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Effect of UV Irradiation and Rebamipide on the Blood Flow and Viability of Rabbit Skin Flap

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This study was undertaken to examine the effects of ultraviolet light (UVL) and rebamipide on the cutaneous blood flow and tissue survival on rabbit skin flap. In a random bipedicle flap, Laser Doppler Flowmetry (LDF) was employed to measure the blood flow of flap (BFF). Wound Margin Strength (WMS) measured by force transducer and Light microscophy were used for evaluation of tissue viability. Single exposure to UVL increased the BFF gradually for more than 15 hours, and decreased the vasoconstrictor effect of intravenous phenylephrine. The UVL-induced increase in BFF regressed after 18 hours of irradiation, and this regression was tended to be enhanced by intradermal injection of L-NAME, a nitric oxide synthase (NOS) inhibitor, but the regression was significantly reversed by acetylcholine, an endothelial constitutive NOS (cNOS) activator and L-arginine, an NO precusor. Rebamipide, a novel antiulcer agent known to scavenge the hydroxyl radical, abruptly reversed the spontaneous regression of the UVL-induced increase in BFF by the same manner as L-arginine. In ischemic skin flap, rebamipide increased the BFF abruptly by the same manner as sodium nitroprusside (SNP), an NO doner, while N-acetylcystein (NAC), a free radical scavenger, gradually increase the BFF. The rebamipide-induced increase in BFF was sustained at the level of the SNP-induced increase in BFF during the late period of experiment. Rebamipide increased the WMS of skin flaps and prevented the tissue necrosis in comparison with L-NAME. Based on these results, it is concluded that in rabbit skin, UVL irradiation increases the BFF by NO release, and rebamipide exerts a protective effect on the viability of ischemic skin flaps by either or both the increase in BFF by NO release and free radical scavenger effect.

Key Words: Ultraviolet light, Rrbamipide, Laser doppler flowmetry, Nitric oxide, Free radical

INTRODUCTION

Cutaneous Blood flow is an important factor for the healing of skin injury and for skin grafts.

Recently, nitric oxide (NO) has been known widely as a regulating factor of cutaneous blood flow that dilates cutaneous vessels (Khan et al, 1993; Pons et al, 1993; Kajekar et al, 1995; Goldsmith et al, 1996). Pang et al (1993) observed that an inhibition of NO synthesis enhanced the vasoconstrictive effect of serotonin and discussed the efficacy of combined serotonergic antagonist and topical nitrovasodilator (that releases NO) treatment for compromised skin circulation in pig skin flap.

A number of investigators reported that ultraviolet light caused erythema and increase in blood flow on skins (Ramsay & Challoner, 1976; Young et al, 1985; Nose & Tsurumi, 1993; Zachariae et al, 1994), and some of them suggested that the vasodilations were related to NO syntheses (Warren, 1994; Goldsmith et al, 1996). Based on the thesis that long wavelength ultraviolet light (UVL) irradiation produces NO from NO_2^- in the solution (Matsunaga and Furchgott,

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1991), Photo-Induced Adequate Nitric Oxide (PIA-NO), a methodology of experimental NO- production has been designated by Chung and Chang (1994).

It is well known that when a tissue is reoxygenated after a considerable period of ischemic condition, oxygen free radical (superoxide, O_2^-)is produced to affect the tissue viability hazardous (McCord, 1985; Price & Pearl, 1994). NO is also a free radical that is highly reactive, and the combination of NO with superoxide forms other radicals including peroxynitrate or nitrogen dioxide with the capacity to injure target cells (Billar, 1995).

Rebamipide(2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl] propionic acid, CAS 11911-87-6), a novel anti-ulcer agent, has been reported that it has an in vitro antioxidant properties as hydroxyl radical scavenger and inhibitor of superoxide production in neutrophils (Yoshikawa et al, 1993), and that it inhibits neutrophils to save both superoxide dismutase and NO synthase activities in gastric mucosa (Kim & Hong, 1995).

In wound healing procedures of either simple skin injury or skin flaps for a purpose of plastic surgery, tissues should experience ischemia and/or reoxygenation more or less extents. In such a situation, NO might contribute to reduce the tissue damage by increasing blood flow, or on the other view point, it might aggravate the tissue damage by building toxic nitrogen-oxygen compound.

