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The Impact of Topical Antiseptics on Skin Microcirculation

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Abstract

Aim: Antiseptics are commonly used in clinical practice to disinfect tissue and to avoid infections. However, topical antiseptics are assumed to have an influence on skin microcirculation, per se. Thus, the aim of the study was to analyse the influence of topically applied antiseptics on the microcirculation of intact skin in vivo.

Materials and Methods: The investigation was carried out on ears of male hairless mice (SKH-1hr, n = 25). The influence of four antiseptics was examined. Sodium chloride 0.9% served as control. An alcohol-based solution with a mixture of ethanol, 2-propanol and purified water (Softasept®), an antiseptic with octenidine dihydrochloride and phenoxyethanol as the main active agents (Octenisept®), as well as hexamethylenbiguanide (Lavasept®) and 70% ethanol were tested. Intravital fluorescence microscopy in combination with intravenous injection of the fluorescence dyes FITC-Dextran as plasma marker and Rhodamine 6G (leukocyte staining) allowed a quantitative analysis of standard microcirculatory parameters (vessel diameter, functional capillary density, red blood cell velocity, FITC-leakage and leukocyte endothelium interaction). Recordings of the microcirculation in several regions of interest (ROI) were made prior to application and after 10 min exposure time and 60 min after the baseline data. Data were evaluated off-line with aid of computer assisted analysis.

R*esults:* The diameter of arterioles decreased after the treatment with the alcoholic solutions. The other two antiseptics (Octenisept® and Lavasept®) caused a significant increase. Functional capillary density (FCD) was significantly reduced after application of ethanol and Softasept®. There was no reduction of FCD following application of Octenisept®. After treatment with ethanol and Softasept® there was a significant decrease in red blood cell velocity (RBCV). The use of Lavasept® revealed a decrease of FCD and RBCV. In the Octenisept® treated group RBCV shows a mild increase after 10 minutes. The application of ethanol, Softasept® and Lavasept® was characterized by a significant increase of leukocyte endothelium interaction (LEI). After treatment with saline and Octenisept® LEI remained constant. All used antiseptics except of Octenisept® caused a significant leakage of FITC-Dextran.

Conclusion: The antiseptics used in this study all showed an influence on skin microcirculation. As expected, our findings show that the alcoholic solutions are most aggressive to skin microcirculation.

Key words: Antiseptics, Softasept®, Lavasept®, Octenisept®, Ethanol, microcirculation, hairless mice, intravital fluorescent microscopy, FITC-dextran, Rhodamine 6G

Abbreviations: FITC = fluoro-isothyocyanate, FCD = functional capillary density, IFM = intravital fluorescent microscopy, LEI = leukocyte endothelium interaction, RBCV = red blood cell velocity, ROI = region of interest

Introduction

Today, antiseptics are an essential part of medical treatment. In all medical fields such as treatment of burns, wound treatment and prophylaxis prior to surgery as well as diagnostic procedures skin disinfectants are routinely used.

Efficacy against bacteria, viruses and fungi plays a key role in the functions of an antiseptic, but in the same manner the tissue compatibility presents an important issue. Sir Alexander Fleming indicated in 1919 that "it also makes it necessary, in the estimation of the value of an antiseptic, to study its effects on the tissues more than its effects on the bacteria" [1].

Antiseptics in daily use in clinical practice may have a negative effect on the microcirculation of the skin - the first barrier of tissue.

It is well known that an inadequate nutritional perfusion of tissue leads to wound healing disorders and skin necrosis [2].

Therefore, the aim of this study was to investigate the influence of clinically used antiseptics on skin by analyzing their interaction with microcirculation after topical application in vivo.

