Evaluation of the Local Synergistic Effect of a Dexketoprofen and Chlorhexidine Combination in the Formalin Test

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Objective: Evidence supports the local application of non-steroidal anti-inflammatory drugs such as dexketoprofen trometamol (DXT) for pain management, but little is known about the potential antinociceptive effect of chlorhexidine gluconate (CHX) and its possible synergistic effect when combined with DXT. The aim of this study was to evaluate the local effect of a DXT–CHX combination using isobolographic analysis in a formalin pain model in rats.

Materials and Methods: Briefly, 60 female Wistar rats were used for the formalin test. Individual dose-effect curves were obtained using linear regression. For each drug, the percentage of antinociception and median effective dose (ED_{50}; 50% of antinociception) were calculated, and drug combinations were prepared using the ED_{50}s for DXT (phase 2) and CHX (phase 1). The ED_{50} of the DXT–CHX combination was determined, and an isobolographic analysis was performed for both phases.

Results: The ED_{50} of local DXT was 5.3867 mg/mL in phase 2 and for CHX was 3.9233 mg/mL in phase 1. When the combination was evaluated, phase 1 showed an interaction index (II) of less than 1, indicating synergism but without statistical significance. For phase 2, the II was 0.3112, with a reduction of 68.88% in the amounts of both drugs to obtain the ED_{50}; this interaction was statistically significant ($P < .05$).

Conclusion: DXT and CHX had a local antinociceptive effect and exhibited synergistic behavior when combined in phase 2 of the formalin model. [*P R Health Sci J 2023;42(1):35-42*]

Key words: Antinociception, Formalin test, Synergism, Dexketoprofen trometamol, Chlorhexidine gluconate

Different clinical scenarios may present a dual challenge to the clinician, especially when infection control and pain modulation are needed simultaneously. This may occur in peripheral circumstances, such as when there are traumatic wounds or during surgical procedures, where the extent of the damage may include the superficial rupture of dermal integrity or even affect the deep subcutaneous tissue or bone structures (1). For acute, local conditions, wounds usually complete the healing process within 5 days to 12 weeks (2,3), while chronic wounds are mostly related to slower metabolic tissues and may heal after 12 weeks, particularly in systemically compromised patients (4). The physiological response of the healing process will include bleeding, vessel contraction, complement activation, swelling, and pain (3). During the inflammatory process, multiple reactions produce the release of chemical mediators such as prostaglandins. In normal conditions, the production of peripheral prostaglandins is low; however, their concentration increases once the inflammation cascade is activated (5).

Oral non-steroidal anti-inflammatory drugs (NSAIDs) are usually used to control the painful inflammatory response present in wounds, but the local administration of these molecules is an attractive alternative with multiple advantages (6), such as the avoidance of systemic pharmacological interactions with other treatments, the attenuation of side-effects, the decrease of metabolic sub-products, and the achievement of an optimal

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clinical effect with lower doses (7). Dexketoprofen trometamol (DXT) is the S(+) enantiomer of ketoprofen (8), a water-soluble NSAID with local antinociceptive effects (9–11).

Surgical infection can contribute to delaying healing and the patient's rejection of the treatment (12), so the use of an antimicrobial agent is desirable to prevent surgical site infections (SSIs). Surgical site infection is a common entity (present in up to 30% of surgical procedures) (13), and the evidence indicates that there is a close relationship between pain and infection in a surgical site (12). Chlorhexidine gluconate (CHX) is a small molecule that comes from organic compounds named chlorobenzenes (14). It is a topical antiseptic compound with a wide action spectrum, effective against multiple microorganisms (bacteria, fungi, and some viruses), with an acceptable safety profile. This molecule has been used in a great number of clinical procedures (15,16), it being one of the most popular antiseptics in the world. Clinical reports suggest the analgesic potential for the local application of CHX (17–20). However, the authors assumed that the observed response was related to the medication's antibacterial effect (18).

In a previous study, the DXT–CHX combination was evaluated in the formalin pain model and showed a higher antinociceptive effect than did DXT, alone. Additionally, the local application of CHX provoked an antinociceptive response (21). To confirm these results and to analyze the possible local synergism between these 2 molecules, the present work aimed to determine the interaction index (II) of the locally applied DXT–CHX combination, using isobolographic analysis in the formalin pain model.

