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Influence of the Antiseptic Agents Polyhexanide and Octenidine on FL Cells and on Healing of Experimental Superficial Aseptic Wounds in Piglets

A Double-Blind, Randomised, Stratified, Controlled, Parallel-Group Study

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Key Words
Polyhexanide · Octenidine · Wound healing · Superficial aseptic wounds · Cytotoxicity

Abstract
The main target of the combination of octenidine with phenoxyethanol (Octenisept®) is the antisepsis of acute wounds, whereas polyhexanide combined with polyethylene glycol in Ringer solution (Lavasept®) is the agent of choice for antisepsis of chronic wounds and burns. Because comparative data for both agents on the effects on wound healing are lacking, we investigated the influence of preparations based on polyhexanide and octenidine versus placebo (Ringer solution) in experimental superficial aseptic skin wounds (n = 108) of 20 mm diameter, using a double-blind, randomised, stratified, controlled, parallel-group design in piglets. Computerised planimetry and histopathological methods were used for the assessment of wound healing. Histologically, no significant differences could be verified at any time between the 3 groups. However, in the early phase (day 9 after wounding), the octenidine-based product retarded wound contraction to a significantly greater extent than placebo and polyhexanide, whereas in the later phase (days 18 and 28), polyhexanide promoted contraction significantly more than did placebo and octenidine. The consequence is complete wound closure after 22.9 days using polyhexanide, in comparison to the placebo after 24.1 days ($p < 0.05$) and octenidine after 28.3 days (no statistical difference to placebo). This may be explained by the better tolerance of polyhexanide in vitro, which was demonstrated with dose and time dependence in cytotoxicity tests on human amnion cells.

Introduction

The combination of octenidine with phenoxyethanol (Octenisept®) is a new, increasingly important antiseptic preparation registered in Germany for adjuvant wound antisepsis as well as for antisepsis for mucous membranes, which is recommended for antisepsis of acute wounds [1]. The combination of polyhexanide with polyethylene glycol in Ringer solution (Lavasept®) is registered as a phar-

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maceutical raw material in Germany, and registered and marketed as an antiseptic in Switzerland. Because of its high tolerance in cell and tissue cultures, polyhexanide is recommended as the agent of choice for treatment of chronic wounds and burns [1, 2]. Documentation of efficacy and tolerance in vitro is available for both formulations [3–7]. Both correspond to the criteria for antiseptics in vitro with a reduction factor of >5 log in the quantitative suspension test without loading and >3 log with loading [6, 7], but octenidine acts more rapidly [4]. In contrast to octenidine, polyhexanide interacts with acidic phospholipids in microbial membranes, resulting in their disruption, while the neutral phospholipids in human cell membranes are only marginally affected [8]. Due to this difference in the selectivity of action, the cytotoxicity and the irritative potential of polyhexanide has been shown to be substantially lower than that of octenidine [5, 7].

The efficacy of polyhexanide in the treatment of contaminated soft tissue wounds has been shown in a randomised double-blind clinical study [9]. However, comparative data for both agents on the effects on wound healing are lacking. Therefore, it was of interest to compare the influence of the two antiseptics on wound healing in a standardised animal wound model before planning clinical studies. Porcine skin was chosen as a model for the wound healing process because of its similarity to human skin regarding blood supply, epidermis and thick dermal papillary body [10–12].

Additionally, the dose-response relationship for cytotoxicity in vitro was examined.

Materials and Methods

In vivo Investigation with Piglets

Study Design and Sample Size

The study was carried out according to a double-blind, randomised (SAS software), stratified, controlled, parallel-group design including 18 piglets with a total of 108 artificial superficial wounds, each 20 mm in diameter.

Each group was divided into three subgroups, with durations of treatment of 9, 18 and 28 days. Six wounds were inflicted on the back of each piglet. The state of each wound was rated and photographically documented on days 9, 18 and 28. At each of these time points, 2 piglets from each treatment group were sacrificed for histopathological examination. Therefore, the histological variables had a sample size of 12 wounds per time point; for wound area, the sample size was 36 wounds for 28 days of treatment, 72 for 18 days and 108 for 9 days.

The experimental part of the study was carried out at the Department of Hypoxia, Bogomoletz Institute of Physiology in Kiev, Ukraine.

Ethics

The study was performed strictly according to the proposed international guiding principles for biomedical research involving animals (17th CIOMS Round Table Conference, Geneva, 1983).