Materials and Methods

Animals

Male hairless mice (SKH-1/hr, bw 18-23g) were obtained from Charles River (Sulzfeld, Germany) one week before the experiments started. The animals, which lose their hair approximately 10 days after birth, were fed with standard laboratory chow (SSNIFF

Spezialdiäten GmbH, Soest, Germany) and water ad libitum. They were housed in cages at 23ºC in a 12 hour dark / 12 hour light cycle. The principles of laboratory animal care were followed (Ruhr-University Bochum, No. 50.8720, 3.65). The tests were carried out in a single-blinded manner in accordance with German law on the protection of animals. After completion of the experiments the animals were killed with an overdose of pentobarbital i.p. given under general anesthesia.

The ear of hairless mice is 300 μm thick. It consists of two full-thickness layers of skin (each ~100μm) separated by a layer of elastic cartilage (~80-100μm) [3]. Three main vessel bundles entering at the base of the ear are responsible for the nutrition of the tissue [4, 5]. The mouse ear was previously used to investigate the pathophysiology of skin ischemia / reperfusion and wound microcirculation by means of intravital microscopy [6, 7].

Test Solutions

Four topical antiseptics (70% ethanol, Softasept®, Octenisept®, and Lavasept®) and 0.9% sodium chloride, serving as control, were tested in this study.

The diluted ethanol in a 70% concentration (Quadflieg GmbH, Gelsenkirchen, Germany) shows his antiseptic effect in denaturation of proteins.

In a similar manner acts Softasept® (B.Braun Melsungen AG, Melsungen, Germany). Softasept® contains 74% of 100% ethanol, 10% of 2-propanol and 16% of purified water.

Octenidine dihydrochloride (0.1%) and phenoxyethanol (2%) are the main active agents of Octenisept® (Schülke&Mayr GmbH, Norderstedt, Germany). Added are 85% glycerole, aqua, sodium chloride, sodium hydroxide, sodium D-gluconate and (3-coconutacidamidopropyl)-dimethylammonioacetate. Octenidine dihydrochloride is a cationactive substance which acts on cell membrane, thus it disturbs cell function. Phenoxyethanol completes synergistically this effect. The other components lead to pH –adjustment.

Lavasept® (Fresenius AG, Bad Homburg, Germany) contains hexamethylenbiguanide as active disinfectant agent. In 1ml Lavasept® 200mg hexamethylenbiguanide with 10mg macrogolum 4000 is solubilised in aqua. The biguanide leads as cationactive substance to the disruption of the inner cell membrane of the anionic bacillus. Macrogolum 4000 (=polyethylenglycol) serves as surfactant.

Study Groups

For the investigations the animals were randomized and assigned to five groups (Lavasept®, Octenisept®, Softasept®, 70% ethanol and sodium chloride 0.9%). Each group consisted of n = 5 mice for the investigations.

Preparation

The mice were anesthetized via spontaneous inhalation of isoflurane - N_2O (FiO_2 0.35, 0.015 L/L Isoflurane, Forene®, Abbott GmbH, Wiesbaden, Germany) and placed on a heated acryl-glass custom-made observation platform (Lobotec, Witten Germany) to maintain body temperature at 37.0ºC. In order to extend one ear of the mice for the experiment three loops (10/0, polyamide thread, monofilament, non-absorbable, Ethilon®, Ethicon GmbH & Co. KG, Norderstedt, Germany) were applied on the ear with microsurgical technique and fixed by three loops (5/0, polypropylene, Prolene®, Ethicon GmbH & Co. KG, Norderstedt, Germany) on the platform [8, 9]. To increase epi-illumination by intravital microscopy the ear had to be flattened using isotonic water applied between the ear and the platform. The fluorescent plasma marker fluorescein isothiocyanate (FITC)-labeled dextran (5 %; MW 150KDa; Sigma Chemicals Co., St. Louis, MO, USA) and Rhodamine 6G (0.2%; Sigma Chemicals Co., St. Louis, MO, USA) were injected in a bolus into a tail vein of the animal (canula ø 0.33 x 12.7 mm, B.Braun Melsungen AG, Melsungen, Germany; 75 μl per injection). Rhodamine 6G is a fluorescent dye for in vivo studies which accumulates in the mitochondria and cytomembranes of the endoplasmic reticulum of cells [10]. Leukocytes can be visualized by means of IFM (Fig. 1).