In this study multiband UVL was employed as a stimulator of NO production. The effects of UVL and rebamipide on the cutaneous blood flow and tissue survival on skin flap were observed.

METHODS

Thirty-five New zealand white rabbits (2.5～3.5 kg BW) anesthetized with intraperitoneal urethane (20% aqueous soln, 1 g/kg) were immobilized on rabbit surgical board. Intravenous catheter into the femoral vein and heating pad for keeping body temperature were prepared.

Random bipedicle skin flaps measuring 1.5×6 cm were created in the chest/abdomen area, along the territories of the thoracodorsal artery. The flaps were undermined and separated from underlying fat tissue by insertion of silicon films so that blood supplies could be done via both ends of the bridge-like skin flap. Blood flow through the flap (BFF) was measured by a surface probe (Type P, Transonic Systems Inc.) of the laser doppler flowmeter (BLF 21D, Transonic Systems Inc. USA), and data were expressed as tissue perfusion unit (TPU) recorded by data aquisition system (Windaq/200, DATAQ Instrument, Inc.) on personal computer.

The survival of skin tissues next to the incised wounds under various conditions after 5 days of incision-suture were observed: Skin strips measuring 3×10 mm including the incision-suture line by perpendicular direction were obtained, and the tensile strengths for separation of the wounds (wound margin strength, WMS) were measured by force displacement transducers (FT-03, Grass) and polygraph (Model 79E, Grass). The histological changes in wound margin were observed by light microscopy with hematoxylin-eosin stain.

Effect of multiband ultraviolet light (UVL) irradiation on the skin blood flow

Alterations in BFF by delayed (17.5 hours) effect of UVL (254～366 nm, 20 mW/cm^2, for 10 minutes) were measured. Phenylephrine (PE, 0.02 mg/kg IV, in 0.5 ml saline) was injected into ear vein 17.5 hours after irradiation of UVL. The reduction of BFF by PE without UVL was control.

Effects of UVL and NO-related drugs on survival of skin flap

UVL irradiation (254～366 nm, 20 mW/cm^2, for 10 minutes) was followed by repeated measurement of BFF in 1, 3, 5, 7 and 15 hours. In 17.5 hours after UVL, acetylcholine (ACh), L-arginine, N^G-nitro-L-arginine methyl ester (L-NAME) or rebamipide was injected intracutaneously (0.1 ml, single) for daily observation of changes in BFF for 5 days.

Hypoxia-reoxygenation and the viability of skin flap

The distal pedicular end of the flap was incised to interrupt blood flow. In two hours after incision, sodium nitroprusside (SNP, 10^{-6} M), L-NAME (10^{-6} M), rebamipide (10^{-6} and 10^{-4} M) or N-acetylcystein (NAC, 10^{-6} M) by volume of 0.1 ml was injected intracutaneously for subsequent measurements of BFF, and the skin was sutured.

In this experiment the skin tissues at the injection site of drug were biopsyed on the 5th day of operation and were examined by light microscopy with Hematoxylin-Eosin stain.

L-NAME, SNP, N-acetylcystein, ACh, and PE were ordered from Sigma biochemical, Inc. L-arginine was ordered from Fluka AG. Rebamipide was donated by Otsuka Korea, which was diluted in 0.5% carboxymethyl-cellulose and water.

Data of changes in BFF and WMS were evaluated by Student's t-test or ANOVA, and the p value less than 0.05 was considered as significant.

RESULTS

Effect of UVL on BFF

BFF of control group was 50.7±0.33 TPU, which was increased by UVL irradiations as much as 0.72± 0.02 TPU ($p<0.05$) in an hour, 1.25±0.02 TPU ($p<0.05$) in 3 hours, 1.5±0.03 TPU ($p<0.05$) in 5 hours, and 2.22±0.07 TPU ($p<0.05$) in 15 hours (Fig. 1).