Materials and Methods

Animals and drugs

This study was carried out according to Official Mexican Standard NOM-062-ZOO-1999 (technical specifications for the production, care, and use of laboratory animals), the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (22) and the manuscript report according to the ARRIVE guidelines (23), with ethics committee (Comité de Ética e Investigación de la Facultad de Estomatología de la UASLP) approval number CEI-FE-004-19. The inclusion criteria yielded a sample of 68 female Wistar rats (n = 5 per group, with 8 control subjects) weighing 200 to 260 g and aged from 6 to 7 weeks. The distribution of the animals is shown in Figure 1. The animals were obtained from the Animal Center of Guanajuato University, México, 2 weeks prior to the experiment, and housed in a 12-hour light–dark cycle environment at 24 (±2) °C, with free access to food and water. On the day of the evaluation and prior to the test, the animals were acclimatized to the laboratory conditions for at least 2 hours. All the animals were used once and sacrificed immediately after the test in a CO2 chamber. Dexketoprofen trometamol (Stein Labs, San José, Costa Rica) was dissolved in deionized water at different concentrations (Table 1), and CHX 20% solution (Sigma Aldrich, USA) was diluted to different concentrations, as well (Table 1). All the solutions were prepared on the day of the experiment.

Measurement of nociceptive response

The antinociceptive response was assessed using the formalin pain model and following a previously validated protocol (21). Briefly, the rats were placed in transparent cylindrical chambers with mirrors placed at a 45° angle to expand the visibility of the animals. The rats were randomized using a simple randomization method with computer generated (R software, using the sample() function) random numbers and then were subcutaneously injected (intradermal route) into the dorsal surface of the hind paw with a total volume of 50 µL of formalin (5%) and the experimental drugs at the same time (Table 1). An independent blinded evaluator observed the pain-related behavior of the rats described as flinches (rapid and brief withdrawal or flexing) of the injected paw in a 1-minute period every 5 minutes to complete 60 minutes of evaluation (Fig. 2). For the 2 phases of the formalin test, a sum method was used to calculate the total amount of response in each phase; the first phase measured flinches during the first 15 minutes (0–20 min) and the second phase, those of the following 45 minutes (25–60 min). The percentage of antinociception (%AN) of each rat was calculated as follows:

$$\%AN = \frac{\text{SUM (Phase) Formalin} - \text{SUM (Phase) Treatment group}}{\text{SUM (Phase) Formalin}} * 100$$

For the control group, 8 rats were used for the formalin group pain-related behavior (Fig. 1), to determine the %AN to be compared with the other experimental groups.

Experimental design

Dose-effect curves for each drug (DXT and CHX) were obtained using linear regression, with 5 rats for each of the 4 doses; the sample size of each drug group and for each dose was based on the findings of previous experiments (11,21); the experimental unit was a single animal. The 50% effective dose (ED_{50}; 50% of antinociception) was calculated for each drug using the least-squares linear regression analysis, and using the log of the dose and the %AN to establish the total amount of the mixture (Zadd). This dose was multiplied by the selected size of fraction (f = 0.5, with Zadd multiplied by f). The dilutions with fixed ratios (1/2, 1/4, and 1/8) of the drug combination were performed. For the Zadd, the ED_{50} of DXT was obtained in phase 2 of the formalin test (Fig. 3A and Fig. 4A); the dose of 1.2% (Table 1) was removed from the linear regression because of the low response obtained in the local environment. The ED_{50} of CHX was obtained in phase 1 of the formalin test (Fig. 4B).

