



Effect of intrauterine application of ozone on neutrophil migration and subclinical endometritis in dairy cattle



Jessica Bárbara González-Aguado ^a

Elisa Ochoa-Estrada ^b

Héctor Raymundo Vera-Ávila ^a

Ma. de Jesús Chávez-López ^{a,c}

Mario Alfredo Espinosa-Martínez ^b

Germinal Jorge Cantó-Alarcón ^a

Claudia Gutiérrez-García ^d

Luis Javier Montiel-Olguín ^{a,b,d,*}

^a Universidad Autónoma de Querétaro. Facultad de Ciencias Naturales, Maestría en Salud y Producción Animal Sustentable. Av. de las Ciencias s/n, 76230, Santiago de Querétaro, Querétaro, México.

^b Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, CENID Fisiología y Mejoramiento Animal. Querétaro, México.

^c Universidad Autónoma de Querétaro. Centro de Extensión e Innovación Regional AC. Querétaro, México.

^d Tecnológico de Monterrey, Escuela de Ingeniería y Ciencias. Querétaro, México.

* Corresponding author: montiel.luis@inifap.gob.mx

Abstract:

The objective was to determine whether an ozonized saline solution (O3SS) increases endometrial polymorphonuclear neutrophils (PMNN) (Exp 1) and to challenge the preventive effect of O3SS on subclinical endometritis (SCE) (Exp 2). In Exp 1, 38 primiparous Holstein cows were used. Cows with (WHM) and no history of postpartum

metritis (NHM) were included; they were then divided into control (CTRL, saline solution) or O3SS (6.7 ± 0.3 ppm) subgroups. At 55 d postpartum, 50 ml of CTRL or O3SS was applied intrauterinely and at 48 h, the quantity of PMNN was recorded by endometrial cytology. In Exp 2, 26 primiparous NHM Holstein cows were used. The cows were randomly distributed in CTRL or O3SS. Two doses of 50 ml were administered with an interval of 7 d (first application 11.3 ± 0.4 d postpartum). At d 30 postpartum, SCE (≥ 6 % PMNN) was diagnosed. WHM cows had higher numbers of endometrial PMNN compared to NHM cows (13.9 ± 6.2 vs 1.0 ± 0.46 , $P < 0.05$). The WHM-CTRL group had a higher number of PMNN than NHM-CTRL (17.0 ± 9.6 vs 0.1 ± 0.1 , $P < 0.05$), while the WHM-O3SS and NHM-O3SS groups (10.4 ± 8.1 vs 1.8 ± 4.8 , $P > 0.05$) and the NHM-CTRL and NHM-O3SS groups (0.1 ± 0.1 and 1.8 ± 4.8 , $P > 0.05$) were not different. A statistical trend ($P = 0.09$) of lower percentage of SCE was observed in the CTRL group compared to O3SS (15.4 and 46.2 %, respectively). In conclusion, transcervical O3SS does not increase the number of endometrial PMNN and preventive treatment with O3SS applied to NHM cows did not decrease SCE. The results suggest a possible anti-inflammatory effect of the ionized saline solution treatment.

Key words: Ozone therapy, Puerperium, Holstein.

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Subclinical endometritis (SCE) is a puerperal disease with high prevalence in dairy farms^(1,2). Cows with SCE are characterized by high polymorphonuclear neutrophil (PMNN) counts in endometrial cytological samples. This disease has a negative impact on reproductive performance because it reduces the conception rate per service by increasing the open days^(1,2). In addition, it is a difficult disease to diagnose at the field level since it requires the use of specialized instruments and the use of a microscope^(3,4). In addition to this, other puerperal diseases, such as placental membrane retention and metritis, have been reported to be risk factors for SCE^(5,6). As for the pathogenesis, the presence of bacteria in the uterus stimulates the release of pro-inflammatory cytokines in the endometrium that favor the migration of PMNN to fight the infection^(7,8). Studies suggest that SCE develops as a result of high production of pro-inflammatory cytokines during the control of the bacterial infection in the uterus^(1,8). Therefore, a strategy to reduce the prevalence of SCE may be to decrease uterine bacterial load during the early postpartum period.

