhibits RNA polymerase. It was also documented that seminalplasmin is a potent inhibitor of E. coli RNA polymerase in vitro (Scheeit et al. 1979) A few years later Rao and Bhargava (1985) isolated from BSP another protein named antiseminalplasmin; that is capable of reversing seminalnplasmin effects. It was postulated that antiseminalplasmin may act by binding to the cell surface and preventing the entry of seminalplasmin into the cells. It has a molecular weight of 39 kDa. Another group of authors (D'Alessio et al. 1972) isolated from bovine seminal plasma a low-molecular weight factor (800-1000 Da) that inhibits RNA synthesis. This substance is a peptide and its inhibitory activity was lost by protease digestion. The authors postulated the mechanism of action to be stabilization of the double stranded DNA molecule.

Fahmi et al. (1985) reported that BSP exerts cytotoxic effects on bovine lymphocytes *in vitro* up to and at 1/100 dilution but not at 1/400. The BSP was immunosuppressive at 1/400 dilution as measured by tritiated thymidine uptake. This finding confirms earlier results suggesting that BSP inhibits deoxyribonucleic acid synthesis in Con A stimulated bovine lymphocytes. The bovine seminal plasma obtained following dialysis in a cellulose dialysis bag (cut off 12 - 14000 da) suppressed thymidine uptake in Con A and PHA stimulated lymphocyte cultures while being noncytotoxic. The same authors (1985 a) isolated two immunosuppressive substances from BSP. One of them had a molecular weight of less than 50 kDa and the other of approximately 150 kDa.

The constituents of seminal plasma can influence viral replication and in 1986, Kuno et al. confirmed that prostaglandin E_2 from human seminal plasma facilitates the replication of the acquired immune deficiency syndrome virus *in vitro*. This prostaglandin is also present in bovine SP but its concentration is much lower (less than 100 mg/ml). Relatively large quantities of PGE and PGF were isolated from incubations of sheep and bull seminal vesicles but their concentration in BSP was low. It is possible that bull spermatozoa bind or metabolize prostaglandins (Kelly 1988.)

Numerous viral agents might be transmited through bull semen and it is of special importance to prevent such transmission in AI (artificial insemination). Bovine herpes virus 1 - BHV 1 (IBR/IPV) can be transmited sexually but under the conditions of natural service, four out of nine cows served by an infected bull, developed clinical signs of the disease, and eight out of nine showed serum neutralizing antibodies (Deas et al., 1973). Kupferschmied et al. (1986) reported transmission of IBR/IPV virus in bovine semen prepared for AI. The problem of BHV 1 in imported semen of high quality can be solved by virus trypsin inactivation without reducing fertilizing capacity (Bielanski et al. 1988). Recently, Oirschout (1995) analyzed the risk of BHV 1 transmission

and concluded that more sensitive methods (e. g. PCR - Polymerase Chain Reaction) should be used for virus detection. The authors preposed that along with the sera, the content of straws for AI should be assayed for the presence of virus.

The aim of our investigations was to estimate the possible influence of BSP on the *in vitro* replication of some sexually transmitted viruses (BHV - 1 and BVD), important for bovine reproduction. In addition, we have tested, in the same model, EHV-1 and Aujeszcky virus replication.

MATERIAL AND METHODS

Preparation of seminal plasma: Semen samples were collected from 10 Holstein bulls by means of an artificial vagina in the Regional Center for Artificial Insemination and Embryotransfer (PKB Agroeconomic, Belgrade). All bulls were seronegative for BHV 1, as estimated by a microserum-neutralization method, as well as for brucellosis, leptospirosis, tuberculosis, enzootic bovine leukemia and parainfluenza. Only those semen samples that possessed normal concentrations, morphology and motility of spermatozoa were used for seminal plasma separation. This was performed by three sequential centrifugations of 15 min. at 400, 800 and 1200 g at + 4 ^{0}C in order to minimize acrosome damage. A pool of seminal plasma was created by taking 1 ml of seminal plasma from 10 ejaculates and was keept frozen at - 20 ^{0}C until used. Before adding to cell cultures the pooled seminal plasma was thawed and sterilized using Millipore filters (0.45 μ). We also used dialyzed seminal plasma samples from the same pool (cellulose dialysis bag, cut off 12 - 14 k Da, 24 hrs at + 4 ^{0}C, 1: 1000).

