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In vitro and *in vivo* evaluation of sodium dodecyl sulfate (SDS) as an inactivator of caprine lentivirus (CLV) in colostrum and milk

[*Avaliação* in vitro *e* in vivo *do dodecil sulfato de sódio (SDS) como inativador do lentivírus caprino (LVC) em colostro e leite*]

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ABSTRACT

The aim of this study was to evaluate *in vitro* and *in vivo* the effect of sodium dodecyl sulfate (SDS) on the caprine lentivirus (CLV) in colostrum and milk. This was performed to develop a practical and efficient method of blocking the lactogenic transmission of the virus. In the *in vitro* experiment, colostrum and milk were treated with 0.25%; 0.50% and 1% SDS. Then, somatic cells of colostrum and milk were submitted to co-culture with caprine synovial membrane cells (CSM). In the *in vivo* test, goats were fed with colostrum and milk provided from CLV-positive goats treated with SDS in the same concentrations used in the *in vitro* experiment. Animals were tested by nested polymerase chain reaction (nPCR) and Western blot (WB) assays. In the *in vitro* experiment, inhibitory activity against CLV without inactivation occurred in colostrum with all SDS concentrations. However, concentrations of 0.25 and 0.5% SDS presented only inhibitory activity against CLV in milk cells, and 1% concentration provided inactivation of the virus. In the *in vivo* tests, none of the three concentrations of SDS was effective in inactivating LVC in colostrum or goat milk, which was confirmed by seroconversion and presence of proviral DNA in animals afterwards.

Keywords: monocyte-phagocytic system, viral inactivation, surfactant, small ruminant lentiviruses

RESUMO

O objetivo da pesquisa foi avaliar in vitro *e* in vivo *o efeito do dodecil sulfato de sódio (SDS) sobre o lentivírus caprino (LVC) no colostro e no leite, a fim de desenvolver um método prático e eficiente no bloqueio da via de transmissão lactogênica do vírus. No experimento* in vitro*, o colostro e o leite de cabras positivas foram tratados com SDS a 0,25%, 0,50% e 1,0%. Em seguida, as células somáticas do colostro e do leite foram obtidas e direcionadas ao cocultivo com células de membrana sinovial caprina (MSC). No teste* in vivo*, os cabritos foram alimentados com colostro e leite providos de cabras positivas para LVC, tratados com SDS nas mesmas concentrações usadas no teste* in vitro*. Os animais foram acompanhados pelos testes de reação em cadeia da polimerase* nested *(nPCR) e* western blot *(WB). Nos resultados* in vitro*,* no colostro*, observou-se que, em todas as concentrações de SDS, ocorreu uma atividade inibitória contra o LVC, sem a inativação. Em relação às células do leite, o SDS apresentou, nas concentrações de 0,25 e 0,5%, atividade inibitória contra o LVC, e na concentração de 1%, houve inativação viral. Nos testes* in vivo*, as três concentrações de SDS testadas não foram efetivas na inativação do LVC no colostro e no leite caprino, o que se comprovou pela soroconversão e pela presença de DNA proviral nos animais.*

Palavras-chave: sistema monociticofagocitário, inativação viral, surfactante, lentiviroses de pequenos ruminantes

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INTRODUCTION

Small ruminant lentiviruses (SRLVs) are diseases caused by viruses of the Retroviridae family, genus *Lentivirus*. These pathogens include ovine lentivirus (OLV) and caprine lentivirus (CLV) (Pisoni *et al.*, 2005). SRLVs cause significant economic losses in goat production (Carneiro, 2011) and clinically cause interstitial pneumonia, arthritis, mastitis, and progressive emaciation, in addition to neurologic conditions in goat kids (Callado *et al.*, 2001).

Lactogenic transmission in either colostrum or milk is a natural model of infection recognized in lentiviruses in general. This form guarantees viral dissemination between generations and persistence of the pathogen in the flock in a population level. In addition, this aspect has a fundamental role in the biology of these viruses, considering that it is one of the main forms of transmission (Pisoni *et al.*, 2010). According to Herrmann-Hoesing *et al.* (2007), animals are exposed to infection by the ingestion of colostrum contaminated with free viral particles and proviruses within monocytes and macrophages, which are then absorbed in the gastrointestinal tract.