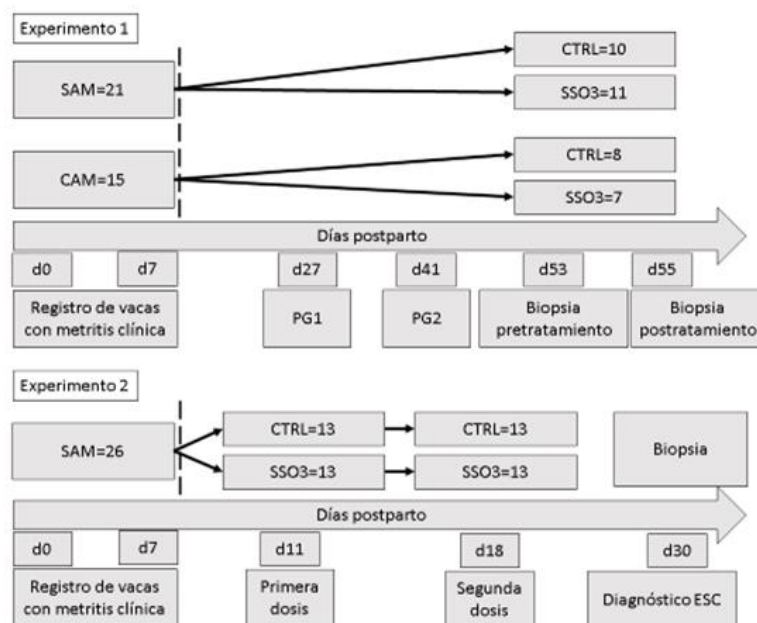
In relation to the above, ozone therapy has been used experimentally to treat puerperal conditions due to its bactericidal and immune response modulating properties. Some examples of the diseases treated are urovagina, birth canal lesions, mastitis, uterine infections and regeneration of the endometrial epithelium (reviewed by Đuričić *et al*⁽⁹⁾). Specifically, ozone therapy in the form of foam applied transcervically has been shown

to be effective in mitigating the negative effects of puerperal diseases on indicators of reproductive performance^(10,11). For example, cows with placental retention, clinical metritis or endometritis treated with ozone had conception rates similar to those of cows without uterine pathologies^(10,11). These beneficial effects have been attributed to the bactericidal properties of ozone^(9,12). In addition to this, although the mechanisms of bactericidal action of ozone are not fully elucidated⁽¹³⁾, reports suggest that the death of microorganisms is induced through a direct oxidative effect by oxygen free radicals released with the therapy^(14,15). On the other hand, there is evidence to suggest that ozone therapy has the ability to stimulate the immune response⁽¹⁶⁾. For example, it has been reported that ozone applied in ppm in the respiratory tract induces the expression of chemotactic factors (IL8 and MIP2) in the epithelium, doubling the number of PMNN compared to the control group^(17,18). All of the above allows supposing that, in addition to the bactericidal effect, ozone treatment could stimulate the expression of chemotactic factors in the endometrium, increasing the number of PMNN, however, this effect in the uterus has not yet been demonstrated. Therefore, due to its immunomodulatory properties, the objective of the present study was to determine the ability of ozonized saline solution (O3SS) to increase the quantity of PMNN in the endometrium and, due to its bactericidal properties, to challenge the preventive effect of this therapy on the prevalence of SCE in dairy cattle.

The study was carried out in a commercial farm in the state of Querétaro (20° 25' N, semi-dry climate), between the months of August and October 2019. The cows were housed throughout the study in roofed pens and earthen areas to sunbathe with free access. Each pen had individual stalls and beds with silica sand. The cleaning of the pens was carried out once a day at 1300 h. A fully mixed ration containing 55 % fodder (alfalfa, oats and corn silage) and 45 % concentrate (ground and rolled corn, wet bran and soybean) on a dry basis was offered. The cows were fed once a day (0700 h) and with free access to the feeders; during the day, the food was repeatedly swept into the feeder to stimulate consumption.

To produce the ozonized saline solution, a commercial medical grade ozone generator (Oxyzonic System Medic, EDE Ozone) was used. Subsequently, 150 ml of saline solution (NaCl 0.9 %) was placed in a gas washing bottle and 0.5 L/min of medical-grade oxygen was passed for 60 sec using a diffuser stone of 25 mm in diameter. The ozone concentration in the generator was set at 70.09 µg/ml. In the laboratory, the residual ozone concentration was determined indirectly with the standard iodometric titration method. The results indicated a concentration of 6.7 ± 0.3 ppm of ozone in the saline solution with this protocol.

