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Use of 0.25% chlorhexidine nanoemulsion as a skin antiseptic for cats

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ABSTRACT: This study evaluated the antiseptic effect of 0.25% chlorhexidine nanoemulsion (NM-Cl) on cat skin and compare its effect with that of 2.0% chlorhexidine digluconate (CS-Cl). NM-Cl was synthesized using the spontaneous emulsification method, and physical and chemical properties were analyzed. The antiseptic effects of NM-Cl and CS-Cl were randomly tested on the thoracic limbs of 10 healthy male cats. After a wide trichotomy of the thoracic limbs, NM-Cl was randomly applied to the trichotomy area of the right (n = 5) or left (n = 5) thoracic limbs. Then, a catheter was inserted aseptically in the cephalic vein. Subsequently, the same procedure was performed using CS-Cl on the contralateral limb. Cutaneous microbiota swab samples were obtained before antisepsis (Tpre); immediately after antisepsis (Tpost); and 4, 8, and 24 h after antisepsis. The collected samples were immediately inoculated on blood agar plates and incubated at 35 ºC ± 2 ºC in aerobiosis. Colony-forming units (CFUs) were manually counted after 24 h of inoculation. The Kruskal–Wallis and Mann–Whitney U tests were performed between groups and within the same group at different sample times, respectively. The NM-Cl and CS-Cl groups showed a reduction in CFUs between Tpre and Tpost in all animals ($P < 0.001$). Both formulations presented an antiseptic effect 24 h of antisepsis ($P < 0.05$), and no difference was observed between formulations at different times ($P < 0.05$). With a lower concentration of chlorhexidine than CS-Cl, NM-Cl presents effective antiseptic action and prolonged residual effect in antisepsis for cat venipuncture.
Key words: antiseptic, chlorhexidine, nanotechnology, nanoformulation, venipuncture.

Avaliação do uso de nanoemulsão de clorexidina a 0,25% na antissepsia da pele de gatos

RESUMO: O objetivo do presente estudo foi avaliar o efeito antisséptico da nanoemulsão de clorexidina a 0,25% (NM-Cl) na pele de gatos e compará-lo com a solução comercial de clorexidina a 2,0% (CS-Cl). A NM-Cl foi desenvolvida através do método de emulsificação espontânea, com posterior análise e caracterização das propriedades físicas e químicas. O efeito antisséptico de NM-Cl e CS-Cl foi testado de forma randomizada nos membros torácicos de dez gatos machos saudáveis. Após ampla tricotomia dos membros torácicos, a antissepsia foi realizada com NM-Cl aplicada nos membros torácicos direito (n = 5) ou esquerdo (n = 5), e um cateter foi inserido assepticamente na veia cefálica. Posteriormente, o mesmo procedimento foi realizado com a CS-Cl no membro contralateral. Amostras da microbiota cutânea foram obtidas antes da antissepsia (Tpre), imediatamente após a antissepsia (Tpos) e quatro, oito e 24 horas após a antissepsia. As amostras coletadas foram imediatamente inoculadas em placas de ágar sangue e incubadas a 35 ºC ± 2 ºC em aerobiose. A contagem manual da unidade formadora de colônia (UFC) foi realizada 24 horas após a inoculação. Os testes de Kruskal-Wallis e Mann-Whitney foram usados entre os grupos e dentro do mesmo grupo em diferentes tempos amostrais. Os grupos NM-Cl e CS-Cl apresentaram redução nas UFC entre Tpre e Tpos em todos os animais ($P < 0,001$). Ambas as formulações apresentaram efeito antisséptico 24 horas após a antissepsia ($P < 0,05$), não havendo diferença entre as formulações nos diferentes tempos ($P < 0,05$). O NM-Cl (com menor concentração de clorexidina que o CS-Cl) apresenta ação antisséptica eficaz e efeito residual prolongado na antissepsia para a punção venosa em gatos.
Palavras-chave: antisséptico, clorexidina, nanotecnologia, nanoformulação, punção venosa.

INTRODUCTION

The practice of antisepsis significantly reduces hospital infections during ambulatory and surgical procedures (ECHOLS et al., 2015; KAMPF, 2016; PRIVITERA et al., 2017). Surgical infections are the main cause of hospital infections in humans (DROHAN et al., 2019) and animals (STULL & WEESE, 2015). Among the available antiseptics, chlorhexidine is widely used to prevent infection in humans and animals.

