Research Report

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Methods

Excisional (full thickness) and scarification (split thickness) wound models in animals, electron spin resonance spectrometry (with spin trap), chemiluminescence, spectrophotometry, enzymatic assays, fluorescence probes, etc.

Animals. Healthy male Wistar rats, weighing 350-400 g were obtained from the animal house of Russian State Medical University. The rats were housed in polypropylene cages on normal food and water *ad libitum*. The rats were anaesthetized prior to infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using ketamine anaesthesia (10 mg/kg). The study was approved by the Ethics Committee of Russian State Medical University.

Wound healing experiments. The rats were inflicted with full thickness excision wounds as described previously [Morton, Nayak]. The rats were anaesthesized prior to wounding, with 1ml of intravenous ketamine hydrochloride (10mg/kg). The dorsal fur was shaved and a full thickness of the excision wound of 1.5 cm in width (square area = 2.25 cm^2) and 0.2 cm depth was done. Each wound was treated daily with 100 µL of either tested sample (0.2 mg/ml) or 0,9% NaCl and Tegaderm$^©$ dressing applied to maintain a moist wound environment and eliminate scab formation. The rats were briefly anesthetized with ether mask daily for 8 days, the dressings were removed, the wounds cleansed with sterile NaCl solution, the topical agents applied and the wounds re-dressed. The animals were divided into two groups of 10 each. The group 1 animals were treated with NaCl (control). Animals of groups 2 and 4 were treated two times a day daily with HIVAMAT for 15 min (the details of the protocol are in the Fig. 1) The measurements of the wound areas were performed on the 4th, and 8th day using transparent paper and a permanent marker. The recorded wound areas were measured by planimetry using special computer program.

The scarification model was performed using device for micro abrasion, which allowed to removing the epidermal layer of the skin without any damage to the dermal layer. All the other experimental procedures and animal treatment were as described above.

Blood samples. Venous blood was drawn by sterile syringe (4 ml) and collected into silicon tubes filled with 25 units/ml of heparin (Richter, Hungary). The blood samples were stored at the temperature +4oC for 4-6 hours before processing and analyses. 100 µl of freshly drawn blood were examined microscopically and the total number of white blood cells (WBC) was counted. The data were expressed as WBC /µl The freshly drawn blood was centrifuged at 400 x g for 10 min. Plasma supernatant was accurately collected, divided into 100ml aliquots, and stored at -20°C before biochemical analyses. For immune assays, the plasma samples were stored at -80°C.

 The pellet containing erythrocytes and WBC was further processed for cell isolation and purification. Granulocytes were isolated using routine method of the double gradient centriguation. In brief, centrifuge tubes were filled up with 2ml of Histopaque gradient (= 1,119 g/cm3) and then, with 2 ml of Histopaque (= 1,077 g/cm3). 2 ml of freshly drawn blood was layered on the top. The tubes were placed into refrigerated centrifuge and rotated at 800 x g for 40 min. The temperature was maintained at +4°C during the centrifugation period. Granulocytes were collected from the interface between two gradients, transferred into another tube with 10ml of Hank's balanced salt solution (HBSS) and centrifuged at 400 x g for 10 min. The washing procedure was repeated three times. Finally, the washed granulocytes were re-suspended into 1 ml of fresh HBSS supplemented with 10% of heat-inactivated calf serum and counted microscopically. The granulocyte suspension was stored at the thawing ice and used for analyses within 4 hours.

Red blood cells. Red blood cells were collected from the sediment and washed three times with cold HBSS. For enzymatic analyses, red blood cells were disrupted by adding of big volume of distilled water. The water lysates of RBC were used for the measurements of glutathione peroxidase and glutathione-S-transferase activities. The RBC lysates were additionally treated with the ethanol:chlorophorm mixture (5:3 v/v), vigorously mixed for three minutes, and centrifuged at 900 x g for 10 min [Grossman]. The final lysates were used for the determination of superoxide dismutase and catalase activities.

Skin, scab, and granulation tissue sampling. The skin samples were taken in the close vicinity (10-20 mm) to the wounded area. The samples were immediately frozen and stored at -20oC not longer than one week before analyses. The scab and granulation tissue samples were taken during surgical necrectomy operations at the 4th and 8th days after wounding. The samples were thoroughly homogenized using the Potter homogenizer and 0.1 M potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 900 x g for 30 min, supernatant was collected

and further used for biochemical analyses and the protein content determination [Lowry]. The supernatants were stored at -20oC, then, analyzed without repeated cycles of freezing-thawing

Edema in the skin was assessed by the measurement of the dry/wet tissue ratio. The fresh skin samples were weighed and then, dried at $+150^{o}C$ until the dry weight did not change.