Animals

A total of 18 female piglets aged 68.6 ± 8.3 days and weighing 9.88 ± 1.06 kg were included in the study (analysis of variance, $p = 0.212$ and $p = 0.343$, respectively). The animals were housed in air-conditioned rooms at a temperature of 22 °C, fed a suitable diet and given water ad libitum. To avoid cross-contamination, only piglets in identical treatment groups were housed together.

Test Agents

To produce the polyhexanide solution of 0.4 mg/ml, 2 ml of a Lavasept concentrate (Fresenius AG, Bad Homburg, Germany; batches: H-10-01 and H-10-06) were added to 1,000 ml of Ringer solution. The octenidine-based product used was Octenisept, a combination of 0.1 g octenidine dihydrochloride with 2 g 2-phenoxyethanol per 100 ml (Schülke & Mayr, Norderstedt, Germany; batch PB 3051). Ringer solution (Fresenius AG; batch HC 1800) was used as the placebo. The medications were visually indistinguishable.

The solutions were applied by identical sprayers (2 × 0.15 ml per spray per day) from a distance of approximately 10 cm directly onto the wound. This represents the recommended dose of Octenisept (30 μg per wound and day) and Lavasept (12 μg per wound and day).

Infliction and Treatment of Superficial Skin Wounds in Piglets

The day of surgery was defined as day 0. Following anaesthesia by an intramuscular injection of atropine sulphate (0.045 mg/kg body mass) and ketamine chloride (11 mg/kg body mass) [12] and shaving, the skin was rubbed with povidone-iodine in 70% ethanol. Each animal received a series of 3 wounds on the right and left sides of the spinal column in the cephalocaudal direction. The epidermis and the superficial portion of the dermis were removed using aseptic surgical techniques. The original wound size was 20 mm in diameter. Wound sites were approximately 10 cm apart to minimise possible interactions of wound healing. Bleeding was stopped by direct pressure only. The first dose of medication was applied blinded. Wounds were covered with sterile cotton compresses. After daily aseptic change of compresses, the wounds were rinsed with isotonic 0.9% NaCl solution. The wound status was determined by visual assessment. Ratings were: 'normal', 'exudative', 'pus' and 'odour'. Finally, 2 spray applications of the allocated medication were administered, and wounds were covered with sterile dressings.

Measurement of Wound Areas

Wound areas were photographed on days 0, 9, 18 and 28 from a distance of 30 cm using a reference scale and evaluated by computerised planimetry. Each photodocument was scanned (Adobe Photoshop™ 3.0) and fed into a planimetry programme (Leica QWin, Leica Imaging Systems, Cambridge, UK). The reference scale and the contour of the wound were manually marked with a pointer on the screen, and wound areas were automatically computerised from these data. The mean of 3 repeated measurements was regarded as the best estimate of the 'real' value. Reproducibility of the measurements was high, as indicated by an intrarater reliability of $r = 0.94$.

Histopathology

On days 9, 18 and 28, the wound sites from 2 anaesthetised piglets from each treatment group were explanted and fixed in 10% neutral formalin. Sections of the wound sites were taken from wound edge to edge, through the centres. Thereafter, these piglets were sacrificed. After fixation, the samples were dehydrated in ethanol of increasing concentrations (from 50 to 100%) and in chloroform, and embedded in paraffin. All histological procedures were carried out according to established techniques [13]. Sections of 5–7 μm were stained with haematoxylin-eosin to determine the number of polymorphonuclear neutrophils, lymphocytes and fibroblasts. Staining with fast blue was used to histochemically localise the non-specific esterase in macrophages. The number of cells was counted in 5 high-power fields of skin samples obtained from each wound site using an oil-immersion lens (×1,000) [14]. To establish the extent of oedema, neovascularisation, necrosis and collagen fibre formation, haematoxylin-eosin-stained wound sections were examined by means of light microscopy (×400) and rated by an experienced pathophysiologist as 'none', 'mild', 'moderate' or 'intense' [15].

Statistics

Results were analysed regarding variability, sensitivity and correlation, using non-parametric methods whenever appropriate, at a 5% level of significance. All p values are of a descriptive nature only. The initial values of day 0 (baseline) were tested for homogeneity between the treatment groups with a one-way analysis of variance (target variables, age, body mass, daily dose per body mass, wound area). Since the measurement of wound area was repeated 3 times per wound, a repeated-measure analysis of variance and an intraclass correlation were analysed to estimate the intrarater reliability. Data on the reduction in wound area and other variables were evaluated by analyses of variance with the factor treatment for each day. Correlation analyses were performed according to Spearman [16].