Isobolographic analysis was used to characterize the drug interaction of DXT and CHX, following previously described method (24). Isobologram analysis involves the graphical representation of the dose-effect curves of 2 antinociceptive drugs in which can be tested their interaction. The points that

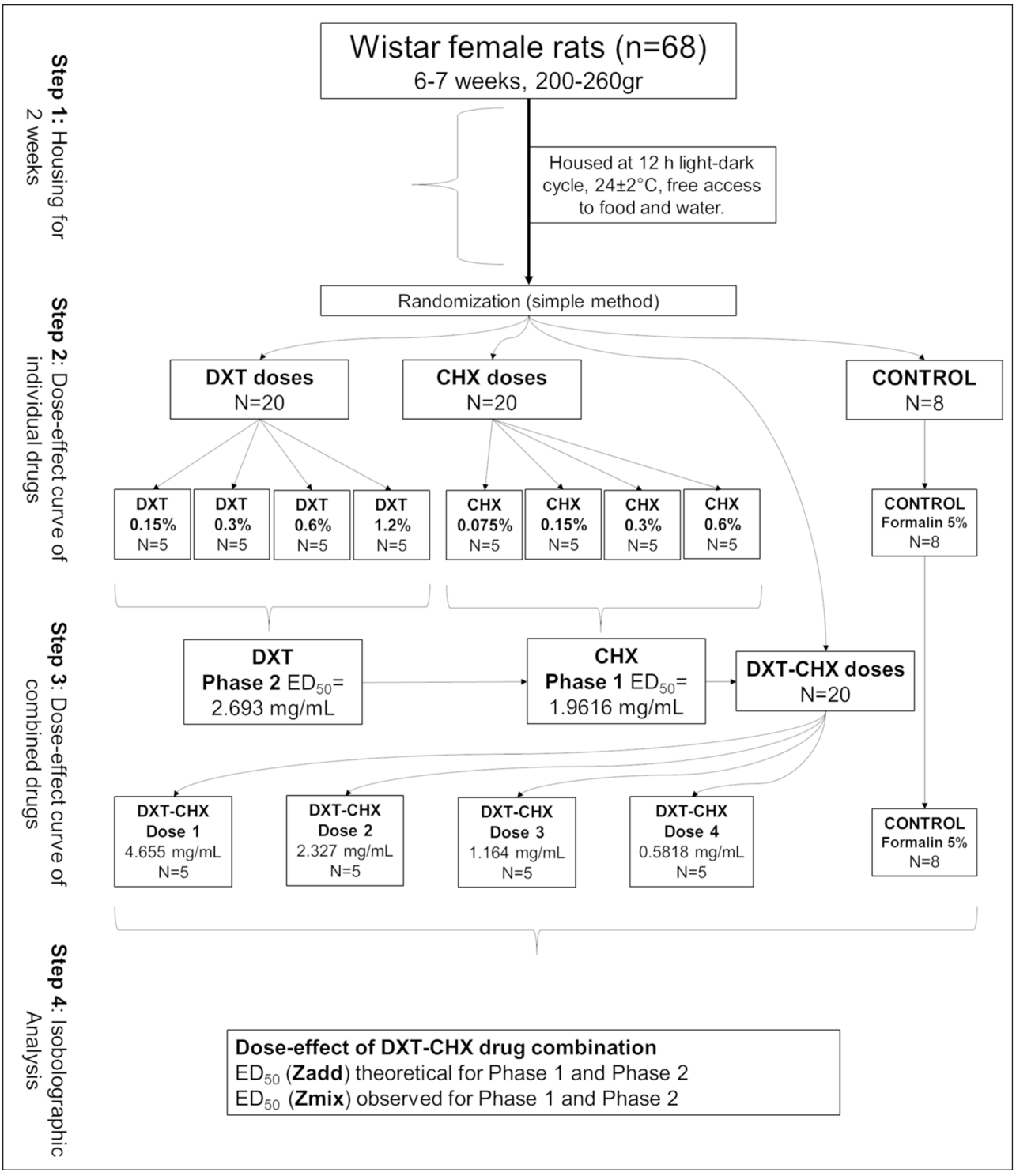

Figure 1. Flow chart showing the animal distribution between drug groups and the experimental design.

constitute an isobole are, therefore, doses that represent the amount of each drug expected to yield an effect of specified magnitude when the 2 compounds are administered together; the curves (lines) use the mathematical approach of the linear regression to compute the ED_{50} and their standard errors (24). The ED_{50} of the combination was assessed with least-squares linear regression analysis using the log of the combination dose and the %AN. The isobolographic analysis was performed for both phases in the drug combination. The experimental ED_{50} (Zmix) for both phases was compared to the theoretical equieffect ED_{50} of the drug combination with the same proportion (Zadd). If the Zmix was lower than the Zadd and