Chlorhexidine, which is a cationic bisbiguanide, acts by binding and rupturing microbial cell membranes (MANGRAM et al., 1999). It has been extensively used in clinical practice from outpatient procedures, such as blood collection, to complex surgical procedures (BOCK et al., 2016; LAI et al., 2016; YAGI et al., 2017). However, antimicrobial agents are associated

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with the development of resistant microorganisms when used inappropriately or indiscriminately (ECHOLS et al., 2015; KAMPF, 2016).

Several bacteria are associated with the development of chlorhexidine resistance, including *Staphylococcus aureus* (ECHOLS et al., 2015) and various gram-negative bacteria such as *Escherichia coli, Klebsiella* spp., and *Pseudomonas aeruginosa* (ECHOLS et al., 2015; KAMPF, 2016). Thus, many studies have attempted to evaluate the antiseptic action of new formulations (KAMPF, 2016), including a combination of nanotechnology and chlorhexidine, to prolong the residual antiseptic effect via the slow release of nanoparticulated molecules (LBOUTOUNNE et al., 2002; LBOUTOUNNE et al., 2004).

A previous study developed and tested 0.25% chlorhexidine nanoemulsion (NM-Cl) in vitro and on rat skin showing promising results promising results (RISSO et al., 2020). Based on these findings and considering that 2% chlorhexidine digluconate (CS-Cl) is frequently used in the maintenance of intravenous devices in veterinary practice (MARSH-NG et al., 2007; MARCHI et al., 2018), the present study evaluated the antiseptic effect of NM-Cl on cat skin and compare its effects with that of CS-Cl.

MATERIALS AND METHODS

Materials

Chlorhexidine gluconate (Sigma Aldrich Laboratory, São Paulo, Brazil) was used to synthesize NM-Cl, and Riohex® 2.0% alcohol base (Rioquímica Laboratory, São Paulo, Brazil), a chlorhexidine solution, was used as CS-Cl. The agar used in the assays was manufactured by HiMedia Laboratories (Mumbai, India), and the defibrinated sheep blood was obtained from Newprov Laboratory (Paraná, Brazil).

Preparation and characterization of NM-Cl

NM-Cl (2.5 mg/mL) was synthesized through the spontaneous emulsification method in accordance with previous studies (BOUCHEMAL et al., 2004; RISSO et al., 2020). The organic phase comprised capric/caprylic triglycerides, Lipoid S45®, and chlorhexidine (2.5 mg/mL) dissolved in acetone (45 °C ± 1 °C). After solubilization, the organic phase was poured under the aqueous phase, which comprised polysorbate 80 and distilled water, and then stirred for 10 min. The organic solvent was evaporated in a rotary evaporator under reduced pressure and the formulation was prepared in triplicate.

The formulations were characterized immediately after preparation by determining the average diameter and polydispersity index, zeta potential, pH, and dosing and encapsulation rates (SANTOS et al., 2021).

Experimental design

The antiseptic effects of NM-Cl and CS-Cl were tested on the thoracic limbs of 10 healthy male cats from a veterinary hospital admitted for elective neutering. The choice of right or left thoracic limb was determined by lot before antisepsis. The number of animals was determined from the sample size calculation. The number of animals and procedures in the study were approved by the Ethics Committee on the Use of Animals (protocol number 037/2019 - CEUA/UNIPAMPA).

Cutaneous microbiota swab samples (CMSS) were obtained before antisepsis (Tpre); immediately after antisepsis (Tpost); and 4 (T4h), 8 (T8h), and 24 (T24h) h after antisepsis (Figure 1).

Inclusion criteria

The inclusion criteria were as follows: absence of abnormalities in clinical and laboratory evaluations (blood count and serum biochemical analyses of urea, creatinine, gamma-glutamyl transpeptidase, and alkaline phosphatase) performed during admission (24 h before the study began).

Antisepsis, catheter insertion, and sample collection

The animals were admitted 24 h before antisepsis and catheter placement to perform wide trichotomy of the right and left thoracic limbs. The elbow joint was considered as the proximal limit, whereas the carpal joint was considered as the distal limit. Surgical gloves, antisepsis forceps, and sterile gauzes were used during the antisepsis of the thoracic limbs. Two sterile gauzes soaked with 7.0 mL of NM-Cl or CS-Cl were randomly applied in the trichotomy area of the right (n = 5) and left (n = 5) thoracic limbs in accordance with the method of RISSO et al. (2020). The gauzes were applied in a proximal to distal direction, and each side of the gauze was used only once, resulting in four movements.

A 24G intravenous catheter was aseptically inserted into the cephalic vein on the distal third of the trichotomy area (Figures 2A and 2C) and fixed with sterile elastic bandages (Figure 2A). Sterile bandages were used to wrap the area of interest to maintain asepsis (Figure 2D). Subsequently, the same procedure was performed using CS-Cl on the trichotomy area of the right (n = 5) or left (n = 5) thoracic limbs.