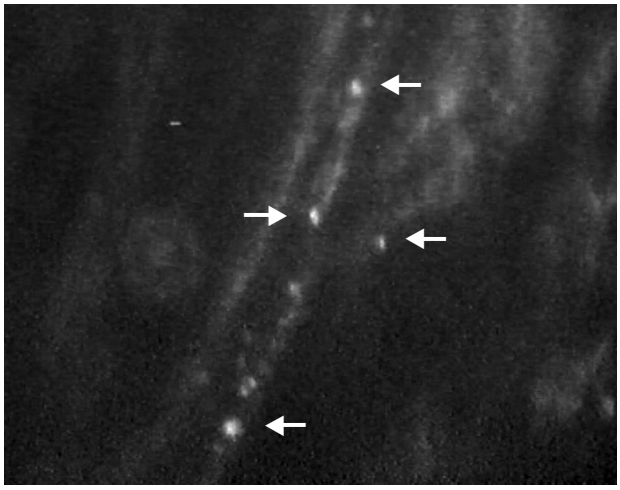

Fig. 1. Leukocytes stained with Rhodamine 6G are visualized by local light exposure at a wavelength of about 520 nm. Note the adherence of leukocytes to the endothelium (white arrows).

Measurements

Intravital Fluorescent Microscopy

For the IFM a custom-made intravital fluorescent microscope (Axiotech vario 100 HD, Carl Zeiss, Göttingen, Germany) with a 4-fold objective (Achroplan 4x, Zeiss) and 20-fold water immersion objective (Achroplan 20x, Zeiss) was used. The microcirculatory pictures taken with the microscope were transmitted by a charge-coupled device video camera (AVT-BC 71, AVT- Horn, Aalen, Germany) on a S-VHS video recorder (Panasonic AG7350, Germany) and recorded on S-VHS video tapes (S-VHS Pro SE-120, FUJI Magnetics GmbH, Kleve, Germany).

Study protocol

After the injection of the fluorescent dyes three to four ROIs within the skin microcirculation were defined using the 4-fold objective. For repeated observations at consecutive points of time the x-y-coordinates of the ROI were saved (AxioVision 3.0, Zeiss, Germany). In addition, drawings of the microcirculatory areas were made to ensure relocation of the ROI.

With the 20-fold objective microscopic pictures of arterioles, venules and capillaries of the ROI were recorded as baseline values. Afterwards the ear was randomly covered with a thin layer of one antiseptic or isotonic saline for an exposure time of 10 minutes. After the careful adsorption of the solution with aid of a synthetic surgical sponge (Fine Science Tools GmbH, Heidelberg, Germany) the identical microvessels in the same ROI were relocated and recorded again. In the same manner the microcirculatory pictures of the same areas were recorded for the third time - 60 minutes after the baseline-microscopy.

Microcirculatory Parameters

From video-taped intravital microscopy pictures the following standard microcirculatory parameters were measured: diameter of arterioles and venules (µm), midstream RBCV (mm/s), functional capillary density (FCD) (cm/cm^2), leakage of the plasmamarker FITC-dextran (intensity intravasal/intensity extravasal) and the venular leukocyte adherence as leukocyte endothelium interaction (LEI) (n/100 µm). FCD used as index for tissue perfusion is defined by the length of red blood cell perfused capillaries per observation field. The determination of FCD was performed by user-assisted redrawing of capillaries from the recorded pictures. To measure the endothelial integrity the leakage of the macromolecular FITC-dextran (150 kDa) out of vessels into the surrounding tissue was analyzed based on the ratio of the fluorescence intensities measured within vessels (li) and outside of vessels (le) [11].