Antioxidant activity. The inhibition of lipid peroxidation (antioxidant activity) was measured spectrophotometrically, using a Shimadzu 1770 UV spectrophotometer, by the thiobarbituric acid (TBA) assay in a system containing egg yolk lipoproteins as a substrate and $FeSO_4$ as a trigger of lipid peroxidation. For the analysis, 0.1mL of freshly prepared egg yolk lipoproteins were mixed with 0.1 mL of the TP extract solutions at different concentrations, and 0.7 mL of potassium phosphate buffer (0.1 M, pH 7.2). In the control probes, 0.1 mL of saline solution (0.155 M NaCl) was added. Lipid peroxidation was induced by 0.1mL of 0.5 mM $FeSO_4$ x $7H_2O$. The probes were incubated for 30 min at room temperature. Then, to stop lipid peroxidation 0.1 mL of 0.01 M ionol was added; the samples were shaken and centrifuged at 400xg for 10 min. Supernatant (0.7 mL) was transferred to another spectrophotometric cell and 0.6 ml of 0.5% TBA was added. The mixture was heated at 100oC for 30 min, then, cooled and the absorbance at 532 nm was measured. The results were expressed as IC_{50}, the concentration of 50% inhibition of MDA formation in the presence of test compounds [Uhiyama].

Oxygen radicals produced in the whole blood. Inhibition of oxygen free radical release from white blood cells (WBC) was assessed by LCL. In brief, 10 µL freshly drawn human blood were mixed with 0.980 mL of Hanks' balanced salt solution (HBSS) containing 5 x 10_{-5} M luminol. To isolate total pool of white blood cells (WBC), 1 mL of freshly drawn blood was laid on the Hipaque gradient (p = 1,119). After centrifugation at 250 x g for 60 min at room temperature, the pellet containing WBC was washed twice in a big volume of cold (HBSS). Finally, washed WBC were resuspended in 0.1 mL of HBSS supplied with fetal calf serum. In each determination, 10_6 WBC were added to the chemiluminometer cuvette. The oxygen free radical generation was induced by 10µL of PMA (final concentration, 10 nM). The LCL response to PMA was registered continuously for 30 min at to=37oC and stirring [Lindena].

Peroxynitrite (OONO-) scavenging properties of skin samples were assessed by electron spin resonance (ESR) method using spin label PTIO and SIN-1 as a source of peroxynitrite [Akaike]. Spin label PTIO (10 µL; 1mM) and 0.1 M KH_2PO_4 buffer (pH 7.4) were placed in the 200 µL cell of a ESR spectrometer (LFR-30 Free radical Analyzer, JEOL, Tokyo, Japan). ESR spectra of spin adduct PTI was repeatedly registered for 1 hour after starting the reaction by the addition of SIN-1 (20 µL; 10mM).

Catalase activity. Catalase activity was determined by the rate of disappearance of hydrogen peroxide (20 µM) measured at 240 nm by spectrophotometer in phosphate buffer pH 7.4 [Aebi].

Superoxide dismutase activity. Superoxide dismutase activity in the RBC extracts and skin homogenates was measured by the method based on the auto-oxidation of adrenaline in the alkaline medium. In brief, 1,5 ml of buffer (0,05 M $NaHCO_3$-Na_2CO_3, pH 10,2 plus 10^{-4} M EDTA) is placed in a 3-ml cuvette with a light path of 1,0 cm at room temperature. The reaction was initiated by adding 0,05 ml of epinephrine stock solution (10^{-2}M) and a rate of absorbance was registered at 480 nm. Another cuvette was then prepared in which the sample to be assayed replaced an equal volume of buffer, and the rate was again recorded after adding epinephrine [Misra].

Myeloperoxidase activity. Peroxidase activity in the supernatant was determined by measuring the rate of conversion of o-dianisidine to its colored product in the presence of H_2O_2. This rate was determined from the absorbance at 560 nm after 5 minute incubation and addition of o-phosphorous acid. [Andrews, Xia]

Statistical evaluation. The results are expressed as mean values (M) of at least five independent determinations. Standard deviation (SD) values were calculated using Excel statistical program for personal computers. Where not reported SD are below 10% variation of the mean value.