In vitro Tests on FL Cells

A suited number of cells (FL, ATCC CCL 62) are seeded into individual wells of a 96-well microtitre tissue culture plate to achieve 60–70% confluence at the time of the addition of the test agents [17, 18]. The medium is then replaced with various concentrations of test agent in phosphate-buffered saline (PBS), 4–8 wells per concentration. The plate is incubated for 10, 30 and 60 min. The test solution is then replaced with neutral-red-containing medium, and after incubation for an additional 3 h to allow for uptake of the dye, the cells are washed carefully with PBS using a microtitre plate washer. Damaged or dead cells lose their ability to retain neutral red, which is then removed during this wash/fixation procedure. The dye is then extracted from the intact, viable cells with a solution of 1% acetic acid:50% ethanol. The plate sealed by a foil is left to stand overnight at 4°C in the refrigerator, then agitated on a microplate shaker for 10 min. The absorbance of dissolved dye is then determined using a spectrophotometer equipped with a 540-nm filter. The mean absorbance of control (medium and/or PBS) is defined as 100%, the absorbance of test solution is calculated in relation to the control.

Statistics

The experiments were done 3 times as triplicates. The results were evaluated by the Wilcoxon-Mann-Whitney U test.

Results

On day 0, the mean body mass of the piglets ranged from 8.5 to 12.1 kg (treatment comparison: $p = 0.3429$). The mean daily increase in body weight was similar for all treatment groups (polyhexanide 0.2 kg/day; octenidine and placebo 0.19 kg/day).

Visual assessment of wound status did not reveal any signs of substantial infection such as pus or foul odour during the entire study. Between day 0 and day 9, wounds were rated 'normal' or 'exudative', thereafter as 'normal' with no differences between the groups.

Initial wound size was nearly identical for all groups. During the early stages of wound healing (days 0–18) octenidine-treated wounds exhibited a significantly slower wound contraction than polyhexanide-treated wounds, whereas placebo-treated wounds were statistically indistinguishable from the polyhexanide group. Linear regression analyses revealed a faster wound closure for polyhexanide compared to placebo ($p < 0.05$), but not for placebo compared to octenidine. The mean numbers of days for complete wound closure were 22.9 days for polyhexanide, 24.1 days for placebo and 28.3 days for octenidine. In summary, the data indicate two distinguishable phases of wound healing: in the early phase, octenidine significantly retards wound contraction, whereas in the later phase (days 18–28), polyhexanide promotes wound contraction to a significantly greater extent than placebo (fig. 1).

Regardless of treatment, the mean number of infiltrating cells (polymorphonuclear neutrophils, lymphocytes, macrophages) was highest on day 9 and decreased over time ($p < 0.001$; table 1). In contrast, the mean number of fibroblasts increased with time and peaked at day 28 in all treatment groups (table 1). For these variables no significant treatment differences were found on days 9 and 28.

The extent of oedema and necrosis decreased continuously with time ($p < 0.001$). With the exception of neovascularisation and collagen fibre formation between Octenisept and placebo on day 28 ($p < 0.05$), there were no significant treatment differences.

In the in vitro experiments, the differences in cytotoxicity between the active agent (polyhexanide, octenidine) and the final formulation (Lavasept, Octenisept) were significant ($p < 0.05$) for each exposure time and with any tested concentration. The effect followed a typical dose-response relation (table 2).