Analysis of the Images

All microcirculatory evaluations were carried out offline from the video tapes using the computer assisted image analysis system CapImage® (Dr. Zeintl, Heidelberg, Germany) [12, 13].

Statistics

The data gained with CapImage® were tested with the aid of the commercially available computer program SigmaStat™ (Jandel Scientific, Erkrath, Germany). The data are presented as mean ± SEM. A non-parametric one way ANOVA test followed by Bonferroni was performed to calculate significance. A $p<0.05$ was considered as statistically significant.

Results

Intravital fluorescent microscopy allows the visualization of the skin microcirculation in vivo. From recorded arterioles, venules and capillaries (Fig. 2) standard microcirculatory parameters were analyzed at several points of time during the investigation.

Arteriolar diameter increased significantly in the Octenisept® ($p = 0.006$) and Lavasept® ($p = 0.03$) treated groups compared to baseline value. The alcoholic preparations cause a statistically not significant decrease in diameter after 10 min exposure time and reached baseline values after 60 min (Fig. 3). As expected, saline showed no effect on arteriolar diameter.

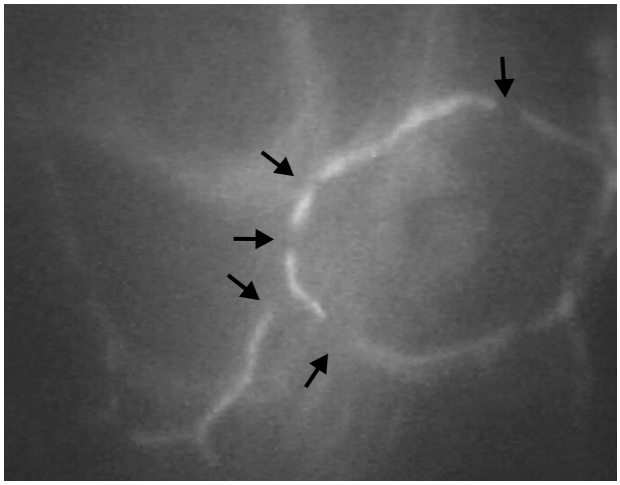

Fig. 2. An observation field of the capillary network of the skin is illustrated via intravital fluorescent microscopy. The FCD is defined by the length of red blood cell perfused capillaries per observation field. After contrast enhancement with FITC-dextran, vessels become visible. Note the five black spots (black arrows) which represent red blood cells.

Arteriolar Diameter [mm]:

solution	**0 min**	**10 min**	**60 min**
saline	24.8 ± 0.8	24.9 ± 0.9	24.5 ± 0.7
ethanol	24.0 ± 1.1	22.8 ± 1.2	23.9 ± 1.0
Softasept®	25.3 ± 1.5	24.2 ± 1.4	25.4 ± 0.7
Octenisept®	26.0 ± 0.8	30.6 ± 0.9	31.1 ± 1.0 *
Lavasept®	25.0 ± 1.2	27.8 ± 1.0	29.7 ± 1.1 *

* $p < 0.05$: 0 min vs 60 min

Fig. 3. Note the significant increase in arteriolar diameter in the Lavasept® and Octenisept® groups. The alcoholic solutions cause a slight decrease in diameter just after their application, but reach baseline value at the 60 min investigation time point.

The FCD decreased after the application of ethanol, Softasept® and Lavasept®. Compared to the saline group a significant difference was seen at 60 minutes ($p<0.05$). The decrease of FCD provoked by Softasept® was after 60 min significant ($p = 0.006$) versus baseline value. Octenisept® influenced the FCD like saline not significantly with a slight increase after 10 min exposure time and with a decrease to baseline values after 60 min (Fig. 4).