Results:

SPLIT THICKNESS EXCISIONAL WOUND MODEL

Wound healing effect

Planimetry results (mm^2, Mean$\pm$SD)

	Intraoperative wound size	4 days after operation	8 days after operation
DEEP OSCILLATION®	856.78$\pm$43.57	528.47$\pm$82.71*;**	37.55$\pm$27.21*;**
CONTROL	851.20$\pm$62.87	619.62$\pm$69.62*	140.67$\pm$79.12*

*- p<0.05 vs. Intraoperative wound size
**- p<0.05 vs. CONTROL

Conclusions: DEEP OSCILLATION® exposure resulted in significant improvement of the wound healing process seen at the 4th and 8th days after wounding.

Biochemical effects

CL, whole blood (mV, Mean$\pm$SD)

	Before operation	**2 days after operation**	**4 days after operation**	**6 days after operation**	**8 days after operation**
DEEP OSCILLATION®	8.2$\pm$2.5	13.8$\pm$3.3*	12.5$\pm$4.4*	14.7$\pm$3.9*	13.8$\pm$2.3*
CONTROL	8.6$\pm$2.3	17.7$\pm$7.7*	14.1$\pm$5.4*	14.9$\pm$4.8*	15.1$\pm$5.3*

*- p<0.05 vs. Before operation

Conclusions: DEEP OSCILLATION® exposure did not affect significantly the free radical production in the circulating blood. Therefore we could suggest it did not have **generalized effects** on the biochemical processes.

MPO, granulation tissue (mkmol/g prot, Mean$\pm$SD)

	4 days after operation	**8 days after operation**
DEEP OSCILLATION®	225.1$\pm$78.0	196.3$\pm$2.3
CONTROL	243.9$\pm$65.1	197.6$\pm$3.7

Conclusions: DEEP OSCILLATION® exposure neither increase nor decrease the recruitment of granulocytes into the granulation tissue. Therefore DEEP OSCILLATION® did not affect the normal process of tissue regeneration.

MPO, new epidermis (mkmol/g prot, Mean$\pm$SD)

	8 days after operation
DEEP OSCILLATION®	**60.2$\pm$6.5***
CONTROL	**90.7$\pm$9.3**

*- p<0.005 vs. CONTROL

Conclusions: DEEP OSCILLATION® exposure resulted in the significant inhibition of myeloperoxidase activity in the new epidermis. Therefore we concluded that DEEP OSCILLATION® possessed the anti-inflammatory effect.

GPx, granulation tissue (un/mg prot, Mean$\pm$SD)

	4 days after operation	8 days after operation
DEEP OSCILLATION®	**0.75$\pm$0.15**	**0.82$\pm$0.03***
CONTROL	**0.75$\pm$0.11**	**1.03$\pm$0.07**

*- p<0.05 vs. CONTROL

Conclusions: DEEP OSCILLATION® exposure resulted in significant inhibition of the glutathione peroxidase activity in the granulation tissue that reflected its anti-oxidant and anti-inflammatory action.

GPx, new epidermis (un/mg prot, Mean$\pm$SD)

	Before operation	8 days after operation
NORMAL EPIDERMIS	0.35$\pm$0.11	
DEEP OSCILLATION®		1.09$\pm$0.15*
CONTROL		0.98$\pm$0.17*

*- p<0.05 vs. NORMAL EPIDERMIS

Catalase, granulation tissue (mkg/mg prot, Mean$\pm$SD)

	4 days after operation	8 days after operation
DEEP OSCILLATION®	9.02$\pm$5.67	9.76$\pm$3.32
CONTROL	10.26$\pm$2.85	5.55$\pm$2.71

Catalase, new epidermis (mkg/mg prot, Mean$\pm$SD)

	Before operation	8 days after operation
NORMAL EPIDERMIS	22.03$\pm$5.09	
DEEP OSCILLATION®		19.81$\pm$5.06**
CONTROL		11.47$\pm$5.26*

*- p<0.05 vs. NORMAL EPIDERMIS
**- p<0.05 vs. CONTROL

SOD, granulation tissue (un/mg prot, Mean$\pm$SD)

	4 days after operation
DEEP OSCILLATION®	2.99$\pm$0.04
CONTROL	3.46$\pm$0.66

SOD, new epidermis (un/mg prot, Mean$\pm$SD)

	Before operation	8 days after operation
NORMAL EPIDERMIS	2.44$\pm$1.32	
DEEP OSCILLATION®		2.76$\pm$0.78
CONTROL		2.68$\pm$1.39

Antiedematous effect

Ratio of tissue weight to dry tissue weight, granulation tissue (g/g, Mean$\pm$SD)

	4 days after operation
DEEP OSCILLATION®	1.977$\pm$0.213*
CONTROL	2.398$\pm$0.266

*- p<0.05 vs. CONTROL

Conclusions: DEEP OSCILLATION® exposure significantly decreased swelling in the wounded area therefore the ratio of dry to wet tissue weight dropped statistically significant.