Table 1. Dose-effect data for DXT and CHX

Dexketoprofen trometamol (n = 5 per dose)			
Final concentration of DXT in 50 µL of solution	**Concentration v/v formalin/DXT**	**Effect in %AN (SEM) Phase 1**	**Phase 2**
1.5 mg/mL (0.0015 mg/µL) 0.15%	25 µL[10%]/ 25 µL[3 mg/mL]	10 (3.24)	27.07 (11.66)
3 mg/mL (0.003 mg/µL) 0.3%	25 µL[10%]/ 25 µL[6 mg/mL]	52.86 (5.84)	35.2 (4.76)
6 mg/mL (0.006 mg/µL) 0.6%	25 µL[10%]/ 25 µL[12 mg/mL]	39.05 (1.35)	53.83 (6.4)
12 mg/mL (0.012 mg/µL) 1.2%	25 µL[10%]/ 25 µL[24 mg/mL]	35.71 (9.38)	29.43 (6.68)
Chlorhexidine gluconate (n = 5 per dose)			
Final concentration of CHX in 50 µL of solution	**Concentration v/v formalin/CHX**	**Effect in %AN (SEM) Phase 1**	**Phase 2**
0.075% (0.00075 mg/µL)	25 µL[10%]/ 25 µL[0.15%]	34.29 (7.31)	27.86 (8.78)
0.15% (0.0015 mg/µL)	25 µL[10%]/ 25 µL[0.3%]	36.67 (4.56)	23.66 (9.64)
0.3% (0.003 mg/µL)	25 µL[10%]/ 25 µL[0.6%]	47.62 (2.86)	48.32 (5.13)
0.6% (0.006 mg/µL)	25 µL[10%]/ 25 µL[1.2%]	55.24 (2.52)	42.02 (7.47)

DXT: dexketoprofen trometamol, CHX: chlorhexidine gluconate, SEM: standard error of mean

the t-test derivation of this difference was significant ($P < .05$), then the drug interaction resulted in synergism.

Statistical analysis

The antinociceptive response is presented as the mean ± standard error of the mean (SEM). The %AN (y-axis) was plotted with the log10 of the doses (x-axis). Linear regression was used to calculate the ED_{50} of each drug and the combination. The dose-effect data analysis was based on the equations reported by Tallarida (with 95% confidence) (24), and the statistical software R. 3.5.2 was used to perform the calculations and construct an isobologram.

Results

The local administration of DXT showed the 2 classical phases in the formalin test (Fig. 1). The ED_{50} in the phase 2 of local DXT was 5.3867 (95% CI: 3.62, 8.02) mg/mL (Table 1, Fig. 2). The local administration of CHX showed the 2 classical phases in the formalin test (Fig. 1); these phases were divided as for DXT to establish the same "cut point" in both drugs. The ED_{50} in the phase 1 for local CHX was 3.9233 (95% CI: 2.79, 5.52) mg/mL (Table 1, Fig 2).

Once the ED_{50} was calculated, the selected fraction size ($f = 0.5$) was multiplied by the ED_{50} of the drugs, and combined doses with a fixed ratio were prepared (Table 2). The %AN (y-axis)—plotted with the log10 of the doses (x-axis)—is shown in Figure 3. Linear regression was used to calculate the ED_{50} of each drug and the combination. The Zadd for the combination was determined to be 4.655 mg/mL, with a potency ratio of 1.37. The combination parameters are shown in Table 3.

For phase 1 of the combination, the dose showed a linear tendency (Fig. 2) with the %AN, except for the second dose (Table 1). The Zmix observed was 2.7423 mg/mL. The II was less than 1, indicating the presence of synergism. However, this interaction was not statistically significant (Table 3, Fig. 3). The amounts of both drugs used to obtain an ED_{50} was reduced to 41.09% for phase 1. For the desired effect of 2.5 mg of DXT