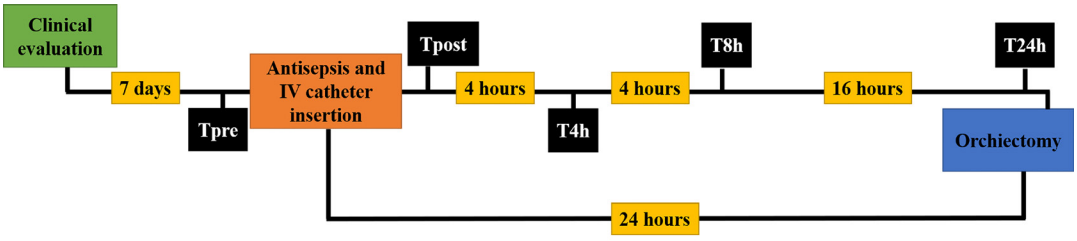

Figure 1 - Experimental design for evaluating the antiseptic effects of 0.25% chlorhexidine nanoemulsion on the skin microbiota of thoracic limbs of felines. IV, intravenous; Tpre, Tpost, T4h, T8h, and T24h = time of sample collection of skin microbiota.

CMSS were obtained before antisepsis (Tpre), and immediately after antisepsis (Tpost). After catheterization, supplementary CMSS were obtained from different positions of the trichotomy area to avoid swabbing repeated sites: at 4 (T4h), 8 (T8h), and 24 (T24h) h after antisepsis. CMSS were collected by rotating a sterile swab over the skin. Each swab was divided into four trichotomy sections to prevent overlapping of samples (Figure 2A). The samples were seeded in plates containing blood agar using zigzag movements (Figure 2B), and incubated at 35 ºC ± 2 ºC in aerobiosis. Colony-forming units (CFUs) were manually counted 24 h after inoculation, as previously described (DAVIDS et al., 2015; RISSO et al., 2020).

The collection of CMSS and removal/replacement of bandages were performed aseptically. Sterile bandage and Elizabethan collar were used to prevent licking and maintain asepsis of the study area. The antisepsis site was evaluated during the time of specimen collection and 48 h after antisepsis to detect redness, skin irritation, and/or edema. The animals underwent routine surgical orchiectomy after the collection of CMSS.

The antiseptic effect of NM-Cl was evaluated by comparing the reduction of CFUs in the CMSS collected before (Tpre) and after (Tpost, T4h, T8h, T24h) antisepsis and comparing these results with those of SC-Cl. The free NM-Cl was not tested

Figure 2 - Use of 0.25% chlorhexidine nanoemulsion in the antisepsis of felines. (A) Samples were collected using a sterile swab, with rotating movements across the trichotomized area. (B) Swabs were seeded using zigzag movements in plates with blood agar. (C) Aseptic insertion of the intravenous catheter. (D) Aseptic maintenance of the area of catheter insertion with sterile bandages.

because it showed no antimicrobial effect (RISSO et al., 2020) and could lead to infection in animals.

Statistical analysis

Statistical analysis was using SPSS software (IBM Corporation, Armonk, NY, USA). The difference in the antiseptic action between formulations was verified using the Kruskal–Wallis test, followed by the Mann–Whitney U test when a difference was detected. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

NM-Cl presented a mean diameter of 344 ± 1 nm. The SPAN of formulations was below 2 (1.45 ± 0.01), which is considered adequate. The pH of nanoemulsions was 7.4 ± 0.1. The nanoemulsions exhibited a positive zeta potential (14.57 ± 1.72 mV). The encapsulation rate demonstrated that the nanoemulsions contained approximately 80% chlorhexidine.

The CFUs of the chlorhexidine solutions are shown in table 1. Cat 6 presented CFUs higher than other animals at different times of asepsis: CS-Cl (Tpre, T24h) and NM-Cl (Tpre, Tpost, T4h, T8h, and T24h). Therefore, cat 6 was excluded from the statistical analysis. The Kruskal–Wallis test demonstrated that the CS-Cl and NM-Cl groups showed adequate antiseptic action, indicated by the difference between Tpre and Tpost CFUs ($P < 0.05$), and prolonged effect maintained at T4h, T8h, and T24h ($P < 0.05$). Furthermore, no difference was observed between the CS-Cl and NM-Cl groups at the evaluated times in the Kruskal–Wallis test, indicating the similarity between chlorhexidine solutions.