Among the blockade techniques of the virus in lactogenic transmission, thermization can be performed in colostrum, transition milk and common milk. Colostrum, for instance, after collection from does soon after giving birth, may be placed in plastic bottles which are sealed and submitted to water bath at 56ºC for an hour. Then, bottles are removed and cooled to room temperature, which may then be stocked at -20ºC until milk feeding (Peretz *et al*., 1993; Andrade, 2008). However, this method may present costs, considering the necessary equipment, such as water bath or heater, and technical labor. Therefore, low cost and practical alternatives that effectively block SRLVs transmission in colostrum and milk are necessary for goat farmers.

Some studies correlating the use of chemical additives in milk with an inhibitory activity of microorganisms have been performed (Muller e Syhre, 1975; Krebs *et al.*, 1999; Urdaneta *et al.*, 2005). Among these, sodium dodecyl sulfate (SDS), a surfactant with cytolytic properties, presents a possible antiviral activity in cell cultures of HIV-1 *(Human immunodeficiency virus type 1*), HSV (*Herpesvirus*) and HPV (*Papillomavirus*) (Piret *et al.*, 2002). Krebs *et al.* (1999) and Urdaneta *et al.* (2005) demonstrated that 0.1% SDS may be effective in inactivating HIV-1 in milk and reported that this concentration is in accordance with safe limits for child consumption. In addition, other studies indicate that 1% SDS in colostrum is an effective biocide and there is no interference in passive transference of immunity or health problems in goat kids (Morales-De La Nuez *et al.*, 2011). Therefore, this study aimed to evaluate *in vitro* and *in vivo* the use of sodium dodecyl sulfate (SDS) in colostrum and milk positive for the caprine lentivirus (CLV) in order to develop a practical and efficient method of blocking the lactogenic transmission of the virus.

MATERIAL AND METHODS

This study was approved by the Ethics Committee for the Use of Animals of the Embrapa Goats and Sheep with the protocol number 012/2014.

The experiment was performed in the same location and divided in two phases (*in vitro* and *in vivo*). In the first phase, colostrum of five adult goat nannies seropositive for CLV, which were tested with Western blot technique, was collected in sterile tubes after giving birth. Approximately 25mL of colostrum was collected from each animal and samples were combined in a pool, which was subdivided in 12 aliquots of 10mL each. Milk collection was performed in the same animals after 30 days following the same methodology.

SDS (CEQuímica®, Brasil) treatment was performed adding the compound to colostrum and milk samples in the concentrations of 0.25%, 0.5% and 1% in triplicate for 15min. Control treatments were used in colostrum and milk without adding SDS. Then, samples were centrifuged at 3,000g for 15min at 4ºC. Somatic cells were obtained following the methodology of Karanikolaou *et al.* (2005).

In order to perform co-culture with caprine synovial membrane cells (CSM), biological samples from SDS treatment step in colostrum and milk were washed with 1000µL of 1X PBS (phosphate-buffered saline: 8g NaCl; 0.2g KCl;

0.2g KH_2PO_4; 1.15g Na_2HPO_4 and 1000mL of H_2O q.s.p.; components: Sigma-Aldrich®, EUA) for removing SDS. Then, 1000µL of minimal essential medium (MEM - Gibco®, EUA), treated with 2% amphotericin B (Sigma-Aldrich®, EUA), 3% penicillin and streptomycin (P/S - Gibco®, EUA), 1% gentamycin (Sigma-Aldrich®, EUA) and 10% fetal bovine serum (FBS - Gibco®, EUA) was added. Then, aliquots of 100µL of samples were distributed in 24 well plates in eight repetitions, in which 1900µL of MEM treated with 5% of FBS was added. A total of 16 control wells were used throughout the culture period. In half, only CSM cells were added as negative control. In the other half of wells, CSM cells infected with standard sample of CLV (CAEV-Cork) with an initial titer of $10^{4.8}$ TCID50/mL were added as positive control. Plates were incubated in 5% CO_2 at 37ºC for 24h. Then, CSM cells were added in eighth passage in the concentration 2.0 x 10^5cells/µL, following incubation in the same previous conditions. In every seven days, media were replaced and cellular trypsinization was performed, and a total duration of 63 days of culture. Supernatants were collected and separated per treatment to be submitted to nested polymerase chain reaction (nPCR). Following culture, four wells of each treatment were stained with violet crystal (0.1%) and the remaining was submitted to trypsinization to collect cellular material.