PE rapidly reduced the BFF immediately after injection. The amount of reduction in BFF by PE

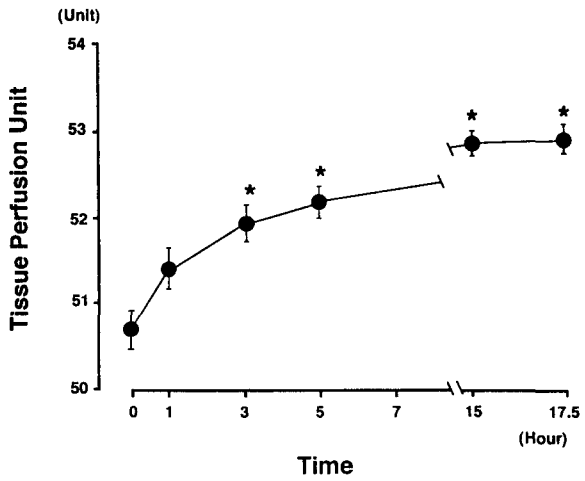

Fig. 1. Time-dependent effect of ultraviolet irradiation on rabbit skin flap blood flow expressed by tissue perfusion unit of laser doppler measuring. Values are expressed as mean±SE of 15 experiments. Time; hours after a single irradiation of ultraviolet light (254~366 nm, 20 mW/cm^2, for 10 minutes).*$p<0.05$: significantly different from 0 time.

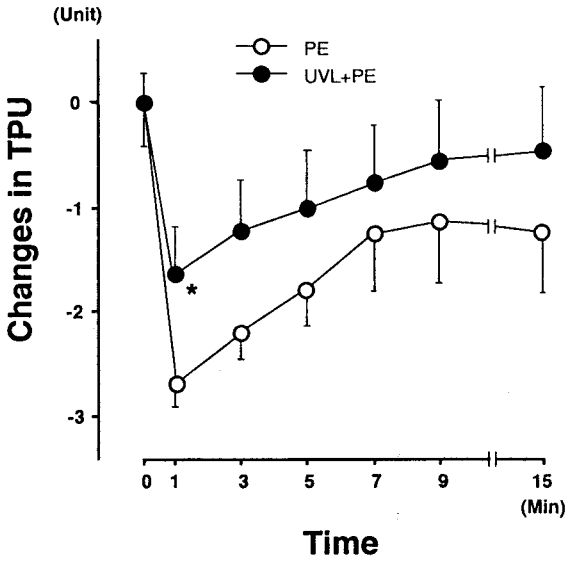

Fig. 2. Delayed effect of ultraviolet irradiation on phenylephrine-induced blood flow change measured by laser doppler. UVL; ultraviolet light (254~366 nm, 20 mW/cm^2, for 10 minutes). PE; Phenylephrine (0.02 mg/kg, IV) injected 17.5 hours after exposure to UVL. Values are expressed as mean±SE of 7 experiments. *$p<0.05$; significantly different from PE only group.

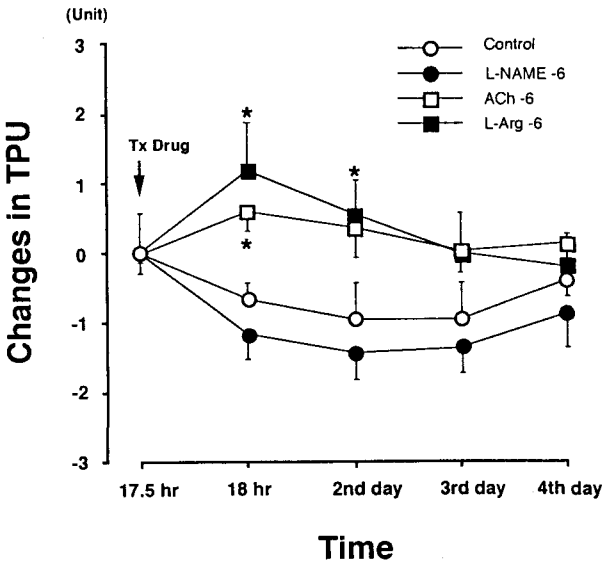

Fig. 3. Effect of nitric oxide donor and nitric oxide synthase inhibitor on cutaneous blood flow of rabbit skin flap, 17.5 hours after ultraviolet irradiation. TPU Means tissue perfusion unit of laser doppler measuring. Values are expressed as mean±SE of 5 experiments. L-Arg -6; L-arginine 10^{-6} M, L-NAME -6; N^G-nitro-L-arginine methyl ester 10^{-6} M, ACh-6; acetylcholine 10^{-6} M. *$p<0.05$; significantly different from control.