Cell culture: The calf kidney MDBK line (Madin Darbi) cell culture was used in the experiment. Cells were preserved in nutrient Eagle MEM culture medium, supplemented with 10% fetal calf serum. The investigation was performed on microtitre plates for cell cultures (Nunc) and in each well 100 μl of MDBK cell suspension was added. During the experiment, cells were incubated at 37 ^{0}C in an atmosphere of 5% CO_2. The cells were observed daily, and the results recorded after incubation for 72 hrs.

Viruses: We used four types of viruses: calf herpesvirus-1 (BHV-1), (EHV-1), three strains of bovine diarrhea virus (BVD) and Morbus Aujeszky's disease virus (VA). All viruses and strains are listed in Table 1, along with their titer, calculated according to Reed-Muench (1938).

Cytotoxicity of seminal plasma: It is well known that BSP has cytotoxic effects. Therefore, it was necessary to establish the dilution of seminal plasma that is not cytotoxic for MDBK cells. For that purpose, double series of seminal plasma dilutions were made on microtire plates in ratios from 1:2 to 1:256 (each dilution was made in triplicate). After that, 100 μl of MDBK cell suspension was added to each well containing 50 μl of BSP. The cells were observed daily, and the results were recorded after incubation for 72 hrs.

Table 1. Strains and titre of viruses

No	Virus	Strain	Titer log_{10}
1	BHV–1	TN–4	4
2	BVD	AD 8	3.75
3	BVD	NADL	4
4	BVD	C24 V	4
5	EHV–1	Kentucky	3.75
6	VA	Ercegovac	4

Seminal plasma influence on virus replication: This investigation was performed in two phases. In the first phase, 50 μl of diluted seminal plasma sample (1:8 to 1:256) was added to each well (in triplicate), along with the same quantity of virus with 100 $CCID_{50}$. The microtitre plates with the seminal plasma dilutions and virus were incubated for 60 minutes at the temperature of 38^{0}C. Afterwards, 100 μl of MDBK cell suspension was added to each well. The cells were observed daily, and the results recorded after 72 hrs. In this phase it was possible to estimate the dilution of seminal plasma that inhibits virus replication, preventing the cytopathic effect.

In the second phase we used the dilution of seminal plasma that neutralized 100 $CCID_{50}$ and ten fold dilutions of the virus. The procedure was performed in such a way that the virus dilutions in triplicate (from 10^{-1} to 10^{-5}) were incubated with the same volume (50 μl) of the selected seminal plasma dilution (the dilution that inhibited virus replication in the previous phase). The microtitre plates were incubated for 60 minutes at 37 ^{0}C. After that, 100 μl suspension of MDBK cells was added to each well. The incubation procedure and recording of results were performed in the same way as in the first phase.

In both phases of our investigation, control cells, control virus suspension and control wells with cytotoxic effects of the seminal plasma were included on every microtitre plate.

RESULTS

The cytotoxic effects of dialyzed and non-dialyzed seminal plasma on the MDBK cell culture are presented in Table 2.

Table 2: Survey of cytotoxic effects of bovine seminal plasma

	Seminal plasma dilution							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Nondialysed BSP	3*/3	3/3	1/3	0/3	0/3	0/3	0/3	0/3
Dialized BSP	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

* The number in front of the slash (/) refers to the appearance of the cytotoxic effect in relation to triplicates

The nondialyzed BSP was cytotoxic in dilutions of 1:2 and 1:4. The dilution of 1:8 was cytotoxic in one out of three samples and higher dilutions were not cytotoxic. Cytotoxicity was completely lost after dialysis. We were thus able to demonstrate that cytotoxicity of BSP on the MDBK cells *in vitro* was dose dependent and, at the dilutions of 1:16 and more, was completely lost. Cells cultured with the above mentioned dilutions of BSP had the same appearance as in the control culture.