The Mann–Whitney U test showed a difference between the CS-Cl and NM-Cl groups at Tpre and Tpost, T4h, T8h, and T24h (Table 2). The evaluation of the antisepsis site showed no irritation, redness, or edema at different times (Tpost, T4h, T8h, T24h, and T48h) in both groups.

DISCUSSION

Considering the type of nanometric formulation chosen in this study, NM-Cl showed physicochemical characteristics suitable for nanoemulsion systems (RISSO et al., 2020). Consistent with the present study, other studies have already reported particle sizes approximately 300 nm for similar formulations (GOMES et al., 2018; MICHELS et al., 2019). In addition, the cationic zeta potential increases the interaction with the cell membrane because of the difference in charges between them, resulting in greater biological performance (ZADYMOVA et al., 2018).

These results suggested that CS-Cl and NM-Cl solutions have adequate antiseptic efficacy for skin antisepsis in cats, and NM-Cl demonstrated an immediate and residual antiseptic action similar to that of CS-Cl, even at lower concentrations of chlorhexidine. CS-Cl is a consolidated formulation used for the antisepsis of intravenous devices (LAI

Table 1 - Action of NM-Cl and CS-Cl on skin antisepsis of cats by manually counting the colony-forming units at different times before antisepsis (Tpre) and after antisepsis (Tpost, T4h, T8h, and T24h).

	----------Tpre---------		----------Tpost----------		----------T4h----------		----------T8h----------		----------T24h---------	
	CS-Cl	NM-Cl	CS-Cl	NM-Cl	CS-Cl	NM-Cl	CS-Cl	NM-Cl	CS-Cl	NM-Cl
Cat 1	38	26	0	2	1	3	0	5	5	3
Cat 2	26	12	0	11	1	0	0	0	0	1
Cat 3	22	300	0	1	0	0	0	5	2	14
Cat 4	10	29	0	0	3	6	0	4	0	0
Cat 5	13	61	0	2	0	3	0	8	0	0
Cat 6	234	300	0	34	1	30	5	96	300	4
Cat 7	13	40	0	4	1	1	0	0	1	0
Cat 8	7	20	0	2	0	1	0	1	2	1
Cat 9	133	60	0	0	0	3	7	0	3	4
Cat 10	85	70	0	1	0	1	1	4	1	7

Abbreviations: CS-Cl, chlorhexidine digluconate; NM-Cl, Chlorhexidine nanoemulsion.

Table 2 - Medians and percentiles of CFUs at different times and P values of the Mann– Whitney U test.

		Median	----------Percentiles----------		Mann– Whitney test (P value)*
			25th	75th	
Tpre	CS-Cl	22.00	11.50	61.50	---
	NM-Cl	40.00	23.00	65.50	
Tpost	CS-Cl	0.00	0.00	0.00	< 0.001
	NM-Cl	2.00	0.50	3.00	< 0.001
T4h	CS-Cl	0.00	0.00	1.00	< 0.001
	NM-Cl	1.00	0.50	3.00	< 0.001
T8h	CS-Cl	0.00	0.00	0.50	< 0.001
	NM-Cl	4.00	0.00	5.00	< 0.001
T24h	CS-Cl	1.00	0.00	2.50	< 0.001
	NM-Cl	1.00	0.00	5.50	< 0.001

*Mann – Whitney U test between Tpre and other times.

et al., 2016; YAGI, 2017) and in surgical procedures of cats (MARSH-NG et al., 2007; JONES et al., 2009). This similarity can be explained using the same molecule in both formulations as various chlorhexidine solutions with different concentrations are commercially available. Our group researched NM-Cl in cats because of a promising study performed *in vitro* and in rats (RISSO et al., 2020), and because the efficiency of the nanostructured formulation has been previously reported as species-dependent (KNORR et al., 2016).

Counting of CFUs is the standard method for counting the number of living microorganisms capable of growth on a specific medium. This versatile method is the standard technique used for measuring the number of living bacteria in medical samples to determine the degree of infection (HOUCHMANDZADEH & BALLET, 2023). However, this technique is time-consuming and labor-intensive (ZHANG, 2022) and can be influenced by colony morphology, colony density, and human error (SUTTON, 2012). In addition, CFU only provides an estimate of the number of cells present as the only cells able to form colonies are those that can grow under the test conditions (i.e., incubation media, temperature, time, and oxygen conditions). These cells do not represent a single cell, but rather those that happened to be well separated on the plate and can thus be distinguished after growth. A colony could arise from one or several thousand cells. Experience has shown that different technicians come up with different counts on the same sample (SUTTON, 2012).