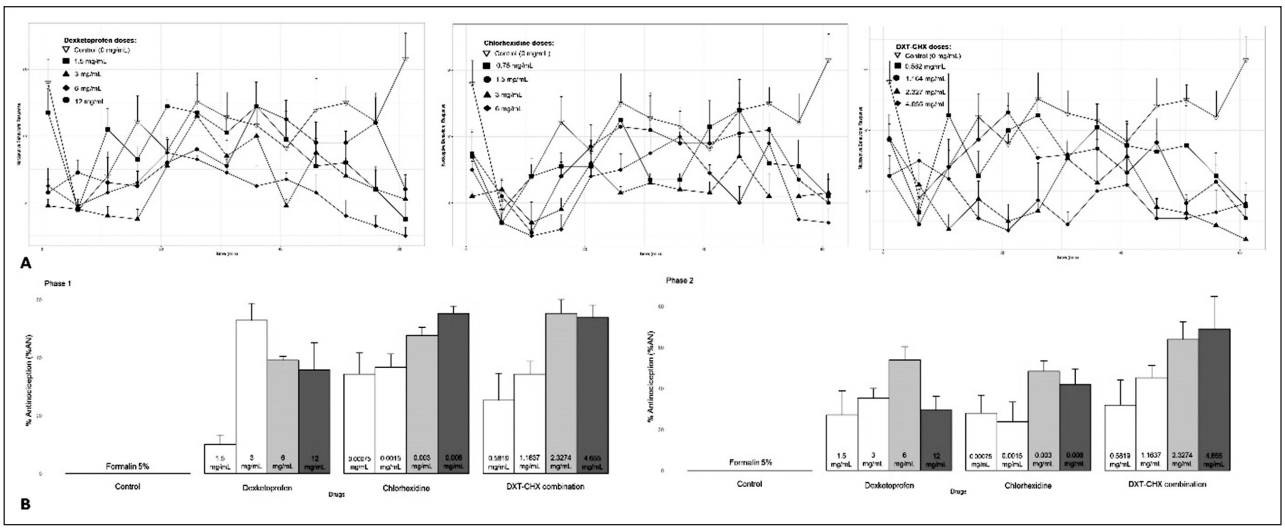

Figure 2. A) Nociceptive behavior curves of different doses of DXT (drug A), CHX (drug B), and the DXT–CHX combination (drug C). B) Dose-effect bars for phase 1 (upper) and phase 2 (lower) of DEX, CHX, and the DXT–CHX combination.

Table 2. DXT–CHX combination dose-effect data (n = 5 per dose)

DXT dose (drug A) (12.5 µL)	CHX dose (drug B) (12.5 µL)	Combined doses (25 µL)	Number of the dose
ED_{50} 2.69335 mg/mL	ED_{50} 1.9616 mg/mL	(ED_{50}A*0.5) + (ED_{50}B*0.5) 4.65495	1
ED_{50}/2 1.346675 mg/mL	ED_{50}/2 0.9808 mg/mL	2.327475	2
ED_{50}/4 0.673375 mg/mL	ED_{50}/4 0.4904 mg/mL	1.1637375	3
ED_{50}/8 0.33666875 mg/mL	ED_{50}/8 0.2452 mg/mL	0.58186875	4

DXT: dexketoprofen trometamol, CHX: chlorhexidine gluconate, ED50: median effective dose (50% of antinociception)

(10% of DXT, oral dose), the necessary dose of both drugs to accomplish the same local effect was 0.7364 mg of DXT plus 0.5363 mg of CHX. In phase 2, the %AN showed linearity (Fig. 3C and Fig. 3D). The Zmix observed was 1.4485 mg/mL. The II was lower than that of phase 1, with a reduction of 68.88% in the amounts of both drugs used to obtain an ED_{50}. The II of 0.3112 in the DXT–CHX combination indicates synergism, and this interaction was significant (P = .021) (Table 3, Fig. 3E). For the desired effect of 2.5 mg of DXT, the necessary dose of both drugs to accomplish the same effect was 0.389 mg of DXT plus 0.2833 mg of CHX.