To achieve the objective of the study, two experiments were designed; Exp 1 to evaluate the ability of O3SS to increase the number of PMNN in the endometrium and Exp 2 to challenge the ability of this therapy to prevent SCE. Figure 1 shows the corresponding experimental designs.

Figure 1: Experimental study designs

SAM= cows with no history of clinical metritis (NHM); CAM= cows with a history of clinical metritis (WHM); CTRL= saline solution treatment; SSO3= treatment with ozonized saline solution (O3SS); PG= prostaglandin; ESC= subclinical endometritis (SCE).

In Exp 1, 38 first-lactation Holstein cows were used, clinically healthy at the time of applying the experimental treatments (cows without systemic signs of disease, with uterus involuted to transrectal palpation and without the presence of exudates indicating clinical endometritis), with an average of 28 L of milk in two milkings (0400 and 1500 h). During the first 7 d postpartum, the presence of cows without (NHM) or with metritis (WHM), diagnosed from the characteristics of uterine secretions (reddish-brown aqueous liquid with a fetid odor⁽¹⁹⁾), was recorded, the latter received treatment with local and systemic broad-spectrum antibiotics (ceftiofur 2 mg•kg⁻¹ sc and two uterine washes with oxytetracycline/saline solution (50:50) at three-day intervals with supportive therapy). As part of the reproductive management of the farm, the cows were applied a pre-synchronization program consisting of two doses of prostaglandin (25 mg of dinoprost tromethamine) at an interval of 14 d. Twelve days after the second prostaglandin (between 50 and 60 d postpartum), a pre-treatment biopsy of endometrial tissue was taken using the Cytobrush technique^(3,4). Subsequently, cows in the WHM and NHM groups were subdivided to receive transcervically 50 ml of saline solution in the control group (CTRL) or ozonized saline solution in the treated group (O3SS). Forty-eight hours after the application of the treatment, samples (post-treatment) of endometrial tissue were taken again with the same technique. In both samples (pre- and post-treatment), slides were mounted and analyzed under a microscope at 400X, 200 cells were counted, and the number of polymorphonuclear neutrophils was recorded. In this experiment, it was necessary to have animals without SCE at the time of the challenges with saline solution. The results of the analysis of the slides indicated that two cows presented SCE ($\geq 6\%$

PMNN⁽²⁾) in the pre-treatment sample, which were discarded from the study to prevent it as a confounding factor.

On the other hand, in Exp 2, 26 first-lactation Holstein cows were used. The cows were randomly divided into two groups; control (0.9 % saline solution; CTRL) and treatment (ozonized saline solution; O3SS). Using infusion pipettes, two doses of 50 ml (with an interval of 7 d) of saline solution or ozonized saline solution were administered intrauterinely. The first dose was administered at 11.3 ± 0.4 d postpartum. The cows included in the study were clinically healthy animals, with no signs of metritis and no history of treatment for puerperal diseases. At d 30 postpartum, the diagnosis of SCE was made with the Cytobrush technique^(3,4). The cows whose samples had ≥ 6 % polymorphonuclear neutrophils (in a count of 200 cells) in the cytological analysis were diagnosed positive for SCE⁽²⁾.