The inhibitory influence of the non-dialyzed BSP on virus replication *in vitro* is presented in Table 3.

Table 3: The inhibitory effects of nondialyzed BSP on virus replication (100 $CCID_{50}$/50 μl) in the MDBK cell culture

Virus	Strain	Seminal plasma dilution					
		1:8	1:16	1:32	1:64	1:128	1:256
BHV–1	TN – 41	0*/3	0/3	0/3	0/3	2/3	3/3
BVD	AD8	0/3	0/3	0/3	0/3	1/3	3/3
BVD	NADL	0/3	0/3	0/3	0/3	1/3	3/3
BVD	C24 V	0/3	0/3	0/3	0/3	1/3	3/3
VA	Ercegovac	0/3	0/3	0/3	1/3	3/3	3/3
EHV–1	Kentucky	0/3	0/3	0/3	2/3	3/3	3/3

* The number in front of the slash (/) refers to the appearance of cytopathic effects in relation to triplicates.

The dilutions of BSP in rang from 1:8 to 1:64 prevented virus replication in all samples, and neutralized 100 $CCID_{50}$ of bovine herpesvirus-1 (BHV-1) and all three strains of bovine diarrhea virus (BVD). The BSP dilution of 1:128 neutralized BHV-1 in one sample and BVD strains in two samples. Further dilutions of the seminal plasma (1:256) had no neutralizing effects, and virus replication, as judged by the cytopathic effect, was completely expressed. The virus of Aujeszkys disease and the equine herpesvirus-1 were completely neutralized with BSP dilutions of 1:8 to 1:32. The dilution of 1:64 neutralized the virus of Aujeszky's disease in two samples, but in the case of equine herpes it did so only in one sample (out of three). Further dilutions of seminal plasma (1:128 and 1:256) had no neutralizing effects and the cytopathic effect was very clear.