Proviral DNA extraction of cellular supernatant from co-culture was performed according to the methodology by Feitosa *et al.* (2011) and nPCR was performed according to Barlough *et al.* (1994).

In phase two of experimentation (*in vivo*), SDS was tested in 31 goat kids, male, born of females negative for CLV by WB and nPCR. Three experimental groups were formed according to the concentrations of SDS, containing seven goat kids each. In addition, two control groups (positive and negative) were formed with five animals. Goat kids were immediately separated after birth, weighted, and submitted to the same diagnostic tests.

SDS was diluted from a standard concentration (SC) to 10% using 100g SDS for every 1000mL of sterile Milli-Q water. To form the concentrations of 0.25%, 0.5% and 1%, the volumes 25mL, 50mL and 100mL of SC were used, respectively, for every liter of caprine colostrum or milk used in feeding the animals of each experimental group.

Inoculum was prepared with SDS using colostrum and milk collected daily from positive nannie goats. Before any treatment, aliquot (10mL) of colostrum and milk were collected for nPCR test to confirm the presence of CLV in the samples. Then, colostrum and milk were treated with SDS in the previous concentrations for 15min and administered to the goat kids. Negative control animals received colostrum and milk treated thermally (56ºC for 60min) and positive control received colostrum and milk from infected goats. Treatments were administered to animals for six days. Then, animals received thermally treated milk until weaning.

Along with milking, experimental monitoring was performed, in which blood collection was performed for diagnostic tests in days 0, 7, 15, 30 and every month for a semester. Blood was collected with venipuncture of jugular vein. Blood serums and leucocytes were maintained in microtubes (*Eppendorf*®, EUA) and frozen at -20ºC until WB was performed, which followed methodology by Pinheiro *et al.* (2011), and nPCR.

RESULTS AND DISCUSSION

Viral cytopathic effects were investigated *in vitro* evaluation with co-culture of CSM and somatic cells of colostrum and goat milk treated with SDS at 0.25%, 0.5% and 1% after 63 days of culture (Table 1). Colostrum treatments of 0.25% and 0.5% SDS revealed a gradual decrease in the presence of syncytium and cell destruction. The concentration of 1% SDS did not cause cytopathic effects, which is an indication of CLV infection. In milk, 0.25% SDS treatment reduced the formation of syncytium and cell destruction. The concentrations of 0.5% and 1% did not produce cytopathic effects.

Table 1. Levels of cytopathic effects in CSM cells after co-culture with somatic cells of colostrum and milk treated with SDS

Cytopathic effects	SDS Treatments											
	Colostrum						Milk					
	C^-	C^+	N/SDS	0.25%	0.5%	1%	C^-	C^+	N/SDS	0.25%	0.5%	1%
Syncytium	-	+++	++	+	-	-	-	+++	++	+(+)	-	-
Cellular Destruction	-	+++	+	(+)	(+)	-	-	+++	++	+(+)	-	-

C^-: negative control; C^+: positive control; N/SDS: no SDS. -: no cytopathic effect; (+): very light cytopathic effect, +: light effect; +(+): light to moderate effect; ++: moderate effect; +++: intense effect.

Figure 1 demonstrates viral cytopathic effects caused by CLV in CSM cells from co-culture with somatic cells from caprine colostrum and milk treated with SDS. The cytopathic effects of multi-nucleated cells and cell destruction were observed in cultures without the addition of SDS (Figure 1A to 1J).