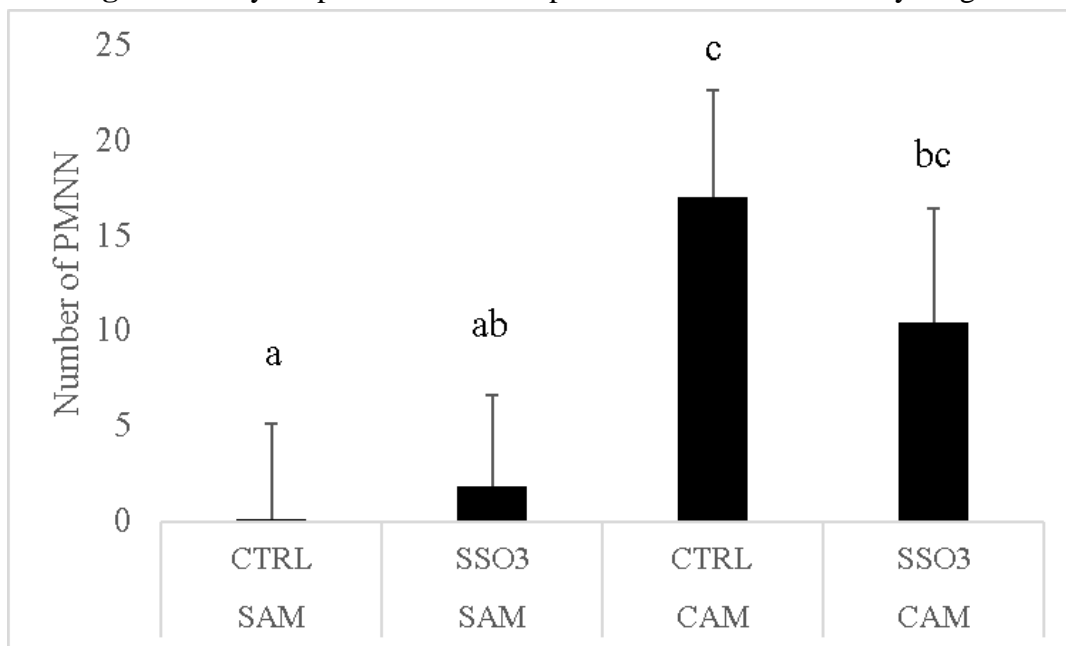
Regarding the statistical analysis, all were carried out using SAS version 9.3 (SAS Institute Inc. Cary, NC, USA). In Exp 1, the statistical analysis consisted of an analysis of variance with a completely randomized design with factorial arrangement (history of diseases (WHM or NHM) x saline solution treatment (CTRL or O3SS)) using the GLM procedure. Residual analysis was carried out with the UNIVARIATE procedure to verify the compliance with the assumptions of the model. To meet the assumptions of the analysis of variance, the response variable (number of PMNN) was logarithmically transformed ($\log Y = \log(Y+1)$). To facilitate the interpretation of the results, means and standard errors are shown untransformed. The comparison between means was carried out using the PDIFF option. In Exp 2, Fisher's exact test was used to determine if there were differences between the percentage of cows with SCE. To determine the risk that a cow had to develop SCE, the odds ratio was obtained through a simple logistic regression analysis with the LOGISTIC procedure. For both experiments, a value of $P < 0.05$ was established as a threshold of statistical significance and a value of $P \leq 0.1$ as a trend indicator.

The results of Exp 1 indicated that the main effect history of metritis was significant ($P = 0.002$), and the interaction history of metritis (WHM or NHM) x saline solution treatment (CTRL or O3SS) showed a statistical trend ($P = 0.08$). In relation to the main effect history of metritis, WHM cows had a higher number of endometrial PMNN than NHM cows (13.9 ± 6.2 vs 1.0 ± 0.46 , $P < 0.05$).

Figure 2 shows the results of the interaction on the number of PMNN in endometrial cytologies by group. The WHM-CTRL group had a higher number of PMNN than NHM-CTRL (17.0 ± 9.6 vs 0.1 ± 0.1 , $P < 0.05$) while the WHM-O3SS and NHM-O3SS groups were not different (10.4 ± 8.1 vs 1.8 ± 4.8 , $P > 0.05$), although WHM-O3SS did have more PMNN than NHM-CTRL (10.4 ± 8.1 vs 0.1 ± 0.1 , $P < 0.05$). For their part, NHM-CTRL and NHM-O3SS were no different ($P > 0.05$). The cow's ability to generate a postpartum immune response is a determining factor in controlling the bacterial proliferation that occurs naturally in the uterus during the puerperium^(19,20,21). In the present study, it was

reported evidence that cows with a history of metritis had higher endometrial PMNN counts compared to the group of cows with no history of metritis. This effect cannot be attributed to the fact that the WHM group had higher PMNN counts because cows without SCE (all with low PMNN counts in the pre-treatment biopsy) were used. Therefore, a possible explanation lies in a differentiated endometrial immune response to the application of treatments in both groups. Saline solution has been reported to have a moderate irritant effect and its administration in uterine washes has been proposed as an effective treatment against SCE⁽²²⁾. On the other hand, in the literature, it is reported that cows with clinical and subclinical endometritis and cows with a history of dystocia (risk factor for SCE) have higher quantities of inflammatory interleukins in the uterus^(23,24). In addition, transcriptomic analyses revealed that cows with a history of clinical endometritis have higher levels of IL-17A at 21 d postpartum⁽²⁵⁾, which stimulates the recruitment of inflammatory cells via IL-8^(26,27). Based on the above, a possible explanation for these results (higher number of post-treatment PMNN in WHM cows) is that the endometrium could have a greater inflammatory response to the irritating effects of saline solution in cows with a history of clinical metritis and endometritis.