FULL THICKNESS EXCISIONAL WOUND MODEL

Wound healing effect

Planimetry results (mm^2, Mean$\pm$SD)

	Intraoperative wound size	4 days after operation	8 days after operation
DEEP OSCILLATION®	418.42$\pm$22.23	178.43$\pm$20.54*;**	50.9$\pm$11.45*;**
CONTROL	418.61$\pm$17.17	241.11$\pm$42.31*	82.93$\pm$14.09*

*- p<0.05 vs. Intraoperative wound size
**- p<0.05 vs. CONTROL

<u>**Conclusions:**</u> DEEP OSCILLATION® exposure resulted in significant improvement of the wound healing process seen at the 4th and 8th days after wounding.

Biochemical effects

CL, whole blood (un, Mean$\pm$SD)

	Before operation	2 days after operation (before procedure)	2 days after operation (after procedure)
DEEP OSCILLATION®	8.7$\pm$4.5	15.5$\pm$5.8	32.7$\pm$9.4*,**
CONTROL	8.7$\pm$4.5	19.7$\pm$5.3*	37.6$\pm$9.6*,**

*- p<0.05 vs. Before operation
**- p<0.05 vs. Before procedure

<u>**Conclusions:**</u> DEEP OSCILLATION® exposure did not affect significantly the free radical production in the circulating blood. Therefore we could suggest it did not have <u>**generalized effects**</u> on the biochemical processes.

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MPO, edge of wound (mkmol/g prot, Mean$\pm$SD)

	Before operation	4 days after operation	8 days after operation
NORMAL SKIN	110.6$\pm$55.4		
DEEP OSCILLATION®		180.9$\pm$42.3**	201.7$\pm$94.8**
CONTROL		293.8$\pm$32.9*	360.5$\pm$146.8*

*- p<0.05 vs. NORMAL SKIN
**- p<0.05 vs. CONROL

Conclusions: DEEP OSCILLATION® exposure resulted in the significant inhibition of myeloperoxidase activity in the wound. Therefore we concluded that DEEP OSCILLATION® possessed evident anti-inflammatory effect.

MDA, edge of wound (mkmol/g prot, Mean$\pm$SD)

	Before operation	4 days after operation	8 days after operation
NORMAL SKIN	0.47$\pm$0.07		
DEEP OSCILLATION®		0.52$\pm$0.11**	0.58$\pm$0.13
CONTROL		0.86$\pm$0.19*	0.54$\pm$0.12

*- p<0.05 vs. NORMAL SKIN
**- p<0.05 vs. CONROL

Conclusions: DEEP OSCILLATION® exposure resulted in the significant inhibition of lipid peroxidation in the wound at the 4th day. Therefore we concluded that DEEP OSCILLATION® possessed both antioxidant and anti-inflammatory effect.

Antiedematous effect

Ratio of tissue weight to dry tissue weight, edge of wound (g/g, Mean$\pm$SD)

	Before operation	4 days after operation	8 days after operation
NORMAL SKIN	**1.908$\pm$0.097**		
DEEP OSCILLATION®		**1.767$\pm$0.142****	**1.921$\pm$0.192****
CONTROL		**2.205$\pm$0.271***	**2.190$\pm$0.147***

*- $p < 0.05$ vs. NORMAL SKIN
**- $p < 0.05$ vs. CONROL

Conclusions: DEEP OSCILLATION® exposure significantly decreased swelling in the wounded area therefore the ratio of dry to wet tissue weight dropped statistically significant.

For photo documentation of the results please see documents:

SPLIT THICKNESS EXCISIONAL WOUND MODEL
s_gb_DO_Mikhalchik2005_excisional_wound_(animal)_photo_split.pdf

FULL THICKNESS EXCISIONAL WOUND MODEL
s_gb_DO_Mikhalchik2005_excisional_wound_(animal)_photo_full.pdf

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