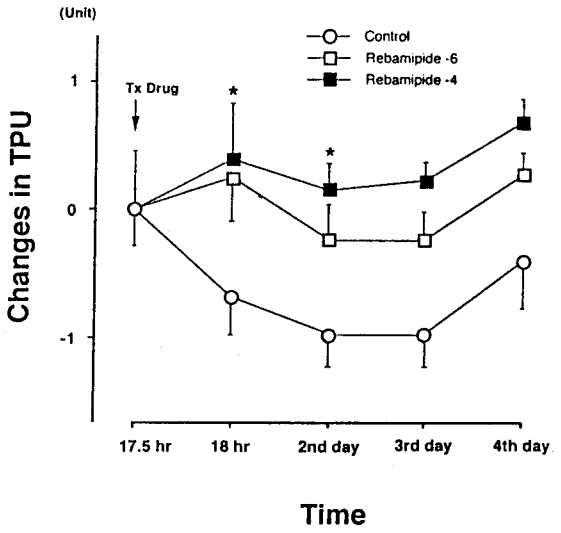

Fig. 4. Effect of rebamipide on rabbit skin flap blood flow 17.5 hour after ultraviolet irradiation. TPU Means tissue perfusion unit of laser doppler measuring. -6 and -4 following rebamipide mean the doses of 10^{-6} and 10^{-4} molar. Values are expressed as mean±SE of 5 experiments. *$p<0.05$; significantly different from control.

after UVL was 1.66±0.03 TPU in 1 minute, which was significantly less than the reduction of BFF by PE only (2.74±0.10 TPU, $p<0.05$) (Fig. 2).

Between 15 hours and 17.5 hours after UVL irradiation, BFF in control group reached the maximum level, and afterward, BFF began to reduce. L-NAME injected 17.5 hours after UVL irradiation tended to enhance this reduction and allowed it to reach −1.28 ±0.02 TPU in 30 minutes, which was not significantly different from the reduction in control group that was −0.68±0.14 TPU. On the other hand, ACh and L-arginine increased the BFF by 0.54±0.28 TPU ($p<0.05$) and 1.16±0.2 TPU ($p<0.05$), respectively in 30 minutes (Fig. 3).

Rebamipide injected 17.5 hours after UVL irradiation by the concentrations of 10^{-6} M and 10^{-4} M increased BFF as much as 0.40±0.03 TPU and 0.26±0.21 TPU ($p<0.05$), respectively in 30 minutes (Fig. 4).

Effects of drugs on BFF

Rebamipide 10^{-6} M and 10^{-4} M at the midtime of incision-suture of the pedicular end of the flap

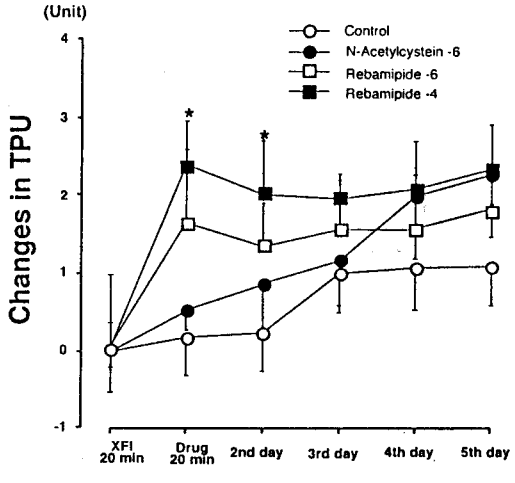

Fig. 5. Effect of antioxidants on cutaneous blood flow of rabbit after 4 hours ischemia. TPU Means tissue perfusion unit of laser doppler measuring. -6 and -4 following rebamipide mean the concentrations of 10^{-6} and 10^{-4} molar (0.1 ml) injected intracutaneously. XFl; interruption of blood flow. Drug; intradermal injection of drugs. N-Acetylcystein-6; N-acetylcystein 10^{-6} M injected (0.1 ml) intracutaneously. (same for rebamipide)
Values are expressed as mean±E of 5 experiments. *$p<$ 0.05: significantly different from control.