Beside the Octenisept® treated group all other groups showed a decrease of RBCV after 10 min exposure time. Significance can be seen in the decrease of RBCV in the ethanol-containing groups compared with the Octenisept® group after 10 min ($p<0.05$) and

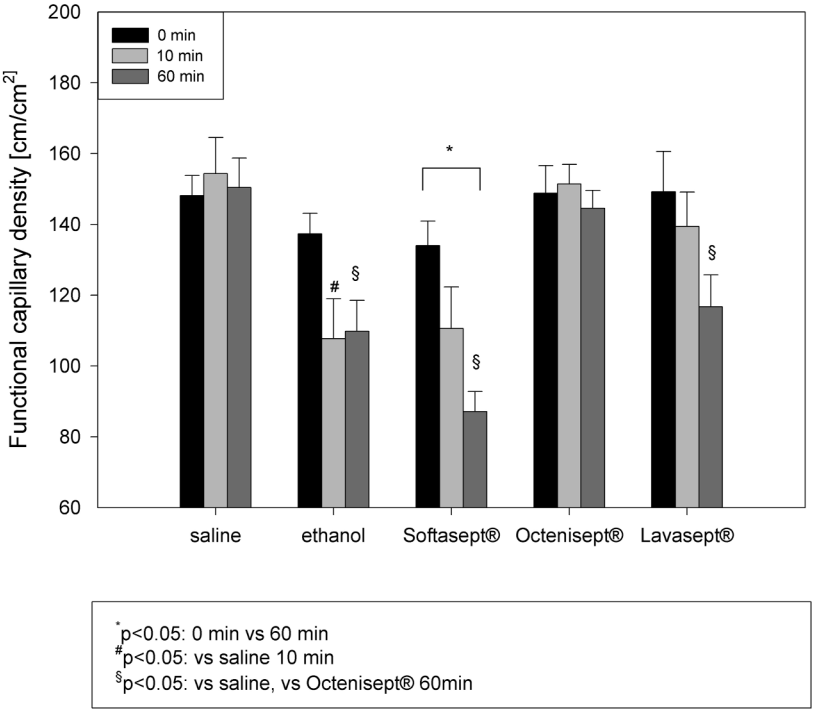

Fig. 4. A significant decrease in FCD from the baseline value to the value at the time point 60 min is observed in the Softasept® group ($p = 0.006$). As well, ethanol and Lavasept ® lead to a significant decrease in FCD at the time point 60 min compared to the control group and Octenisept® ($p<0.05$).

Red Blood Cell Velocity [mm/s]

solution	**0 min**	**10 min**	**60 min**
saline	0,44 ± 0,04	0,39 ± 0,06	0,40 ± 0,05
ethanol	0,53 ± 0,05 *	0,38 ± 0,05 #	0,28 ± 0,03 *§
Softasept®	0,55 ± 0,04 *	0,39 ± 0,05 #	0,32 ± 0,02 *§
Octenisept®	0,59 ± 0,04	0,61 ± 0,04	0,52 ± 0,03
Lavasept®	0,47 ± 0,05	0,41 ± 0,05	0,42 ± 0,04

* p < 0.05 : 0 min vs 60 min
# p < 0.05: vs Octenisept 10 min
§ p < 0.05: vs Octenisept 60 min

Fig. 5. 10 min and 60 min after application of ethanol and Softasept® the RBCV measured in venules is decreased significantly compared to baseline values and Octenisept®. The Saline and Lavasept® treated groups showed a trend in decreased RBCV.

