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Use of bupivacaine infiltrative local block in bitches submitted to ovariohysterectomy by celiotomy or video-assisted surgery by two-portals access. Uso de bloqueio local infiltrativo de bupivacaína em cadelas submetidas à ovariohisterectomia por celiotomia ou cirurgia videoassistida por acesso de dois portais.

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Abstract
This randomized blinded study objected to investigate a local anesthesia (0,5% bupivacaine) in the surgical incision site in dogs underwent to ovariohysterectomy by celiotomy or video-assisted by two-portal. Twenty-eight healthy adults were selected. Dogs were divided into celiotomy control group, celiotomy blocking group (BC), video-assisted control group and blocking video-assisted group (BV). In the BC and BV, bupivacaine was administered SC in the incision line or port sites. Control groups received NaCl 0,9% at the same sites. Three evaluators blinded evaluated postoperative pain, using Melbourne Pain Scale and visual analog scale. The anesthetic infiltrative blockade used was efficient in promoting postoperative comfort in both celiotomy and video assisted OVH.
Keywords: Analgesia. Block incision line. Spay. Video surgery.

Resumo
Este estudo randomizado cego teve como objetivo investigar a anestesia local (bupivacaína a 0,5%) no local da incisão cirúrgica em cadelas submetidas à ovariohisterectomia por celiotomia ou videoassistida por dois portais. Vinte e oito adultos saudáveis foram selecionados. Os cães foram divididos em grupo de controle de celiotomia, grupo de bloqueio de celiotomia (BC), grupo de controle videoassistido e grupo de bloqueio assistido por vídeo (BV). No BC e BV, a bupivacaína foi administrada SC na linha de incisão ou locais dos portais. Os grupos controles receberam NaCl 0,9% nos mesmos locais. Três avaliadores avaliaram cegamente a dor pós-operatória, utilizando a escala de dor de Melbourne e a escala analógica visual. O bloqueio anestésico infiltrativo utilizado foi eficiente em promover conforto pós-operatório tanto na celiotomia quanto na OVH videoassistida.
Palavras-chave: Analgesia. Bloqueio linha de incisão. Castração. Videocirurgia.

Introduction

Advances in ovariohysterectomy (OVH) techniques have been observed over the years (BRUN, 2015; LASCELLES et al., 2008). Minimally invasive approaches revolutionized surgery and have been used to replace conventional procedures. Besides incorporating innovative modalities for diagnostic and therapeutic surgical procedures, other benefits are observed: access through small incisions, less tissue trauma, less discomfort and postoperative pain, shorter hospitalization, faster post-surgical recovery, lower costs and better aesthetic results (BRUN, 2015; CULP, MAYEW, BROWN, 2009; DEVITT et al., 2005; HELLYER et al., 2007). Among the techniques for OVH in dogs, the video-assisted techniques have gained popularity in veterinary medicine, since it maintains the characteristics of minimally invasive surgery despite performing complex procedures outside the abdominal cavity. It involves an intra and extracavitary procedures. This characteristic, which differs from the other laparoscopic techniques, significantly reduces its complexity, making it more versatile for routine use (BRUN, 2015; DEVITT et al., 2005).

According to authors, in animals, pain is defined as a negative sensory and emotional experience that produces protective motor actions, resulting in a conditioned aversion, and modifies behavioral traits specific to the species (MORTON et al., 2005). As animal patients are unable to self-report, pain management is challenging, thus, the ability to treat and diagnose it effectively becomes subjective and the methods of pain assessment are crucial for identifying and monitoring the efficacy of analgesic treatment. A multiple-aspect approach to pain assessment is generally accepted as the option that provides better results (FIRTH, HALDANE, 1999; HANCOCK et al., 2005).

Multimodal pain management designates the use of analgesic drugs with different mechanisms of action, with the objective of reducing or avoiding the perception of pain by the patient following nociceptive stimuli through different forms of nerve stimulation. Skinner (2004) demonstrated the efficacy of multimodal analgesic protocols in reducing postoperative morbidity and mortality pain-related in human, increasing quality of life and patient satisfaction. The association of locoregional anesthesia combined with systemic analgesia is a simple way of applying the multimodal protocol. Local anesthetics act on the interruption of neural transmission of afferent sensory nerves or sensory tracts (LASCELLES et al., 2008; MARTINS et al., 2010; SLINGSBY, MURREL, TAYLOR, 2010). The infiltration of local anesthetic before the surgical procedure has been demonstrated to decrease the volume of general anesthetics and opioids to be used in surgeries (CUVILLON et al., 2009), being an efficient manner to prevent or reduce nociceptive impulses or pain during and after surgery.