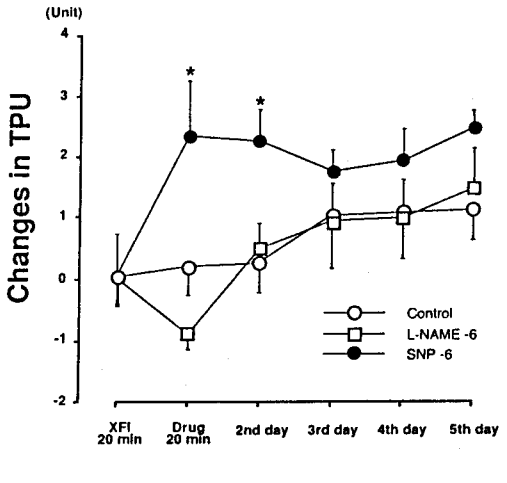

Fig. 6. Effect of nitric oxide donor and nitric oxide synthase inhibitor on rabbit skin flap blood flow after 4 hours ischemia. TPU Means tissue perfusion unit of laser doppler measuring. XFl 20 min; 20 minutes after interruption of blood flow. Drug 20 min; 20 minutes after intracutaneous injections of drugs. Data are expressed as Mean±SE of 7 experiments. SNP -6; sodium nitroprusside 10^{-6} M, L-NAME -6; N^{G}-nitro-L-arginine methyl ester 10^{-6} M. *$p<0.05$: significantly different from control.

increased the BFF as much as 1.58±0.04 TPU ($p<0.05$) and 2.36±0.18 TPU ($p<0.05$) in 20 minutes of injection, and such increase were sustained to the 5th days. Acetylcholine 10^{-6} M gradually increased the BFF throughout the observation period, and reached to 2.22±0.14 TPU ($p<0.05$) (Fig. 5).

SNP 10^{-6} M after 2 hours of interruption (incision-suture) of the pedicular end of the flap increased the BFF as much as 2.28±0.18 TPU ($p<0.05$) in 20 minutes of injection, and this increase was sustained to the 5th days. L-NAME 10^{-6} M transiently decreased the BFF as much as 0.92±0.26 TPU than recovered to the level of control in 1 day (Fig. 6).

Changes in Wound Margin Strength (WMS)

The control value of WMS 5 days after incision-UVL-suture was 2.61±0.16 g. The WMS of rebamipide 10^{-6} M was 2.88±0.34 g, rebamipide 10^{-4} M group was 2.93±0.31 g, L-arginine 10^{-6} M group was 2.78±0.30 g, and L-NAME 10^{-6} M group was 2.40±0.22 g (Fig. 7).

The control value of WMS 5 days after interruption (incision-suture) of the pedicular end of the flap was 2.70±0.21 g. The WMS of rebamipide 10^{-6} M group was 2.88±0.19 g, rebamipide 10^{-4} M group was 3.01±0.29 g ($p<0.05$), N-acetylcystein 10^{-6} M group was 2.86±0.18 g, SNP 10^{-6} M group was 2.82±0.33 g, and L-NAME 10^{-6} M group was 2.40±0.22 g (Fig. 8).

Histological changes

On the 5th day after interruption of blood flow of the skin flap, the light microscopy showed the histological features as shown on Figure 9. The skin preparations treated with L-NAME (10^{-6} M, 0.1 ml IC) showed a wide range of destruction of hair