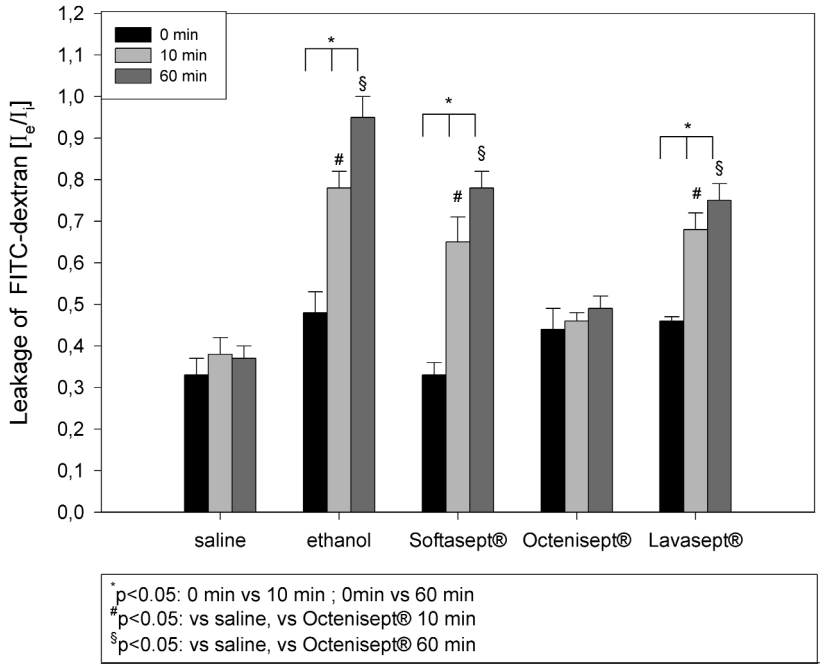

Fig. 6. Data on macromolecular leakage of the plasmarker FITC-dextran (150.000 Da) indicates the loss of endothelium integrity. The two alcohol-based preparations and Lavasept® cause a significant increase of the extravasation in FITC-dextran versus the baseline value after 10 min exposure time ($p<0.03$) and after 60 min ($p\leq0.001$) and versus the saline ($p<0.002$) and Octenisept® ($p<0.02$) treated groups.

Adherent Leukocytes [n/100mm]

solution	0 min	10 min	60 min
saline	2,6 ± 0,7	3,0 ± 0,7	2,9 ± 0,9
ethanol	3,6 ± 0,8 *	7,8 ± 0,6 *	6,0 ± 0,7
Softasept®	3,6 ± 0,5 *	8,1 ± 1,0 *	7,5 ± 0,6 *
Octenisept®	4,3 ± 0,8	4,3 ± 1,0	3,7 ± 0,5
Lavasept®	3,2 ± 0,8 *	5,6 ± 0,9	7,4 ± 0,7 *

* $p < 0.05$: 0 min vs 10 min ; 0 min vs 60 min

F*ig. 7*. The leukocyte adherence is defined as leukocytes, which stuck on endothelial cells along a 100µm section of the observed blood vessel. The Lavasept®, the ethanol and the Softasept® treated groups show a significant increase in the number of adherent leukocytes. The LEI shows hardly any difference in both the control and Octenisept® group as compared to baseline.

60 min ($p<0.01$) and their own baseline values ($p<0.01$). Octenisept® showed a slight not significant increase of RBCV at 10 min time point, but after 60 min the RBCV was also not significantly decreased compared to baseline value. In the control groupa significant alterations could not be seen neither at 10 min nor after 60 min (Fig. 5).

The leakage of FITC-dextran, as index for endothelium integrity, did not grow much after the treatement with saline. However it increased significantly after application of ethanol ($p<0.01$), Softasept® ($p<0.03$) and Lavasept® ($p<0.03$) after 10 min and continuing after 60 min compared to the baseline values in these groups. The significant increase was also compared to Octenisept® and saline data.. Octenisept® provoked slight leakage of the plasmamarker out of the vessels as well (Fig. 6), but here leakage was not significant.

The number of adherent leukocytes to the endothelium increased significantly compared to baseline value after the use of the two alcoholic solutions, ethanol ($p = 0.004$) and Softasept® ($p = 0.003$), measured at the time point of 10 min. Groups treated with ethanol and Softasept® showed a decrease towards baseline value after 60 min in contrast to the Lavasept® group in which LEI continued to increase significantly ($p=0.017$) compared to baseline value. Octenisept® affects no significant increase in the number of adherent leukocytes just after its use and shows even a decrease at the time point 60 minutes, but not statistically significant. The control group treated with saline showed also in no time point of investigation a significant change in the number of adherent leukocytes (Fig. 7).