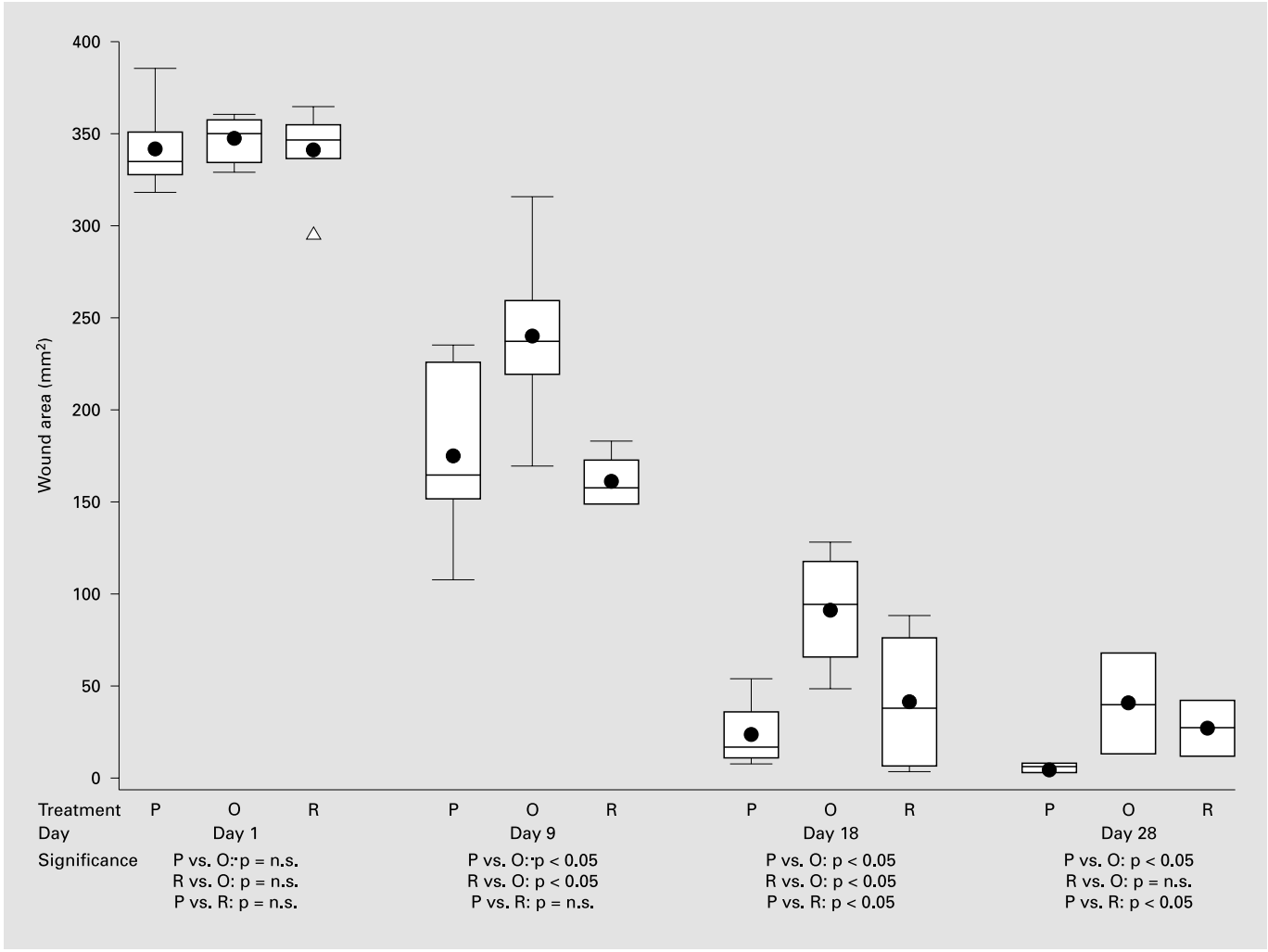

Fig. 1. Wound areas. Box and whisker plots for wound areas (mm^2) over time after experimental wounding (day 0). P = Polyhexanide; O = octenidine; R = Ringer solution; n.s. = non-significant (p values are of descriptive nature).

Discussion

The study was not designed to compare the antiseptic potential of octenidine and polyhexanide but to compare the influence of the substances on aseptic wound healing. Furthermore, the vast majority of superficial skin wounds heal aseptically without complication when treated properly.

Our data represent results of identical and comparable wounds, which could not be realised in humans. However, further studies in infected wounds are necessary to provide additional data on the interaction of antiseptic efficacy, wound tolerance and consequences for wound healing. It can be estimated that the inferiority of Octenisept to Ringer solution found in this study would not be found in a study with septic wounds.

Apparently, the most remarkable result concerns the different rates of wound healing. Wound closure was more rapid with polyhexanide than with octenidine at all times. Additionally, octenidine-treated wounds took about 1 week longer to reach the results of polyhexanide. Since there was no difference in the rate of wound closure between polyhexanide and Ringer solution up to day 18, the conclusion must be drawn that octenidine retards wound closure in the early healing phase. However, in the final phase of wound healing, polyhexanide promotes