A gradual reduction of cytopathic effect was observed with the progression of the experiment according to the SDS concentration of treatments. In addition, 1% SDS concentrations in colostrum did not present characteristics of viral infection (Figure 1F). In milk, this decrease was observed in SDS concentrations of 0.5% and 1% (Figure 1K and 1L). In both co-cultures, cells from mammary glands characterized by rounded forms and increased nuclei in comparison to CSM were observed (Figure 1E, 1F).

Viral cytopathic effects were more clearly observed in samples of colostrum in comparison to milk. This difference may be related to the defense cells (monocytes and macrophages) and lipid molecules, which are found in greater rates in colostrum. These characteristics decrease SDS efficiency due to the superior proportions of fat in comparison to surfactant, causing a possible reduction in processes of cell suffering and lysis (Partearroyo *et al.*, 1990; Kalmanzon *et al.*, 1992). In this context, monocytes/macrophages when are not destroyed may carry the virus in a cellular and flock level (Herrmann-Hoesing *et al.*, 2007).

Table 2 present analyses of cell suspension and supernatant with nPCR test in 63 days of co-culture. Colostrum samples presented positive results at least once in tests, independent of SDS treatment. Milk samples presented positive results in reactions of SDS treatments 0.25% and 0.5%. However, 1% SDS concentration did not present positive results in any of the samples submitted to nPCR test throughout the experiment.

Data found in this study demonstrated that SDS presented inhibitory activity against CLV in colostrum, considering the decrease in cytopathic effects as concentrations increased. However, a complete viral inactivation was not possible. In milk, SDS presented partial inhibitory activity against CLV in 0.25% and 0.5% concentrations, considering the decrease in cytopathic effects as concentrations of the surfactant were increased. However, 1% SDS presented a probable viral inactivation of CLV in culture, considering the absence of cells with infectious characteristics and negative nPCR results.

Comparing the viral activity of SDS in CLV and data found in literature with HIV-1 revealed that SDS concentrations needed for a significant result of CLV inactivation were ten times higher than what Urdaneta *et al.* (2005) used. These authors evaluated samples in a rapid *in vitro* system (MAGI - *Multinuclear Activation of Galactosidase Indicator*), which quantifies viral infectiveness after microbiocide treatment. They found that SDS concentrations of 0.1 and 0.5% for 10min in HeLa cultures were sufficient to cause full inhibition of viral infection caused by HIV-1 in milk. Similar findings were observed used in studies performed by Krebs *et al.* (1999) with 0.025% and 0.05% SDS in HIV-1, which are 20 and 40 times lower than 1% concentration used in this study.