Discussion

The direct effect of topical antiseptics on the microcirculation of the intact skin was analyzed in vivo. Intravital fluorescent microscopy was used to visualize skin microvessels and to assess standard microcirculatory parameters.

Many previous studies deal with microcirculation because of its contribute to the healing of flaps [14, 15], wounds [16] and burns [17]. In operative and conservative procedures, such as the treatment of pathological skin situations, topical antimicrobial agents are in permanent use. The question came up about the extent to which these antiseptic solutions influence microvascular perfusion – the only nutritive access to tissue. To date, innumerable investigations have been carried out analyzing the efficacy of disinfectants against microorganisms and their tissue compatibility in terms of cell growth inhibition and cell toxicity. However, investigations concerning the interaction of antiseptics and microcirculation are rare. The effect of wound ointments on muscle microcirculation was investigated using the model of the rat cremaster muscle [18]. The influence of antiseptics on the healing wound was previously determined by means of the rabbit ear chamber [19].

In 1980, Errikson et al. established the model of the hairless mouse ear as a method for in vivo microscopy of the skin microcirculation [3]. Since then, many microcirculatory studies have been carried out using this model to investigate skin microcirculation [15, 20], wound healing [5, 8] and burns [17]. It is the most suitable tool for in vivo investigations of the skin microcirculation particularly for measuring repeatedly short-term changes of microcirculatory parameters. The sensitivity of this method allowed us to ascertain the short-term effects of topically applied antiseptics on the cutaneous microcirculation by continuous, direct observations and quantitative analysis.

The antiseptic for skin and mucous membrane, Octenisept®, consists mainly of 0,1% octenidine dihydrochloride which is an bispyridinamine supplemented by 2% phenoxyethanol. Octenisept® acts as cation active substance on the cell membrane and leads with it to the destruction of the cell function. Our results show that Octenisept® causes the slightest impact on microcirculatory parameters compared to the other tested antiseptics. It causes a vasodilatation in both arterioles and venules, which increased tissue perfusion. An increase in FCD and RBCV corresponding to results concerning the chemically related chlorhexidine [21] underlines the positive effect of this antiseptic on skin microcirculation. Octenisept® affects a minimal leakage of FITC-dextran and almost no increase in the number of adherent leukocytes. This behavior resembles the results of the control group of saline treated animals. Consequently, this antimicrobial agent does not stimulate leukocytes more than saline.

The concentrate Lavasept® is a biguanide (polihexanide) as the disinfection part with addition of polyethylenglycol for reduction of the surface energy. Many investigations concerning the tissue compatibility of Lavasept® have shown a non-toxicity and acceptable tissue tolerance [22]. Surprisingly, we have observed a negative influence of this antiseptic on skin microcirculation (e.g. reduction in FCD and RBCV, respectively).

Softasept® is an alcoholic preparation for skin antiseptic. Like 70% ethanol it is mainly used as a surface disinfectant resulting from wound cauterization. In general, it is assumed that alcoholic solutions act as a vasodilator [23] and thus positively influence the microvascular system [24]. Our findings show the oppo-

site. A vasoconstriction, both in arterioles and in venules, was similarly reported by Altura et al. and Mayhan et al. [25, 26]. The different effect of ethanol, compared to other studies mentioned above, may be due to the alteration of its concentration. Even the FCD and RBCV decrease enormously after the application of both Softasept® and 70% ethanol. With the lower RBCV wall shear rate is reduced, consequently, in the investigated vessels. A diminution in wall shear rate may be the reason for increased leukocyte adhesion. In the same manner, the endothelial cell damage demonstrated by the enormous leakage of FITC-dextran after the use of the alcoholic solutions may determine the increase in the number of adherent leukocytes, as well [27].

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