This study aimed to investigate the use of infiltrative blockade with bupivacaine in the surgical incision line or port entry sites and the effectiveness of this technique as part of a multimodal approach in postoperative pain control in dogs submitted to OVH by video-assisted approach or conventional celiotomy technique.

Material and methods

After approval by the Ethics Committee on Animal Use (CEUA) of the UFSM (protocol 021/2014), there were selected twenty-eight bitches to participate in the study. The inclusion criteria included the age of 2-4 years, 10-15 kg of body weight (BW), American Society of Anesthesiologists (ASA) physical status of I and informed owner written consent about the study participation. All

animals were healthy according to clinical and laboratory examination, including platelet counts, hematological and biochemical tests (albumin, alkaline phosphatase, alanine aminotransferase, creatinine and urea levels). Serum glucose and cortisol were also evaluated. Animals were hospitalized 24 hours prior surgery to provide an appropriate adaptation to the evaluation site and evaluators.

Food and water were withdrawn 12 and 6 hours before surgery, respectively. Dogs were premedicated with an intramuscular (IM) injection of acepromazine maleate (0.05 mg/kg BW, Apromazin 2 mg/m*l*, Syntec, Brazil). After 15 minutes, the right cephalic vein was catheterized with a 22G catheter using an aseptic technique and abdominal hair was clipped. General anesthesia was induced using intravenous (IV) propofol at 4.0 mg/kg BW (Propovan® 10 mg/m*l*, Cristália Prod. Quím. Farm. Ltda., Brazil) and maintained with isoflurane (Isoflurano, Instituto BioChimico Ind. Farm. Ltda. Brazil) vaporized in 100% oxygen, with an adequate concentration to maintain the animals in anaesthetic plane by partial rebreathing system. The intraoperative analgesia was promoted by the continuous intravenous infusion of fentanyl citrate (10 μg/kg/hr, Fentanest® 0.05 mg/m*l*, Cristália Prod. Quím. Farm. Ltda., Brazil) by peristaltic infusion pump, preceded by a 2.5 μg/kg BW IV loading dose of the same drug. Fentanyl infusion initiated immediately after anesthesia induction and was discontinued at the end of the surgery. Sodium ampicillin (20 mg/kg BW, IV, Ampicilina Sódica 1g, Teuto Brasileiro S/A, Brazil) was administered as antimicrobial chemoprophylaxis.

Respiratory frequency (*f*), systolic, diastolic and mean blood pressures by a non-invasive device, partial O_2 saturation (SpO_2), end-tidal CO_2 ($EtCO_2$) were monitored by a multiparameter monitor (PM 9000, Mindray) continuously during all the surgery and registered every 5 minutes.

Dogs were randomly allocated into two large groups according to the type of OVH surgery to be submitted: celiotomy or video assisted. Then, each group was subdivided into two smaller groups according to the proposed analgesic procedure: with or without incision line block. Namely, the four groups were: control celiotomy group (CC: n=7), blocking celiotomy group (BC: n=7), control video-assisted group (CV: n=7) and blocking video-assisted group (BV: n=7). The randomization was performed by withdrawing pieces of paper with group identifications from a bag.

After stabilization of the anesthetic plane, the experimental protocol was applied. Dogs from control groups received 3 m*l* of saline solution (Sodium Chloride 0,9%, Halex Istar Ind. Farm., Brazil) subcutaneously in the incision line. The blocking groups were administered bupivacaine hydrochloride at 0,5% (2 mg/kg BW, Neocaína® 5 mg/m*l*, Cristália Prod. Quím. Farm. Ltda., Brazil) in the incision line. In the video-assisted group, half of the total volume of bupivacaine or saline solution was injected in each of the two-port entry points. A dermatographic pen was used to mark the site of injection.