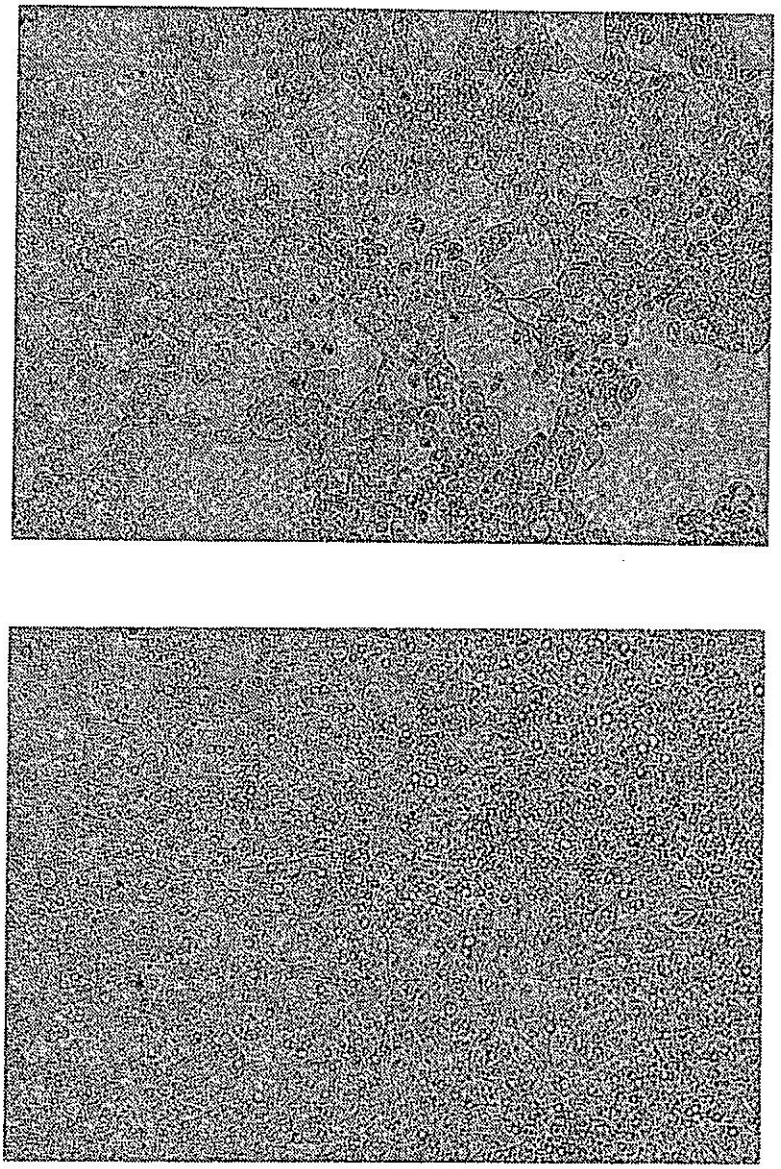


Figure 1 and 1 a: Control of BHV-1 and the effects of nondialyzed BSP (1:64) - cytotoxicity and its inhibition.

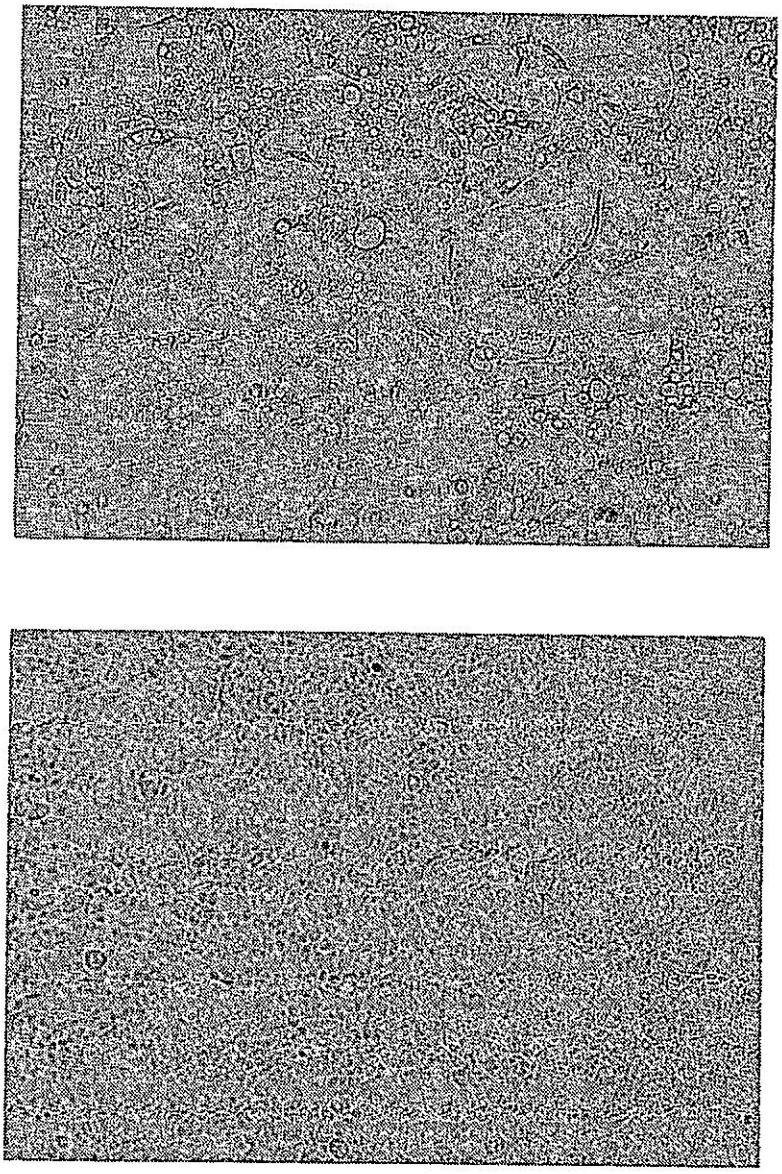
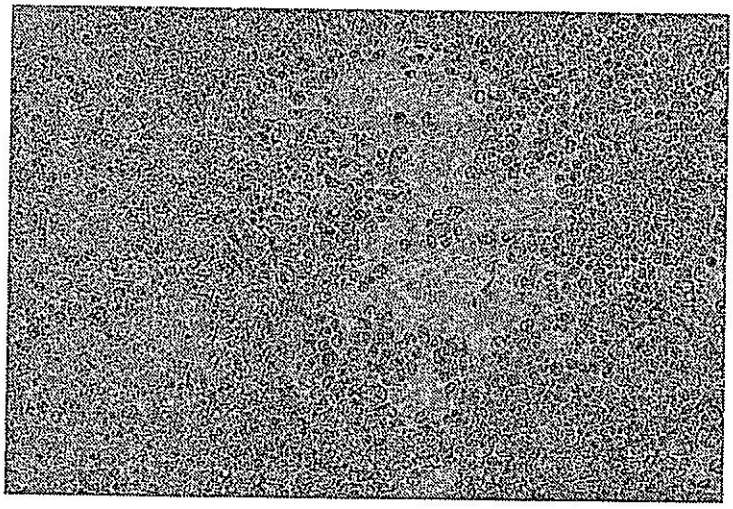