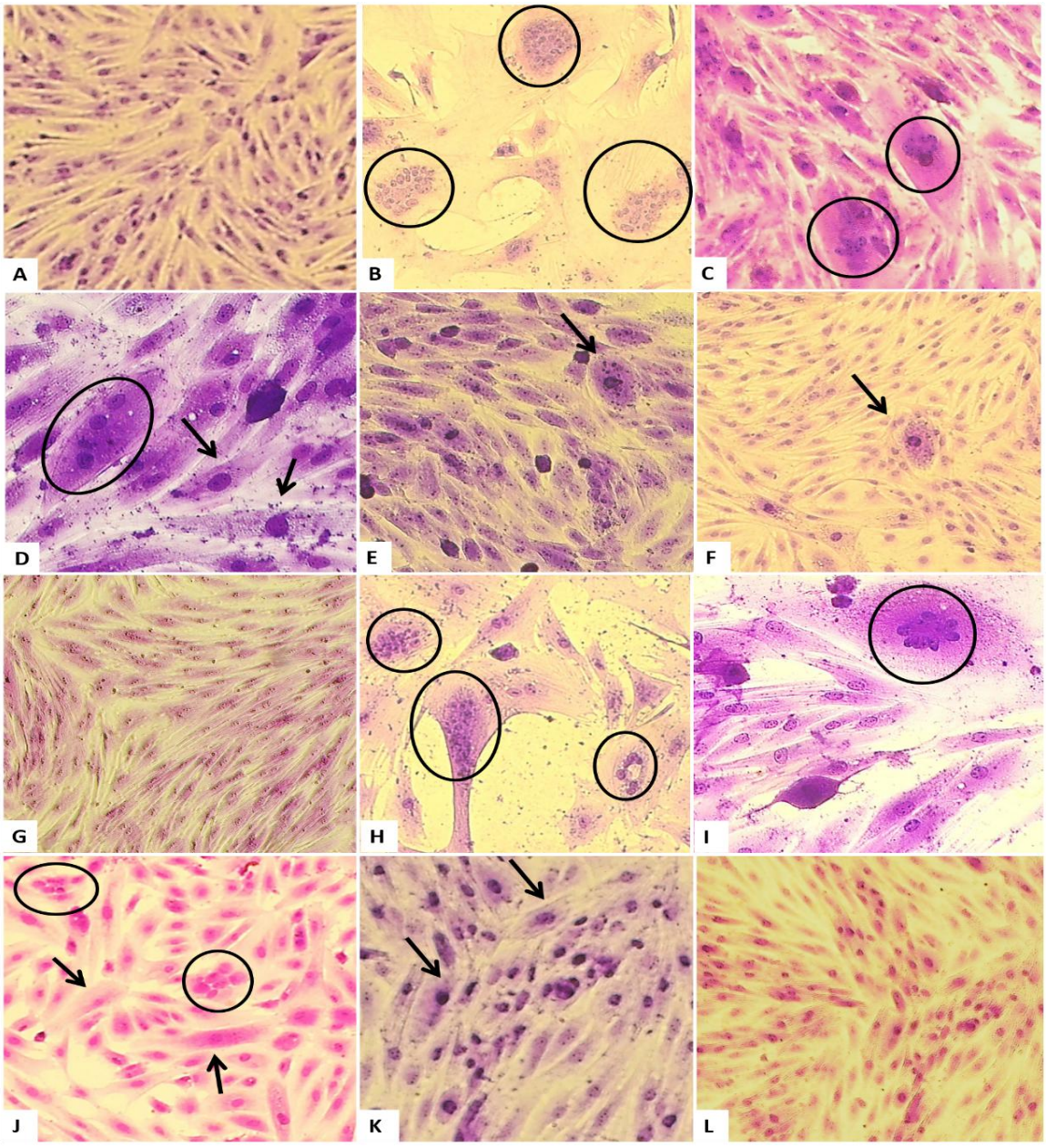

Figure 1. Co-culture of CSM and somatic cells in colostrum and milk. A and G: negative control of CSM cells (100x magnification). B and H: positive control of culture (100x magnification); C and I: control treatment (N/SDS) with colostrum and milk cells, respectively, with cytopathic effects (200x magnification). D, E and F: SDS treatment at 0.25%, 0.5% and 1% in colostrum, respectively. D: 0.25% SDS with presence of syncytium (circle) and cells visually increased in size (magnification 200x). E: SDS treatment at 0.5% with presence of cells (arrow) visually increased in size and rounded (magnification 200x). F: SDS treatment at 1% without the presence of cytopathic effects and only some cells (arrow) with a differentiated morphology in comparison to the others in colostrum (magnification 100x). J, K and L: SDS treatment at 0.25%, 0.5% and 1% in milk, respectively; J: 0.25% SDS treatment with presence of syncytium (circle) and cells visually increased in size (200x magnification); K: 0.5% SDS treatment with presence of cells (arrows) visually increased in size (200x magnification); L: 1% SDS treatment without the presence of cytopathic effect (100x magnification).