Conventional celiotomy technique was performed in the groups CC and BC, while the two-port technique described by Brun (2015) was performed in CV and BV. Meloxicam at 0.2 mg/kg BW was administered subcutaneously (SC) at the end of the surgery (Maxicam 2%, Ourofino, Brazil). Postoperative analgesia was composed of a commercial formulation containing metamizole sodium (25 mg/kg BW) and hyoscine N-butylbromide (0.2 mg/kg BW, Buscofin Composto, metamizole sodium 500 mg/m*l* and hyoscine N-butylbromide 4.0 mg/m*l*, União Química Farmacêutica Nacional S/A, Brazil) administered intravenously immediately after the end of surgery. Subsequent doses were given SC every eight hours for 2 days.

The postoperative pain measurement was performed by three experienced evaluators blinded to the anesthetic protocol and surgery technique using two scales: the visual analog scale (VAS) and the Melbourne Pain Scale (MPS). Animals were evaluated hourly in the first eight hours (T1 to T8)

and, then, at 12 (T12), 18 (T18), 24 (T24), 36 (T36) and 48 hours (T48) postoperative. Each of the evaluators assessed every dog and agreement among their evaluation was required to rescue analgesia be administered.

The guidelines described for VAS and for Melbourne scale were strictly followed (Firth, Haldane, 1999; Mich, Hellyer, 2008). Scores above 5 cm or 6 points in VAS or MPS, respectively, were used as the threshold for the administration of anesthetic rescue which consisted of tramadol (4 mg/kg BW, IM, Tramadon® 50 mg/m*l*, Cristália Prod. Quím. Farm. Ltda., Brazil). The threshold score in MPS used in this study is in accordance with a study by Pohl et al. (2014) who identified that the moderate pain score on VAS scale (5 cm) is compared in equivalence to 5.88 in Melbourne Pain Scale due a high correlation. All animals that received anesthetic rescue were retested hourly after administration of the supplemental analgesia until reaching scores below threshold point in both scales. All these animals were removed from the statistical analysis. The abdomen of the dogs was covered with bandages to prevent the evaluators to identify the groups. The bandages were changed according to the need, by a non-participatory team considering analgesic evaluation.

In parallel with pain assessment, blood samples were collected to measure serum cortisol and glucose levels by chemioluminescence technique and blood glucometer (G-Tech free 1, G-Tech, Brazil) at the following times: T0 (before the surgical procedure) T1, T6, T12, T24, and T48.

During the experiment, animals were hospitalized in individual cages, receiving commercial food and water *ad libitum* for 48 hours postoperatively and then subsequently discharged from the hospital with postoperative care prescription.

Statistical analysis

All variables analyzed followed a non-normal distribution. Kruskal-Wallis test was used to access difference among the groups, followed by Dunn test *post hoc* to perform multiple comparisons. The difference of mean scores according to the time of evaluation for each group was assessed by t-test after utilizing bootstrap to build confidence intervals. Kruskal-Wallis at 5% of significance was also used to compare serum glucose and cortisol levels among groups in the different assessment periods of time. Correlation between the pain scores acquired by VAS and MPS were obtained by the Pearson Coefficient Correlation. Statistical tests were performed using R (R Core Team. R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria.).

Results

A total of 28 dogs were selected and completed the study. One dog was excluded due to pyometritis. There was no difference among groups concerning age, weight or duration of anesthesia and surgery (34±8.24 and 22.10±5.02 min respectively).

The anesthetic infiltrative blockade performed with 2 mg/kg of bupivacaine promoted less postoperative pain when used in both celiotomy and video-assisted surgeries, regardless the pain scale used (VAS, P= 0.043; MPS, P= 0.0129). However, mean pain scores between BC and BV assessed by VAS or MPS were not different (P=0.3021 and 0.1518, respectively). Celiotomy control group demonstrated higher levels of pain than BC (P=0.043) and CV (P<0.001) by VAS, and CV (P=0.0379) and BV (P=0.0017) in MPS evaluation. Blocking celiotomy group also experienced less pain than CV in both VAS and MPS (P=0.0002 and P=0.0129, respectively).

Regarding the pain scales used, a strong correlation between VAS and MPS was not demonstrated (table 1), except in case surgery was performed by celiotomy under bupivacaine block. Only the MPS scale elicited enough punctuation for performing an analgesic rescue.

Table 1 - Correlation between pain scores obtained by VAS and MPS.