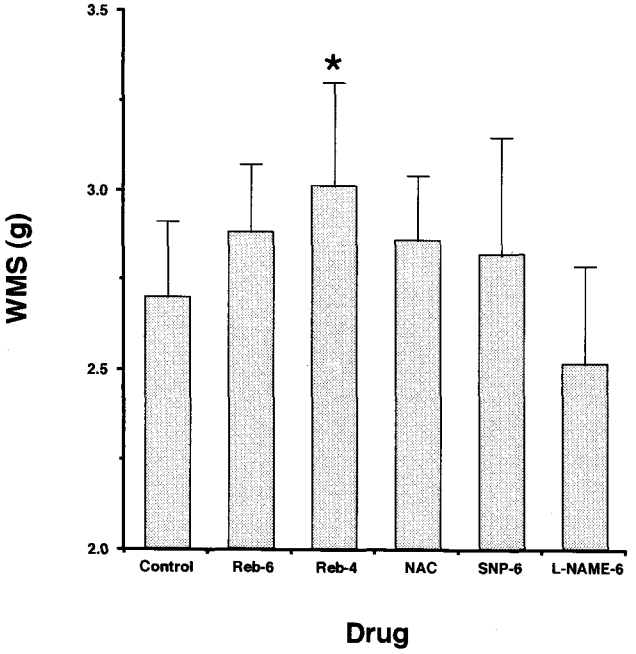

Fig. 7. Effect of rebamipide, acetylcholine, L-arginine and N^G-nitro-L-arginine methyl ester on the wound margin strength of the rabbit skin flap 5 days after ultraviolet irradiation.WMS; Wound margin strength measured as the tensile strength of specimen under force displacement transducer. Values are expressed as mean± SE (n=5). Control; ultraviolet light only. Reb-6 and Reb-4; rebamipide 10^{-6} M and 10^{-4} M. ACh; acetylcholine. L-Arg; L-arginine. L-NAME; N^G-nitro-L-arginine methyl ester.

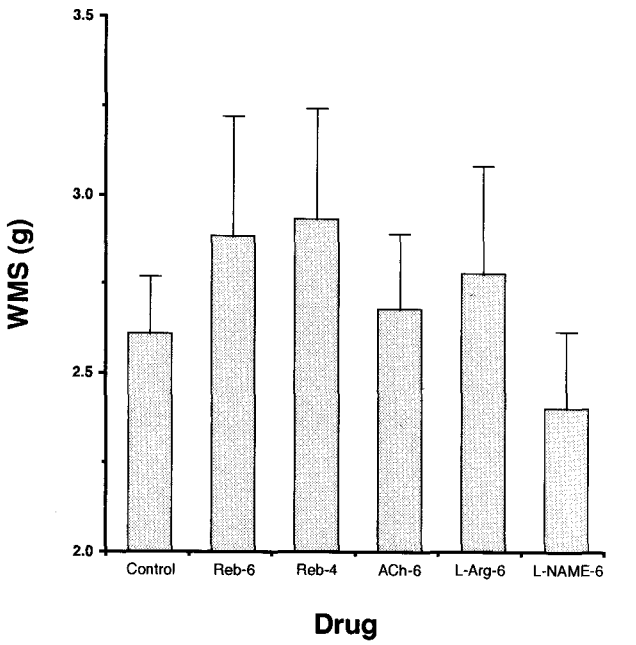

Fig. 8. Effect of rebamipide, N-acetylcystein, sodium nitroprusside, and N^G-nitro-L-Arginine on the wound margin strength of the rabbit skin flap 5 days after 4 hours ischemia. Values are expressed as mean±SE (n=5) of tensile strength of the margin of specimen under force displacement transducer. Reb-6 and Reb-4; rebamipide 10^{-6} M and 10^{-4} M. SNP; sodium nitroprusside. L-NAME; N^G-nitro-L-arginine methyl ester. *$p<0.05$; significantly different from control.