Figure 2: Polymorphonuclear neutrophil count in endometrial cytologies



PMNN= polymorphonuclear neutrophils (PMNN); CTRL= control subgroup with saline solution treatment; SSO3= treatment subgroup with ozonized saline solution (O3SS); SAM= cows with no history of clinical metritis (NHM); CAM= cows with a history of clinical metritis (WHM).

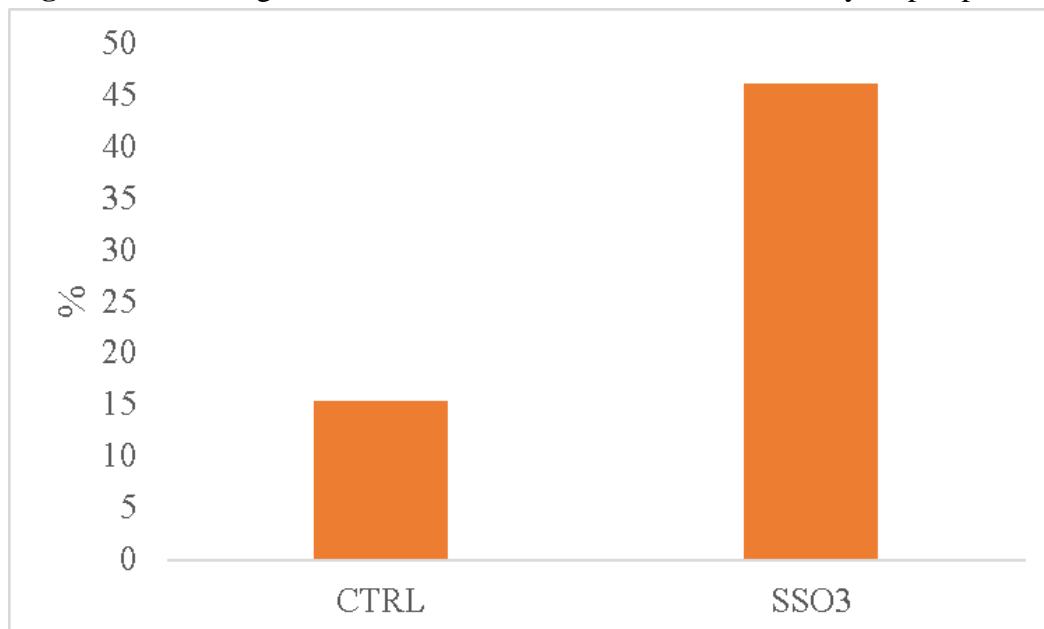
^{abc} Columns with different letter indicate statistical differences ($P < 0.05$).

Several studies report that intrauterine ozone application is effective in the treatment of metritis and placental retention, an effect that has been associated with its bactericidal property^(10,11). In addition, it has been observed that ozone stimulates the expression of chemotactic factors in the respiratory epithelium^(17,18), therefore, it was hypothesized that ozone treatment would stimulate the migration of a greater quantity of PMNN to the endometrium in our experiment. However, the results indicated that the number of

endometrial PMNN in WHM-O3SS cows was not statistically different from that presented by the NHM-O3SS group, that is, the treatment tended to decrease the inflammatory over-response in WHM cows. A possible explanation for these results is in recent studies reporting anti-inflammatory properties of ozone therapy⁽¹⁶⁾. Ozone treatment has been reported to be effective in reducing the severity of pelvic inflammatory disease in a rat model; an effect presumably associated with decreased expression of inflammatory factors IL-6 and TNF- α ⁽²⁸⁾. In another study, patients with osteoarthritis who received local ozone treatment were reported to have higher IL-10 expression (anti-inflammatory factor) and lower levels of TNF- α ⁽²⁹⁾. Based on this, it is possible that, with the protocol used, ozone therapy has decreased the local inflammatory response in cows with a history of metritis. These results suggest that the ozone therapy used may also have an anti-inflammatory effect on the endometrium. In addition, based on other studies^(28,29), the mechanism could be through the downward modulation of the expression of inflammatory factors in cows with a history of metritis, a hypothesis pending to be challenged.