Furthermore, chlorhexidine has better action on Gram-positive bacteria, since Gram-negative bacteria have intrinsic resistance mechanisms, such as the presence of an external membrane that limits the action of drugs (FOSSUM, 2014; MCDONNELL & RUSSEL, 1999). The results demonstrated the efficacy of NM-Cl at significantly lower concentrations than that of CS-Cl. Although, nanotechnology is expensive (INDELLI et al., 2021), nanostructured formulations can decrease the concentration of substances while maintaining an equal or superior effect compared with conventional formulations (BOCK et al., 2016; SAGAVE et al., 2015), as previously demonstrated with NM-Cl (BOUCHEMAL et al., 2004; RISSO et al., 2020), chlorhexidine nanocapsules (LBOUTOUNNE et al., 2002; LBOUTOUNNE et al., 2004; KNORR et al., 2016; VISWANATHAN et al., 2016), and vegetable oil (WANG et al., 2017).

Another advantage of nanoformulations is that they can efficiently control drug release, and can slowly release substances in order to increase the time of action (LBOUTOUNNE et al., 2004; KNORR et al., 2016; WANG et al., 2017). Even with a lower chlorhexidine concentration, NM-Cl showed prolonged residual antiseptic effect (T24h) probably because of the controlled and sustained release mechanism (LBOUTOUNNE et al., 2004; WANG et al., 2017; RISSO et al., 2020). Despite this, no differences were observed between CS-Cl and NM-Cl groups at all sample times. The limitations of this study included the inability to assess cutaneous penetration mechanisms, potential toxicity, bacterial

growth after 24 h, and stability over a minimum period of 90 days.

None of the tested cats had any skin irritation, redness, or edema for 48 h after antisepsis. This is because chlorhexidine has low toxicity and irritability and high absorption rate and low concentrations are sufficient to slow and inhibit the development of bacteria. Moreover, its effect was not significantly altered by the presence of organic matter (MULLANY et al., 2006; VISWANATHAN et al., 2016; MARCHI et al., 2018). As cephalic catheters are becoming more accessible, there is an increased risk of licking, chewing, or soiling with food in the studied area (MARSH-NG et al., 2007). Hence, sterile bandages and Elizabethan collars were used to wrap the limbs and prevent licking, reduce contamination and aseptically maintain the studied area. However, there were some fluctuations of CFUs over time, especially in cat 6, which was removed from the study.

Although, probable contamination occurred in cat 6, the method of antisepsis and aseptic protection using sterile bandages was effective in avoiding recontamination after antisepsis. Moreover, the antisepsis efficacy was demonstrated by the absence of a significant increase in CFUs during the study period. This method mimicked antisepsis procedures that maintain the sterile site or allow the minimum possible contamination, for prolonged periods, such as in the maintenance of intravascular devices (used here) and surgical procedures in which an antiseptic is applied and the sterile surgical field is maintained. However, this method may not represent the inpatient environment, such as when intravascular devices are maintained for prolonged periods without using sterile gases and bandages for protection.

Concerns about systemic infections related to intravenous catheterization, which are caused mainly by multi-drug-resistant microorganisms in hospitalized patients, are growing in humans (EDGEWORTH, 2009; LAI et al., 2016; YAGI, 2017) and animals (MARSH-NG et al., 2007; JONES et al., 2009; MANN, 2018). Thus, further research is needed to develop more effective antiseptic products in order to prepare patients for intravenous catheterization and surgical procedures (MARSH-NG et al., 2007; ECHOLS et al., 2015; LAI et al., 2016; YAGI, 2017; PRIVITERA et al., 2017; MARCHI et al., 2018; DROHAN et al., 2019).

CONCLUSION

NM-Cl showed antiseptic efficacy immediately and 24 h after antisepsis in intravenously catheterized cats, as evidenced by the reduction in counting of CFUs, and exhibited similar antiseptic action to CS-Cl. Nevertheless, NM-Cl contained lower chlorhexidine concentrations than CS-Cl. The antiseptic nanoformulation may be used as an alternative to antiseptics employed in routine veterinary medicine.

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DECLARATION OF CONFLICT OF INTEREST

The authors declared no conflict of interest.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This work involved the use of experimental animals and the study therefore had ethical approval from an established committee as stated in the manuscript. The research was approved by the Ethics Committee on the Use of Animals of UNIPAMPA (CEUA 037/2019). All animal experiments and protocols followed the normative resolutions of the National Animal Experimentation Control Council of Brazil (CONCEA) and international guidelines for animal welfare.

Informed written consent was obtained from the owner or legal custodian of all animals described in this work for the procedures undertaken in this prospective study. No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

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