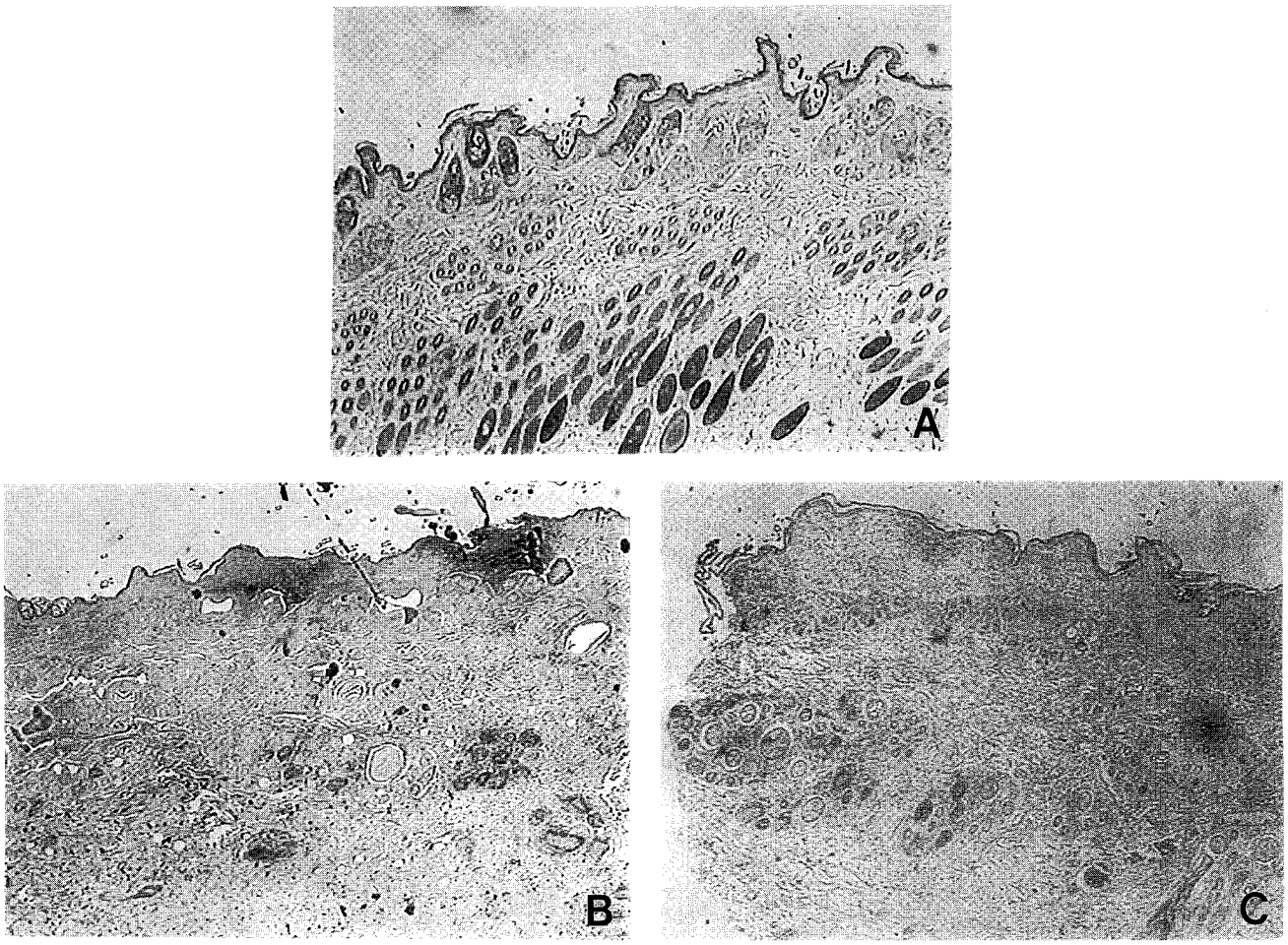

Fig. 9. Light micrographs (Hematoxylin-Eosin Stain, 40X) of rabbit skin flap on the 5th day after 4 hours interruption of blood flow. A. Healthy skin. B. L-NAME treated skin (10^{-6} M, 0.2 ml IC) showing severe necrosis. C. Skin tissue treated with rebamipide (10^{-4} M, 0.2 ml IC). Epithelium, some of hair follicle, skin adnexa are preserved with intact epithelium, and mild coagulation necrosis is shown.

follicles and skin adnexa and moderate coagulation necrosis in broad area. The skin preparations treated with rebamipide (10^{-4} M, 0.1 ml IC) showed only partial destructions of hair follicles and skin adnexa and slight necrotic changes. Epithelium of L-NAME group was destroyed but that of rebamipide group was intact.

DISCUSSION

On skin injury or flap formation, it is inevitable that the tissues experience hypoxia more or less. As a parameter to predict the tissue viability and to evaluate the usefulness of UVL and rebamipide, the Laser Doppler Flowmetry (LDF) was employed in this study. LDF has been proposed as a continuous, noninvasive method to assess superficial tissue blood flow (Watkins & Holloway, 1978; Nilsson et al, 1980; Hallock & Altobelli, 1992). Actually, LDF measurements were able to follow dynamic blood flow variations in cutaneous blood circulation, and able to detect flow impairment earlier and well within the critical ischemic period (Lanthier et al, 1990).

In rat skin, UVL Type B caused a delayed onset of increase in skin blood flow that was only visible as a slight reddening of the skin between 15~24 hours after exposure, and in this phenomenon, NO was suggested to be involved (Warren et al, 1993). Pang et al (1993) described that there were several factors

that influence the vasospasm and ischemic necrosis of skin flaps, and suggested NO would contribute to prevent such a phenomena. In human skin, endogenous NO was generated by intradermal injection of ACh and the exposure to UVL type B by activations of constitutive and inducible nitric oxide synthase (NOS), respectively (Warren, 1994). The concept that UVL generates NO was expressed as PIANO in vitro (Chung & Chang, 1994).