Discussion

DXT behavior in the formalin pain test

The present study evidences the antinociceptive response in the formalin pain model in rats for the combination of CHX and DXT. The antinociceptive behavior of DXT was evaluated, and only those doses that showed linearity between the %AN and the dose were considered for this matter (Table 1, Figs. 2 and 3). The ED_{50} of DXT was in concordance with Isiordia-Espinoza et al., which team reported an ED_{50} of 5.93 mg/mL for local DXT in a formalin pain model (11). This behavior was expected for an NSAID; its action mechanism induced local cyclooxygenase inhibition (7,8) and has previously been discussed by different authors (11,25,26). Briefly, phase 1 of the formalin pain model responds to the direct effect on nociceptors, and phase 2 is mediated by prostaglandins, leading to inflammatory nociception. In consequence, the antinociceptive effect obtained by DXT was demonstrated in phase 2.

The CHX behavior in the formalin pain test

The ED_{50} for local CHX was calculated using the data from the formalin phase 1 because only in this phase did CHX show a linear relationship between %AN and the dose applied (Table 1, Figs. 2 and 3) The challenge in the experiment was to analyze and evaluate the chlorhexidine molecule, which, in theory, does not have antinociceptive properties. In this context, the linearity of the 2 classical phases in the antinociceptive model may not apply with this kind of molecule. This behavior can be explained by the different nerve fibers involved in the immediate response to formalin. Phase 1 corresponds to direct nociceptor stimulation mediated by small, myelinated A-delta fibers. Nociceptive stimulation can be affected by local anesthetics such as lidocaine and procaine (27). Röed suggests that CHX acts like procaine in the nerve action potential like membrane-stabilizer agents (28). However, the mechanism to stabilize excitable cell membranes is different from that of procaine, so further research must be carried out to determine the exact mechanisms involved in the membrane effect of CHX. Additionally, Röed evidenced the selectivity of CHX for myelinated A-fibers and the unaffected slow component (C-fibers) of nerves. Based on that, the author assumed that CHX has an affinity for specific receptor sites in the nerve membrane (28,29). Shaihutdinova et al. reported that CHX can inhibit the evoked endplate currents by plugging the open ionic channel, by increasing desensitization, or by favoring molecule trapping in a voltage-dependent channel; their results proposed that CHX's antinociceptive effect may be caused by an open-channel modulatory effect with allosteric inhibition (30).

The behavior of the DXT–CHX combination in the formalin pain test

The isobologram of both phases is shown in Figure 3. The additivity line (solid line) contains the Zadd point, with its standard error, representing the calculated additivity numbers for this proportional combination (f = 0.5). The Zmix point (with its standard error) is the DXT–CHX combination point that was determined experimentally, with the same proportional mixture. The Zadd and Zmix points in both phases are separate; however, only in phase 2 was the difference between the Zmix and the Zadd significant. The local effect of DXT showed the classic NSAID behavior in the formalin pain model and, in combination with CHX, had a better antinociceptive response (21). This finding was remarkable

Table 3. DXT–CHX interaction parameters

Formalin phase	Parameters	Dose combination DXT–CHX
Phase 1	Zmix: 2.7423 mg/mL 95% CI (1.58, 4.76) mg/mL Potency ratio: 1.373 Interaction index: 0.5891 *P* value > .05	1.587/1.156 mg/mL 57.8595%/42.1405%
Phase 2	Zmix: 1.4485 mg/mL 95% CI (1.13, 1.85) mg/mL Potency ratio: 1.373 Interaction index: 0.3112 *P* value < .05	0.838/0.61 mg/mL 57.8595%/42.1405%