Table 1. Histopathological results

Marker	Day	Polyhexanide	Octenidine	Placebo
Cell number				
PMNs[1]	9	7.7 ± 3.7	13.8 ± 10.1	9.6 ± 7.1
	18	4.4 ± 2.6	4.4 ± 3.1	3.5 ± 4.0
	28	1.6 ± 1.3	2.4 ± 1.8	2.8 ± 1.6
Lymphocytes[2]	9	4.4 ± 3.2	4.6 ± 2.4	3.2 ± 1.7
	18	1.8 ± 1.9	2.0 ± 1.7	1.3 ± 0.9
	28	1.7 ± 1.6	2.8 ± 2.3	3.4 ± 2.5
Macrophages[1]	9	9.3 ± 3.3	11.1 ± 2.8	11.7 ± 4.4
	18	7.1 ± 3.0	5.3 ± 2.0	6.2 ± 2.6
	28	7.6 ± 3.3	8.0 ± 2.1	9.0 ± 4.0
Fibroblasts[2]	9	40.5 ± 20.2	28.8 ± 15.5	41.0 ± 21.3
	18	36.8 ± 8.2	28.6 ± 11.2	33.1 ± 13.4
	28	53.0 ± 16.7	45.8 ± 10.8	55.5 ± 19.5
Extent				
Oedema[1]	9	2.6 ± 0.7	3.0 ± 1.0	2.6 ± 0.7
	18	1.3 ± 0.5	1.4 ± 0.5	1.5 ± 0.7
	28	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3
Neovascularisation[2]	9	2.8 ± 1.0	2.7 ± 0.8	2.3 ± 0.6
	18	1.5 ± 0.5	1.8 ± 0.9	1.7 ± 0.7
	28	1.8 ± 0.8	2.8 ± 1.3[3]	1.6 ± 0.8
Necrosis[1]	9	2.3 ± 0.9	3.1 ± 0.9	2.7 ± 0.8
	18	1.3 ± 0.5	1.3 ± 0.7	1.4 ± 0.7
	28	1.1 ± 0.3	1.2 ± 0.4	1.1 ± 0.3
Collagen fibre formation[2]	9	3.3 ± 0.8	3.1 ± 1.0	3.5 ± 0.8
	18	2.2 ± 0.8	1.8 ± 0.8	1.8 ± 0.9
	28	2.2 ± 0.8	2.6 ± 0.7	2.1 ± 0.8

n = 12 wounds per treatment and time point, F tests for treatment differences at a given time point. Cell number = Number of infiltrating cells and fibroblasts (means ± SD); PMNs = polymorphonuclear neutrophils; extent = means ± SD of 12 wounds.

[1] Differences between days (9 > 18 > 28): $p < 0.001$.

[2] Differences between days (9 > 18, 28): $p < 0.001$.

[3] Difference between Octenisept and placebo: $p < 0.05$.

Table 2. Cytotoxicity of polyhexanide/octenidine and Lavasept/Octenisept to FL cells (mean of 3 separate examinations as triplicates)

Concentration of active agent	Cell vitality, %		
	after 10 min	after 30 min	after 60 min
10 µg/ml	89.3/52.3	64.7/18.6	39.2/20.4
	85.3/54.9	60.6/24.3	37.2/10.4
20 µg/ml	88.2/17.5	61.3/8.2	37.3/20.0
	81.4/26.8	58.5/8.5	30.4/2.4
30 µg/ml	60.9/9.7	33.8/11.4	25.3/15.6
	55.3/14.3	31.8/2.8	18.7/1.9

Cell vitality = First line polyhexanide/octenidine, second line Lavasept/Octenisept (mean values; control = 100%). $p < 0.05$ for all data pairs (polyhexanide/octenidine; Lavasept/Octenisept).

healing, since at that time polyhexanide-, but not octenidine-treated wounds had contracted more than Ringer-solution-treated wounds.

The inferiority of octenidine to polyhexanide in terms of wound closure rates cannot be explained by the content of phenoxyethanol in Octenisept. Our in vitro studies using human amniotic cells showed that the cytotoxic potential of octenidine, i.e. Octenisept, and polyhexanide, i.e. Lavasept, both were time and dose dependent. The cytotoxicity seemed to be provoked by octenidine, since no significant difference between octenidine and Octenisept could be demonstrated. Obviously the delayed wound healing (closure time) is depending on the higher cytotoxicity of Octenisept as demonstrated in vitro by cell culture. Surprisingly this delay was not accompanied by any histopathological alteration, therefore additional tests to elucidate the mode of delayed wound healing are of further interest.

An additional result was the finding that the best treatment effects are seen between days 9 and 18, which is of importance for the biometrical planning of further studies. Another finding was that it was not necessary to sacrifice the piglets, as no valuable histopathological data were gained, and wounds will heal completely.

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