In this study, we observed that single exposure to UV increased the BFF gradually, reaching a significant level in 5 hours and going on increasing for more than 15 hours. Intravenous PE, 17.5 hours after UVL exposure, decreased the BFF to the significantly less extent than PE without UVL. These findings coincide the delayed increase in blood flow in rat skin (Warren et al, 1993) and attenuation of PE-induced vasoconstriction by NO (Rocha et al, 1995; Griffiths et al, 1995), respectively. The increased BFF regressed after 18 hours of irradiation, and this regression was tended to be enhanced by intradermal injection of L-NAME, a typical NOS inhibitor. On the other hand, the regression was tended to be reversed by ACh, an endothelial constitutive NOS (cNOS) activator. L-arginine, an NO precusor which can be the substrates of both endothelial cNOS and of inducible NOS (iNOS) in the extraendothelial cells. These results verify that the UVL irradiation in this study produced NO in the skin tissue and dilated small vessels to increase BFF.

After a hypoxic period by interruption of circulation, there comes a period of revascularization or reoxygenation by some other means of vasodilation as an inflammation or so on. During the reoxygenation, superoxide radicals (O_2^-) are produced from polymorphonuclear leukocytes and macrophages (Gabig et al, 1978). It is an old concept that the toxic effect of O_2^- is mediated by the hydroxyl radical (McCord & Fridovich, 1978; McCord, 1985). Price and Pearl (1994) reported that pathological vasospasm and free radical production contribute to skin flap necrosis, and single agent therapy with either transdermal nitroglycerin that induced vasodilation or intravenous allopurinol that inhibited xanthine oxidase significantly enhanced the flap survival. The above descriptions suggest that a vasodilation which improve the BFF or removal of free radicals may increase the survival rate of skin flap.

In this study rebamipide prevented the spontaneous regression of the UVL-induced increase in BFF. There are many reports that rebamipide scavenges hydroxyl radical thus prevent tissue damage in variable situations (Ogino et al, 1992; Sakurai & Yamasaki, 1994; Kim & Hong, 1995), but there is only one report that rebamipide exerts an inhibition of mobilization and activation of neutrophils in association with an attenuation of the decreases in both superoxide dismutase and nitric oxide synthase activities, thereby preventing the gastric microcirculation from deterioration (Kim & Hong, 1995). Apparently the result of this study coincides Kim and Hongs report as far as the increase in BFF by UVL-irradiation is due to NO release.

To compare the rebamipide-induced increase in BFF with that of NAC, an oxygen free radical scavenger, the blood supply into the skin flap was interrupted by incision of a pedicular end of the skin flap. NAC injected in this ischemic state gradually increased the BFF after the incision site were sutured, and the increase in BFF continued throughout the observation period for 5 days. Contradistinctively, the increase in BFF by rebamipide was abrupt, and the BFF reached to the peak level just in 20 minutes after injection and the increase was sustained for 5 days. Rebamipide looks having dual vasodilating effects, immediate and gradual. In the same circumstances, SNP which liberates NO in smooth muscle cells increased BFF by the same manner as rebamipide, and L-NAME, an NOS inhibitor totally reversed the effect of SNP. Supposedly, rebamipide has a mechanism evoking an NO activity that needs further investigations focusing the possibility of rebamipide action being inhibited by some blockers of NO releasing pathways. The gradual vasodilation by rebamipide may be oxygen free radical scavenging.

The viability of skin flap was extrapolated by WMS and observations by light microscopy. All the values of WMS in every experimental group went parallel with the data from the experiments of BFF. ACh, L-arginine and rebamipide tended to increase the effect of UVL, and L-NAME affected negatively. The data from the ischemic skin flaps also showed the same trends. NAC and SNP tended to increase the WMS, rebamipide with higher dose significantly improved the viability of the skin flaps, and L-NAME tended to affect negatively. The light microscopic observation showed that rebamipide preserved the hair follicles, skin adnexa and epithelium more than

L-NAME.

Based on these results, it is concluded that in rabbit skin, UVL irradiation increases the BFF by NO release, and rebamipide exerts a protective effect on the viability of ischemic skin flaps by either or both the increase in BFF by NO release and free radical scavenging.

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