DXT: dexketoprofen trometamol, CHX: chlorhexidine gluconate

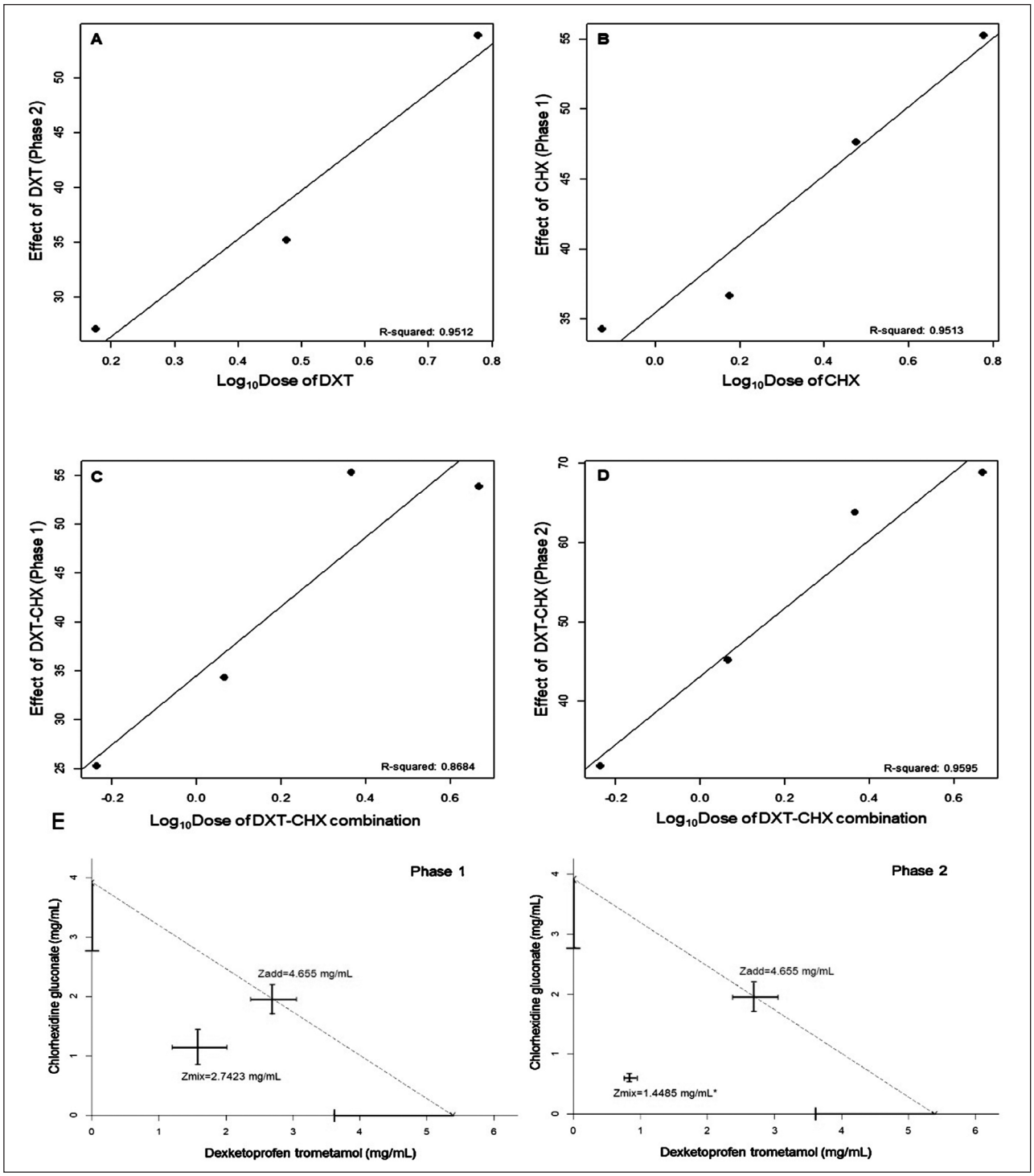

Figure 3. Log10 dose-response curves of the drugs. A) DXT, phase 2, B) CHX, phase 1, C) DXT–CHX combination, phase 1, D) DXT–CHX combination, phase 2, E) isobolograms of both phases for the DXT–CHX combination.

since CHX is an antiseptic that is mostly known for its antimicrobial properties but not its antinociceptive potential (16,31–33). CHX is a cation molecule that binds non-specifically to negatively charged membrane phospholipids of bacteria. At low concentrations, CHX affects the osmotic balance of potassium, phosphorus, and other low-weight molecules. At sublethal concentrations, this drug can incur as much as a 50% loss of potassium ions, an irreversible condition that may lead to cell death by cytolysis (34). This mechanism in cellular membranes can play a possible role in nerve signal

transmission, and thus, antinociceptive response. However, further experiments are needed to verify this hypothesis.