On the other hand, the results of the second experiment indicated that the overall percentage of cows with SCE at d 30 was 30.7 %. A statistical trend ($P=0.09$) was also observed for differences in the percentage of cows with SCE between CTRL and O3SS groups (15.4 and 46.2 %, respectively, Figure 3). The results of the logistic regression analysis indicated a statistical trend ($P=0.1$); cows that received ozone treatment (O3SS) had an odds ratio of 4.7 (0.73-30.3, 95 % CI) to develop SCE compared to cows that received only saline solution (CTRL).

Figure 3: Percentage of cows with subclinical endometritis at day 30 postpartum



CTRL= control group with saline solution treatment; SSO3= treatment group with ozonized saline solution (O3SS). ($P=0.09$).

The pathogenesis of SCE has not been fully explained, however, there are reports indicating that the origin of the disease is an exacerbated immune response in response to a high number of bacteria in the uterus^(1,8). The reasoning behind the second experiment was that the percentage of cows with SCE could be decreased by applying ozone therapy during the early puerperium, this to reduce the bacterial load present at that time. However, the results indicated that cows that received O3SS treatment had a higher prevalence and higher risk of suffering SCE at 30 d postpartum. These results should be taken with caution, since, although the group with O3SS had 3 times more animals with SCE and the odds ratio indicated 370 % higher probability of developing SCE, the statistical significance was at the trend level. Now, a possible explanation for these results is that the two doses administered at a 7-d interval have not been effective in reducing the bacterial load, but in modulating the immune response downwards. In the laboratory of this research, it was observed that the application of a single dose of 50 ml of O3SS (6.7 ± 0.3 ppm) improves the characteristics of uterine secretions in cows with metritis 24 h after the application of treatment (unpublished results). However, the treated cows again present secretions with characteristics of metritis in later days. This allows supposing that the protocol used in the present study may have been insufficient to reduce the bacterial load in the uterus. On the other hand, it is known that the presence of bacteria in the uterine lumen stimulates the expression of pro-inflammatory factors that favor the migration of PMNN to fight the infection⁽⁸⁾. In addition, it has been reported that an increase in the number of pro-inflammatory cytokines predisposes to the development of SCE^(1,8). If treatment with O3SS was insufficient to decrease the bacterial load, but decreased the local immune response, a more severe uterine infection may have been induced. Subsequently, this uterine infection may have stimulated an even more aggressive immune response, thus predisposing cows to an increased risk to develop SCE, a hypothesis pending to be challenged. It is not omitted to mention that the present is a preliminary study, and the results of preventive treatment are applicable to cows with a healthy puerperium and without risk factors for SCE. In addition, the statistical trend obtained invites one to take the results with caution.

Modulation of the immune response has been proposed as a strategy to reduce the prevalence of SCE⁽⁷⁾. In the first experiment, the results suggest that there may be a decrease in immune response in response to O3SS treatment. In the second experiment, the results indicated that cows treated with O3SS tend to have a greater predisposition to develop SCE, which may have been made possible by an immunosuppressive effect induced by the therapy. However, a limitation of the present study is that in the second experiment only cows that were not suffering from metritis (risk factor for SCE^(5,6)) were used and the statistical trend obtained. As observed in the first experiment, there is a differentiated response to treatment depending on whether the animals have infectious uterine disease. Therefore, it is still pending to challenge the preventive effect of ozone treatment in cows with risk factors for SCE. Despite this limitation, the results presented here are foundational to continue exploring the effects of local uterine therapy with ozone, mainly due to the apparent anti-inflammatory effect of this element.

In conclusion, with the protocol used, transcervical O3SS does not increase the number of PMNN in the endometrium after its application. In addition, treatment with O3SS applied in cows with no history of early metritis increases the risk of developing SCE. One implication of this work is that O3SS may have a local anti-inflammatory effect, which justifies exploring the effectiveness of the preventive protocol in cows with risk factors for SCE.

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Compliance with ethical standards and conflict of interest

The protocol of this study was approved by the Bioethics Committee of the Faculty of Natural Sciences of the Autonomous University of Querétaro (84FCN2018). The authors declare that they have no conflicts of interest.

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