Figure 2 and 2 a: Control of BVD-1 and the effects of nondialyzed BSP (1:64) - cytotoxicity and its inhibition



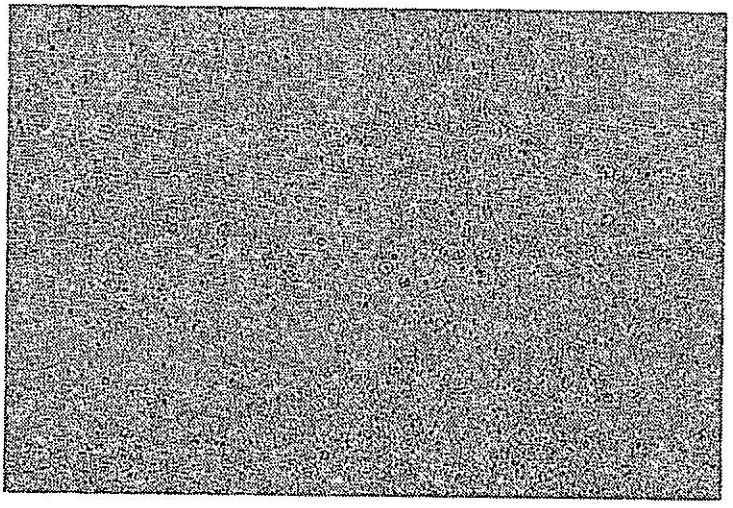


Figure 3. and 3 a: Control of Aujeszky's disease virus and the effects of nondialyzed BSP (1:32) - cytotoxicity and its inhibithion