Comparison between scales	r	CI
BC_{VAS} vs. BC_{MPS}	0.7417	[0.32, 0.91]
CV_{VAS} vs. CV_{MPS}	0.2684	[-.33, 0.71]
BV_{VAS} vs. BV_{MPS}	-0.148	[-0.64, 0.43]

a) The presented values refer to Pearson coefficient two-by-two. Values are sample correlation and intervals to 95% of confidence. CI: confidence interval; BC_{VAS}: Blocking celiotomy group evaluated by Visual Analogue Scale; BC_{MPS}: Blocking celiotomy group evaluated by Melbourne Pain Scale; CV_{VAS}: Control video-assisted group evaluated by Visual Analogue Scale; CV_{MPS}: Control video-assisted group evaluated by Melbourne Pain Scale; BV_{VAS}: Blocking video-assisted group evaluated by Visual Analogue Scale; BV_{MPS}: Blocking video-assisted group evaluated by Melbourne Pain Scale.

Rescue analgesia was administered to one animal in CV after one hour postoperative, while the seven dogs in CC received additional analgesia in T1 and T2. Animals in CC were excluded from subsequent pain evaluations. None of the animals from groups receiving anesthetic blockade (BC and BV) were administered analgesic rescue.

The t-test performed two-by-two with confidence intervals built by a bootstrap demonstrated significant difference among treatments at each time point (table 2). At T1 pain scores were greater in CC, BC, and CV than in BV according to VAS scale (P<0.444). In Melbourne Pain Scale, BV showed the statistically smaller score (P=0.001). Two hours postoperatively, CV presented the smaller pain score among all groups in VAS measurement (P<0.016) but not different than BC (P=0.845) and BV (P=0.051) in MPS. Control celiotomy group obtained statistically higher pain scores in both scales (P<0.001). At T3, there was not difference among the means presented by BC (P=0.312) and BV (P=0.089) in VAS. In MPS, BV demonstrated less pain than all the other groups (P<0.012). At this time point, CV and BV showed the significant smaller pain scores in VAS and MPS, respectively.

CV maintained the smaller score among groups when evaluated by VAS from T4 until the end of the observations at T48 (P<0.008). According to VAS evaluation, BC scores were not statistically different from BV at any time points, except for T4 (P=0.046).

For MPS assessment, there was not difference among groups at T4 to T5 (P>0.197), T7 to T8 (P>0.205) and at T18 (P>0.289). Different from the evaluation by VAS, CV presented the higher scores in comparison with the other groups along T6, T12, T24, T36 and T48 evaluations using MPS (P<0.044). At T6, T12 and T24 BC showed the smaller scores. BV presented statistically similar pain scores to BC at T12 and T24, showing significant smaller pain score in relation to CV and BC at T36 (P=0.058; 0.021) and T48 (P=0.001; 0.026).

The glucose concentration of all groups in this study increased considerably in the first hour after surgery, decreasing gradually until the twelfth hour when it returned to baseline values, although there was no statistical difference at any time point (Table 3). Regarding cortisol, no statistical difference among groups was observed only at T1 (P=0.05).

Table 2 - Mean pain scores of the four groups on the visual analogue scale (VAS) or the Melbourne Pain Scale (MPS) of dogs submitted to ovariohysterectomy by celiotomy or video-assisted surgery in different time points of evaluation.

	VAS				MPS			
	CG		BG		CG		BG	
Time (h)	CC	CV	BC	BV	CC	CV	BC	BV
1	3.03[a]	1.85[b]	3.20[a]	2.52[ab]	7.33[a]*	3.85[b]	3.78[b]	1.62[c]
2	2.82[a]	1.48[c]	2.32[ab]	2.11[b]	5.38[a]*	2.09[b]	2.28[b]	1.13[b]
3	2.38[a]	1.34[b]	1.96[a]	2.12[a]	2.71[a]	2.19[a]	2.44[a]	1.32[b]
4	1.88[b]	1.21[c]	1.76[b]	2.51[a]	1.86	1.76	1.50	1.72
5		0.80[b]	1.71[a]	1.89[a]		1.39	2.00	1.58
6		0.77[b]	1.75[ab]	1.89[a]		1.83[a]	1.11[b]	1.38[a]
7		0.87[b]	1.73[a]	1.98[a]		1.83	2.17	1.60
8		0.48[b]	1.63[a]	1.85[a]		1.78	1.50	0.87
12		0.62[b]	1.69[a]	1.99[a]		2.78[a]	1.56[b]	2.29[b]
18		0.52[b]	1.51[a]	1.40[a]		1.67	1.44	2.11
24		0.35[b]	1.25[a]	1.20[a]		2.78[a]	1.11[b]	2.16[b]
36		0.42[b]	1.14[a]	1.15[a]		2.05[ab]	2.28[a]	1.39[b]
48		0.31[b]	0.97[a]	0.90[a]		2.94[a]	1.67[b]	1.49[c]
Means		1.74	1.91	0.85		1.81	2.23	1.59
Confidence		[1.3; 2.1]	[1.5; 2.3]	[0.5; 1.1]		[1.5; 2.1]	[1.8; 2.6]	[1.3; 1.9]