Table 2. Results of nPCR in supernatant samples of CSM and somatic cells co-culture from colostrum and milk

SDS Concentrations (%)	Colostrum			Milk		
	21d*	42d	63d	21d	42d	63d
0.25	+	+	+	+	+	+
0.5	+	+	-	+	+	+
1	+	-	+	-	-	-

*: days of culture

A greater resistance of SRLV in comparison to HIV was also observed by Thormar *et al.* (1995), which used several viral inhibitors, among these: plant derived lectins (LOA, GNA, NPA, CA, GlcNAc, UDA, Gal-4, GalNAc, SNA-II, BPA, IRA, SBA, Neu5Ac, Gal / GalNAc), TIBO-RT (*tetrahydroimidazo-[4,5,1-jk][1,4]-benzodiazepin-2-(1H)-one*, reverse transcriptase inhibitor) and AZT (3'-Azido-2',3'-dideoxythymidine). These authors verified that concentrations 12 to 40 times higher of lectin, 200 to 300 times higher of TIBO-RT and 5 to 10 times higher of AZT were necessary to cause a full inactivation of Maedi-Visna virus (MVV) in comparison to concentrations used for HIV.

In the second experimental phase (*in vivo*), molecular (nPCR) and serological (WB) follow-ups were performed in experimental animals, which were fed with colostrum and milk of infected goat nannies treated with SDS at 0.25%, 0.5% and 1% (Table 3).

Table 3. Individual monitoring with nPCR and WB of experimental animals

		0d		7d	15d	30d	60d	90d		120d		150d		180d	
Groups	Animal	nPCR	WB		nPCR			nPCR	WB	nPCR	WB	nPCR	WB	nPCR	WB
Cont(-)	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cont(+)	F	+	-	+	+	+	-	-	+	-	+	+	+	+	+
	G	-	-	-	-	+	+	-	-	-	-	-	-	+	-
	H	-	-	-	-	-	-	-	+	-	+	-	+	+	+
	I	-	-	-	+	+	-	-	+	-	-	-	+	+	+
	J	-	-	-	-	-	+	-	+	-	+	-	+	-	+
	K	-	-	-	-	-	-	-	+	-	+	-	+	-	+
G1 (0.25%)	L	-	-	+	+	+	-	+	-	*					
	M	-	-	-	-	-	-	-	-	-	-	+	-	+	-
	N	-	-	-	+	-	-	-	+	-	-	-	+	-	+
	O	-	-	-	-	+	-	-	-	-	-	+	-	+	-
	P	-	-	-	+	-	-	-	-	-	+	+	-	+	-
	Q	-	+		#										
G2 (0.5%)	R	-	-	-	-	-	-	-	+	-	-	-	-	+	-
	S	-	-	+	-	-	-	+	-						
	T	-	-	-	-	+	-	-	+	-	+	-	+	+	+
	U	-	-	-	+	-	-	-	-	-	-	+	-	-	-
	V	-	-	+	-	-	+	-	-						
G3 (1%)	W	-	-	-	-	-	+	+	+	-	+	-	+	-	+
	X	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	Y	-	-	-	-	-	-	-	-	-	-	+	-	+	-
	Z	-	-	-	-	-	-	-	+	-	-	-	+	+	+
	A1	-	-	-	-	-	-	-	+	-	-	-	-	+	-

+: positive diagnostic, - : negative diagnostic, *: animals that died before collection of 120 days (suggestive of clostridiosis), #: animal eliminated from group due to positive result in 0h in WB test.

Positive results were found soon in the second collection (7 days) for nPCR after experimentation in G1 and G2. In collections performed after 180 days, 60% (3/5) of animals in G1 and 40% (2/5) of G2 were positive in nPCR. Animals of G3 were positive for CAEV only in collection performed at 150 days, when one animal (33%) was positive. However, at 180 days, 100% (3/3) of animals were positive. Data found in this study demonstrated that SDS concentrations tested in caprine milk and colostrum did not promote *in vivo* inactivation of the lentivirus, exposing animals to infection.

At 90 days of evaluation, antibodies for CLV were identified in WB test of all groups that received colostrum/milk (G1- 33.3%; G2 – 42.8%; G3 – 66.7%; and positive control group - 80%), confirming the infection and activation of immune system. In addition, animals from these groups were also positive in all collection thereafter (120 days, 150 days, and 180 days). Antibodies for CLV found until 70 days of life detected by WB are very likely to have been originated from colostrum (Souza *et al.*, 2015). Antibodies were detected after 120 days of life and proviral DNA after seven days of experimentation, which indicates that SDS concentrations were not effective in viral inactivation, confirming results obtained with molecular and serological diagnostic tests.