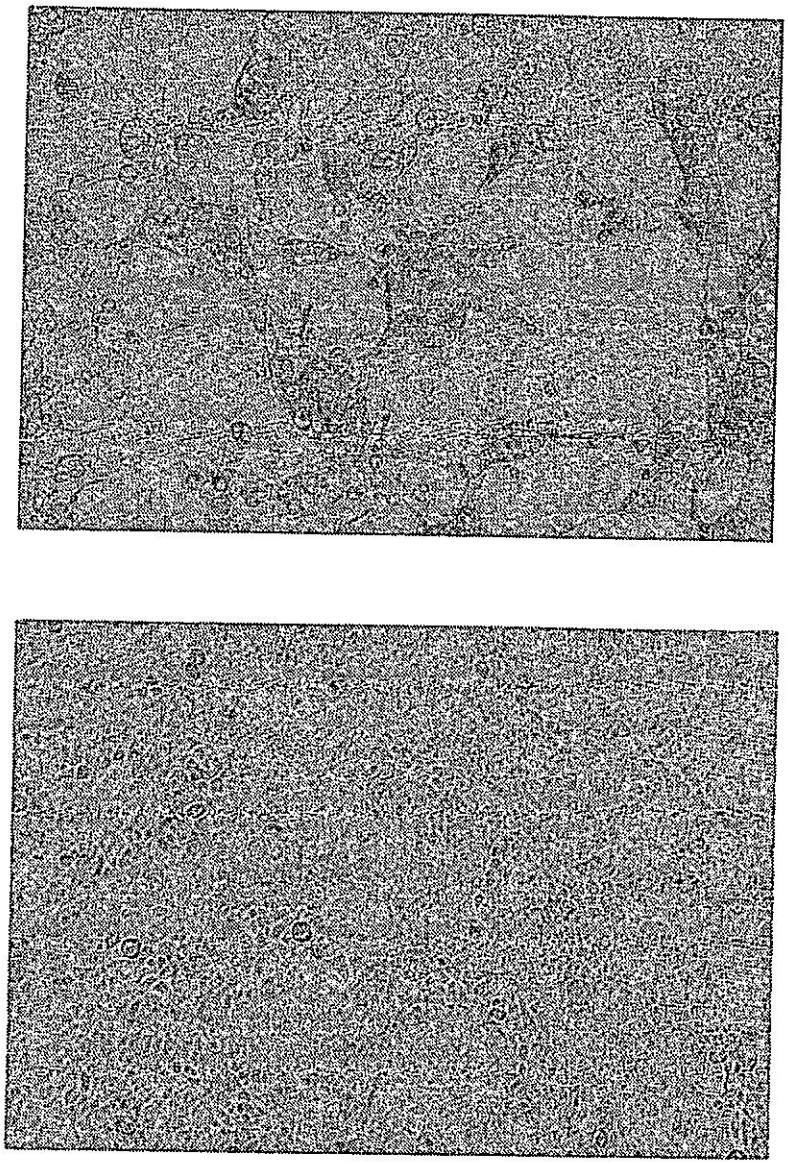


Figure 4 and 4 a: Control of EHV-1 and the effects of nondialyzed BSP (1:32) - cytotoxicity and its inhibition

Figures 1, 2, 3 and 4 illustrate the cytopathic effects of the viruses on MDBK cells in vitro. The inhibitory effects of nondialyzed BSP on virus replication *in vitro* are presented in Figures 1a, 2a, 3a and 4a.

The results of the second plase of the investigation are presented in Table 4, and they confirmed the results in Table 3. Namely, it was documented that the seminal plasma dilution of 1:64 neutralized 100 $CCID_{50}$ of all examined viruses.

Table 4: The inhibitory influence of the BSP dilution 1:64 on virus replication in vitro

Virus	Strain	Virus dilution				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
BHV-1	TN – 41	3/3	0/3	0/3	0/3	0/3
BVD	AD 8	1/3	0/3	0/3	0/3	0/3
BVD	NADL	1/3	0/3	0/3	0/3	0/3
BVD	C24V	1/3	0/3	0/3	0/3	0/3
VA	Ercegovac	3/3	1/3	0/3	0/3	0/3
EHV-1	Kentucky	3/3	1/3	1/3	0/3	0/3

* The number in front of the slash (/) refers to the appearance of cytopathic effects in relation to triplicates.

The investigation of dialyzed seminal plasma was methodologically, performed in the same way as for the nondialyzed samples. Even the lowest dilution (1:2) of dialyzed BSP did not prevent virus replication. The cytopathic effect was expressed with all dilutions of BSP as well as in all concentrations of the examined viruses (results not shown here).

In another survey of this phenomenon we used inactivated (56 ^{0}C, 30 min) samples of nondialyzed BSP from the same pool. The inhibitory effects of the inactivated BSP sample on virus replication were nearly the same as presented in Table 4 (results not shown here).

DISCUSSION

As with many other authors (Matoušek and Grozdanović, 1973, Souček et al. 1978 Fahmi et al. 1985), we were able to demonstrate that bovine seminal plasma exerts cytotoxic effects. Cytotoxicity was dose dependent and decreased according to the degree of dilution. It was documented that the cytotoxic effect was lost following dialysis, which is in general agreement with the findings of Fahmi et al. (1985). The main difference between the two sets of results is in the degree of dilution. We found that the cytotoxic effect of BSP on MDBK cells was lost in dilutions higher than 1:16, while Fahmi et al. 1985 stated that cytotoxicity towards bovine lymphocytes still exists at the dilution of 1:100. This could be explained by differences in cell sensitivity (MDBK vs. lymphocytes).