a) CG: Control groups; BG: Blocking groups. CC: Control Celiotomy group; CV: Control video-assisted group; BC: Blocking celiotomy group; BV: Blocking video-assisted group; CI: confidence interval.

Table 3 - Means of serum glucose and cortisol of the four groups of bitches submitted to conventional and video-assisted ovariohysterectomy.

	Glucose					Cortisol				
	CG		BG			CG		BG		
Time (h)	CC	CV	BC	BV	p-value	CC	VC	BC	BV	p-value
0	88.29	85.86	89.67	115.63		3.93	2.74	4.71	4.60	
1	157.14	127.71	125.16	129.28	0.44	12.64	5.5	7.94	5.81	0.05
6		104.33	108.83	116	0.34		2.38	3.64	2.66	0.29
12		95.33	98.16	101.14	0.47		2	2.11	2.29	0.91
24		89.83	102.5	99.28	0.18		1.83	1.81	2.01	0.88
48		91.33	104.5	99.14	0.08		2.86	1.98	2.78	0.68

a) Values are expressed as median (range) and analysed by Kruskal-Wallis test at 5% of significance.
CG: Control groups; BG: Blocking Groups. CC: Control Celiotomy group; CV: Control video-assisted group; BC: Blocking celiotomy group; BV: Blocking video-assisted group.

Discussion

In the present study, the anesthetic protocol administered, besides the administration of metamizole sodium with hyoscine N-butylbromide, as postoperative analgesia did not provide adequate analgesia for the animals submitted to OVH by celiotomy without the use of incisional block (CC). This fact is evidenced by the analgesic rescues received by 100% of the animals from this group (n=7) until the third postoperative hour (T3). The dogs submitted to the local incisional blockade with

bupivacaine (BC and BV), regardless the surgical technique applied, experienced less pain (lower scores on MPS and VAS scale) when compared to CC.

In the first hour of postoperative evaluation, which is considered by Desborough (2000) as a critical period in anesthetic and surgical recovery, animals from BV group presented lower pain scores by the MPS associated with serum cortisol levels near baseline, which indicates the effectiveness of the blockade until this moment, a fact expected because it is a potent local anesthetic drug (CUVILLON et al., 2009). Besides that, this group had the lowest scores on this scale most of the time. The BC group, which also received infiltrative anaesthetic block, obtained similar scores from the sixth postoperative hour, corroborating the efficiency of the pre-incisional anaesthetic block for both celiotomy and video-assisted surgeries.

The short-term analgesia period given by the administration of fentanyl and the absence of residual effect (LAMONT, MATHEWS, 2007) might explain the ineffective analgesia in the CC group in the first hours of evaluation, due to this drug period of action. This is associated with the fact that celiotomy surgeries promote pain of moderate intensity (CARPENTER et al., 2004) justifying the need for rescue analgesia in the third hour of evaluation in this group. On the other hand, bupivacaine has onset of effect of approximately 16±9 min after the administration and duration of up to 4 hours (CUVILLON et al., 2009), explaining the lower scores reached by the BC group compared to CC group.