The nerve fiber selectivity of CHX and its membrane-stabilizing effect may explain the linear response in the formalin phase 1. This effect will be favorable to combine with the obtained in phase 2 with DXT, thus covering both phases of nociception. CHX can inhibit the initial action potential in the A-delta fibers, while DXT may modulate the late inflammatory nociceptive reaction. Röed reported selectivity for the A-fibers, but long desensitization in the nociceptors of CHX might explain the remaining effect in phase 2 of the formalin test (28,29). Shaihutdinova et al. also reported that the possible long-lasting action of CHX in nociceptive fibers might be made possible by the CHX partition coefficient (CHX base, 0.754; and CHX digluconate, 0.037) (35), which would provide an amphiphilic character to CHX, favoring the penetration of the membrane lipid milieu. The authors speculate that these mechanisms are responsible for desensitization, the lower number of functioning receptors, and, consequently, decreased postsynaptic responses, for a long period (30). Combined with the antinociception induced by DXT, this effect results in the synergistic interaction of both drugs in the second phase of the formalin test.

Since the results in the present work cannot yet be extrapolated to a clinical human application, further clinical research must be conducted to validate this evidence beyond animal experiments. As mentioned before, some clinical trials (17–20) suggest that there is an analgesic effect present in the CHX groups, but the authors support the observed effect based on the antibacterial activity of CHX; although CHX potentially has different mechanisms involved in the antinociceptive response, the dose should be selected carefully, first evaluating its biocompatibility to avoid possible toxic reactions (36,37). To enhance the best behavior, the design of a drug delivery system to control the release of the CHX–DXT combination is advisable. Such a device would allow the local administration of the combination, reducing the systemic intake of NSAIDs by acting directly on the target zone (38). If the observations presented in this report can be reproduced in the clinical environment, the local combination of CHX–DXT, might be useful beyond the classical clinical application of these drugs—specifically, in a surgical context. The translational approach to promoting the use of this combination relies on the fact that surgical wounds include external tissue damage and subcutaneous tissue modification. Before the suturing and complete healing of the defect, this innovative proposal may be used directly as a peripheral drug delivery system, possibly controlling the analgesic and antibacterial properties of bacterial infection in an analgesic environment and with fewer systemic effects. Further studies will be done to obtain the basic scientific and clinical evidence needed to support this hypothesis. In conclusion, this work confirms a novel peripheral multimodal analgesic combination (39) to prevent and treat nociceptive responses in a contaminated environment where disinfection is mandatory. In conclusion, DXT and CHX showed, individually, local antinociceptive effects (especially in phase 2 and phase 1 of the formalin test, respectively), and synergistic behavior when combined in phase 2.

Resumen

Objetivo: La evidencia soporta el uso de analgésicos no esteroideos (AINES) como el dexketoprofeno trometamol (DXT), sin embargo, existe poca evidencia sobre el potencial efecto antinociceptivo del gluconato de clorhexidina (CHX), y su posible efecto sinérgico. El objetivo de este estudio fue evaluar el efecto local de la combinación de DXT-CHT usando el análisis isobolográfico en un modelo de dolor de formalina en ratas. Métodos: Fueron utilizadas un total de 60 hembras de ratas Wistar en la prueba de formalina. Las curvas dosis-efecto fueron obtenidas mediante una regresión lineal. El porcentaje de antinocicepción (%AN) y el 50% del efecto (ED_{50}) fueron calculados para las drogas individuales. Las dosis de la combinación DXT-CHX fueron preparadas utilizando la ED_{50} para DXT en la fase 2 del modelo de formalina y la ED_{50} para CHX en la fase 2. La ED_{50} de la combinación DXT-CHX fue calculada, y el análisis isobolografico se realizó para ambas fases del modelo. Resultados: La ED_{50} local para DXT fue de 5.3867 mg/mL en la fase 2, y para CHX fue de 3.9233 mg/mL en la fase 1. Cuando la combinación fue evaluada, la fase 1 mostró un Índice de Interacción (Ii) menor que 1, indicando sinergismo sin significancia estadística. Para la fase 2, el Ii fue de 0.3112, con una reducción del 68.88% en la cantidad de ambas drogas para obtener la ED_{50}; esta interacción fue estadísticamente significativa con $p<0.05$. Conclusión: El DXT y la CHX mostraron un efecto antinociceptivo local, y mostraron un comportamiento sinérgico cuando fueron combinados en la fase 2 del modelo de formalina.

Acknowledgments

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