Our results indicate that the BSP factor responsible for the inhibition of *in vitro* virus replication is of low molecular weight because this activity was lost after dialysis. In our study all tested viruses belonged to the group of DNK viruses and inhibitors of nucleic acid synthesis are candidates for the inhibitory effects on their replication. However, AS RNAse (Stanek et al. 1978), factors isolated by Fahmi et al. (1985a) and Reddy and Bhargava (1979) have high molecular weights and they were not separated during dialysis in cellulose bags. That means that substances from BSP exerting embryotoxic and cancerostatic effects and also those that inhibit lymphocyte proliferation are not responsible for the inhibition of *in vitro* virus replication. There is a possibility that the low molecular weight peptide from BSP, isolated by Lugaro and colleagues (1984) is the substance that inhibits virus replication by acting on RNA synthesis.

The seminal plasma inhibition of virus replication *in vitro* can be of biological immportance because it will reduce chances for spreading sexually transmissible diseases in a population. Deas and Johnson (1973) reported that during natural service by an infected bull all cows did not develop signs of the disease. At the same time, during the preparation of bull semen for A.I. of cows, the ejaculates are diluted several dozen times and the volume of the straws for A.I. is usually 0.45 ml (compared to the approximately 5 ml of ejaculate in the natural service). For that reason the amount of seminal plasma in A. I. is significantly reduced (about 300 - 500 times). This could enhance chances for virus replication and it is extremely important to use only semen from seronegative animals as well to check for the possible presence of viruses in the insemination straws. Some recent experiments showed that trypsin treatment of BHV-1 infected semen reduced semen quality and that this procedure must be carefully applied (Silva et al. 1999). Therefore, it is necessary to use only semen from seronegative bulls in the AI centers, because with the decreased amount of SP in AI straws the possibility for infection might be enhanced. It is of interest to explore if the BSP activity on virus replication is lost during semen preparation for AI and to estimate individual differences between various bulls.

The biological importance of the described *in vitro* phenomenon is still unclear but it seems reasonable that along with the strong, well known, immunosuppressive properties, SP prevents viral and bacterial growth. If this were not the case, all genital infections might have deleterious effects on the female's health. Some other antiviral substances are also present in bull's semen. One of them is the iron-binding glycoprotein, lactoferrin, a constituent of many biological secretions such as milk, tears, semen, neutrophyl granulocytes and plasma. Lactoferrin is able to inhibit replication of HIV-1 in a dose dependent manner (Puddu et al. 1998). It is postulated that lactoferrin prevents virus binding to C 8 166 T - cells. At the same time human seminal plasma might enhance Epstein-Barr virus replication by inhibiting activity of cytotoxic T lymphocytes (Turner et al. 1990).

It was also documented in our study that the virus replication inhibitory factor from BSP was heat stable (as the above-mentioned peptide) because its activity was still present after inactivation and repeated freezing and thawing.

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UTICAJ SEMENE PLAZME BIKA NA REPLIKACIJU VIRUSA BHV-1, EHV-1, BVD I VIRUSA AUJESKIJEVE BOLESTI U *IN VITRO* USLOVIMA

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SADRŽAJ

Cilj ovih istraživanja je bio da se ispita mogući uticaj semene plazme bika (BSP) na *in vitro* replikaciju nekih virusa koji se prenose polnim putem: BHV - 1 (Bovine herpes virus - 1) i BVD (Bovine viral diarrhoea) a značajni su za reprodukciju goveda. Dodatno smo, na istom modelu, ispitali uticaj BSP na replikaciju EHV-1 (Equine herpes virus - 1) i Morbus Aujeski virusa. Naši rezultati su pokazali da BSP ima inhibitorne efekte na replikaciju svih testiranih virusa i njihovih sojeva. Ova aktivnost se gubi posle postupka dijalize što ukazuje da je aktivni faktor male molekulske mase (manje od 12 kDa). Šta više, inhibicija je bila očuvana posle ponovljenih otapanja i zamrzavanja i posle inaktivacije u trajanju od 30 min na 56 ^{0}C što dokazuje da je inhibitorni faktor termostabilan.