It is suggested that the effect of bupivacaine started before the first incision and lasted over the first four hours postoperatively. Moreover, the metamizole and hyoscine N-butylbromide association was sufficient to maintain a low nociceptive threshold once the pain receptors could be already downregulated by the bupivacaine action (LANITIS et al., 2015). This result disagrees with Campagnol et al. (2012) who reported that there was not difference in the administration of bupivacaine instilled intraperitoneally or at the incision line in bitches submitted to OVH by celiotomy. For these authors, after the first postoperative hour, both pain scores and the number of analgesic rescues administered were similar for all groups. Local anaesthetic block prior OVH surgery was also tested by Mckune et al. (2014), who reported a non-significant difference between the block and non-block groups. This result was attributed to the non-use of systemic analgesia in association with local blockade during the surgical procedure. The expected action of local anaesthetics is to block nerve conduction by preventing the nociceptive signal to reach the central nervous system and therefore the hypothalamic-pituitary-adrenal (HPA) axis. The activation of this axis explains the changes caused by stress, which includes the postoperative increase of plasma levels of cortisol and glucose (DEVITT et al., 2005).

The glucose concentration of all groups in this study increased considerably in the first hour after surgery, decreasing gradually until the twelfth hour when it returned to baseline values. Since there was not difference between the groups at any time point of assessment and the increased glucose levels were accompanied by a concomitant rise in cortisol levels, it is possible to infer that the association of anaesthetic blockade with continuous infusion of fentanyl did not block the HPA axis, although it might have desensitized the pain receptors. This result agrees with Romano et al. (2016), suggesting that, in clinical doses, fentanyl administration is not able to control adrenal and glycemic responses to surgical stimulation since the HPA axis may still be activated.

In relation to cortisol, there was no difference among groups at different times. However, there was an increase in the first postoperative evaluation time point (T1), corroborating with Desborough (2000), who reported that the magnitude of the metabolic response may be attributed to the perception of pain and intensity of the surgical trauma, with cortisol concentrations increasing in a sustained

manner during the beginning of the surgery until the maximum of 4 to 6 hours. This increase was more strongly evidenced in CC, while in the other groups, which received an anesthetic block or underwent a low magnitude trauma procedure, the increase was less expressive, demonstrating that pain may be the cause of cortisol increase.

The results of the variables of animals from the control video-assisted group (CV) were similar to those of the blockade groups (BC and BV). This similarity is attributed to the less invasiveness of the video-assisted procedures, which promote less somatic stimulation since both the surgical incision as the tissue damage of the skin and musculature are significantly smaller when compared to the celiotomy. This data corroborates with comparative studies between conventional and video-assisted OVHs using two-port, concluding that video surgery promotes lesser pain and surgical stress (CASE et al., 2015; DALMOLIN et al., 2016).

Metamizole is widely used for acute postoperative pain treatment of moderate to severe intensities. Pohl et al. (2014) reported that the use of metamizole combined with hyoscine N-butylbromide as an analgesic protocol provided adequate analgesia for dogs submitted to videolaparoscopic OVH but not for those undergoing celiotomy. The present study obtained similar results. Although there was an increase in the pain scores of all the groups in the first postoperative hour, the threshold score requirement for analgesic rescue was not reached by any group, except by CC.

According to the literature, the latency period of intravenous metamizole is about 30 min (NIKOLOVA et al., 2013). In this study, it was inferred that the onset of effect of this drug occurred within this period in all groups. However, a residual anesthetic and tranquilizer effect in the first postoperative hour may have interfered in the evaluation of pain, increasing the scores by the behavioral factor. The CC scores were attributed to the non-administration of anesthetic blockade and to the more invasive procedure in the animals of this group. Probably, the isolated therapy with metamizole is inefficient for controlling pain given this intensity of nociceptive stimuli.

The present study concludes that 2 mg/kg of 0,5% bupivacaine administered in infiltrative blockade at the incision line and combined with the postoperative administration of metamizole, meloxicam and hyoscine N-butylbromide constitutes a suitable multimodal postoperative protocol for OVH performed by both celiotomy and the video-assisted techniques.

Conclusion

The present study concludes that 2 mg/kg BW of 0,5% bupivacaine administered in the surgical incision line or port entry sites combined with intraoperative constant rate infusion of fentanyl and the postoperative administration of metamizole, meloxicam, and hyoscine N-butylbromide constitutes a suitable multimodal postoperative protocol for OVH performed by both celiotomy and the video-assisted techniques.

Declaration of conflicting interests

The authors declare no potential conflicts of interest; no author of this article has a commercial or financial interest or partnership for commercial purposes with this work.

Authors' contribution

LRM designed the study, LGT, MTL, JPSF, GPC, CM and RCB contributed to the development of this study, MVB and AVS approved the final version to be published.

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