Despite the report by Piret *et al.* (2002), which demonstrates that SDS is a potent inhibitor of infectiveness of enveloped and non-enveloped viruses due to protein denaturation properties, the *in vivo* study here reported did not present CLV inactivation. In addition, this data contradicts the *in vitro* results by Urdaneta *et al.* (2005), which assessed infectiveness level of HIV. According to these authors, concentrations and time of action of SDS were sufficient to inhibit in full the viral infection index of HIV-1 in milk. This study does not corroborate with Krebs *et al.* (1999), which identified inhibition of HIV-1 by SDS concentrations of 0.0025% to 0.05%.

In addition to evaluating the SDS effect as viral inactivator, the second experimental phase (*in vivo*) allowed the assessment of receptiveness of goat kids to treated colostrum and milk. During milk feeding, animals from G1 did not present clinical alterations, demonstrating a good receptiveness in consuming colostrum and milk treated with SDS at 0.25%. Animals from G2 and G3 presented good acceptance of colostrum treated with SDS at 0.5% and 1%, respectively, without clinical signs. However, during the milk-feeding period, both groups presented rejection to treated milk in the same concentrations as SDS. To improve palatability, 2g of strawberry flavored Nesquik (Nestle®) was added to every 200mL of milk. After 24h of treatment, all animals from G2 group (7/7) presented light diarrhea. However, these animals remained in the experiment until the period of six days administering milk was ended. In G3 group, after 24h of ingestion of milk treated with SDS at 1%, 57.1% (4/7) of animals died and the remaining presented intense diarrhea and apathy. Therefore, treatment was suspended, and animals were maintained and monitored until the end of the experiment.

Hartmann *et al.* (2006) described a method of removing SDS with a commercial resin, which is based in the ion-exchange chromatography. This is considered an efficient method for removing SDS, considering that protein recovery of 4 out of 5 samples of human milk treated with 1% SDS was 100%. However, this method would not be applicable or profitable for goat farmers, considering the equipment, reagents and specialized labor necessary to execute. In this sense, SDS was not removed from milk or colostrum provided for goat kids in the second experimental phase.

These adverse effects that occurred in G2 and G3 probably occurred due to the lytic activity of SDS, which was not completely emulsified to lipids found in milk, causing possible lesions in gastrointestinal epithelial cells. SDS in direct contact with cells may cause permeability alterations in plasma membrane, which is the target structure for this substance (Benoit *et al.*, 1987; Partearroyo *et al.*, 1990).

According to Morales-De La Nuez *et al.* (2011), SDS treatment at 1% in caprine colostrum promoted a reduction in bacterial load and lower destruction of immunoglobulin G (IgG) in comparison to what is observed in pasteurization. In addition, these authors reported that goat kids fed with 1% SDS in colostrum did not present pathological effects of alterations in passive immune transference.

Kimura and Yoshida (1982) observed the reduction of enzymatic activities in pharmacological studies after administration of SDS at 0.25% in diet of mice. During experimentation, diarrhea, exfoliation of gastrointestinal membrane and malnutrition were observed in animals.

Data observed in this study with the sole administration of colostrum treated with SDS corroborate with Morales-De La Nuez *et al.* (2011) in health status of the animals. However, SDS may cause diverse effects when administered in diet, corroborating with data by Kimura e Yoshida (1982).

CONCLUSIONS

The *in vitro* study promoted inhibition of the caprine lentivirus in milk only in the 1% concentration of SDS. In the *in vivo* evaluation, SDS in colostrum/milk in none of the tested concentrations promoted inactivation of the caprine lentivirus. In addition, gastrointestinal complications occurred, such as profuse diarrhea. However, more studies are necessary in the chemical inactivation of virus to elucidate the activity of SDS in small ruminant lentiviruses. Considering the promising *in vitro* results found in this study, more studies involving the action of this compound in colostrum and milk should be performed.

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