

Wound healing in rat skin subjected to loud noise stress; a light microscopic study

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Abstract

Objective: To determine the effect of loud noise stress on wound healing in a skin tissue.

Methods: The randomised control trial was conducted at the Department of Anatomy, College of Physicians and Surgeons Pakistan Regional Centre, Islamabad, from September 2007 to September 2008. The study comprised 240 male Sprague Dawley rats who were randomised into control group A and experimental group B. Each group comprised 120 animals. Main groups were further subdivided into four subgroups of 30 animals each. After induction of local anaesthesia a linear full thickness skin incision paravertebral to thoracic spine was made. The experimental group B was exposed to loud noise stimulus. The animals were decapitated 1, 3, 5 and 7 days after surgery. Histological data was collected in the incisional space of the wound. Polymorpho-nuclear leukocytes, macrophages, fibroblasts and blood vessels, were analysed quantitatively, whereas re-epithelialisation and content of collagen fibres in the incisional space were analysed qualitatively.

Results: Macrophages were decreased initially 3 days after surgery and were increased 5 and 7 days after surgery in the experimental subgroups. Similarly, blood vessels and fibroblast were significantly decreased in experimental subgroups 3 days after surgery, but were increased significantly in experimental subgroups 7 days after surgery. Nothing significant was found regarding re-epithelialisation and collagenisation of wound.

Conclusion: Loud noise stress affects the important cells involved in the healing of the wound. Therefore, it is expected to have an impact on the stages of wound healing.

Keywords: Loud noise, Wound healing, Stress, Skin. (JPMA 64: 1265; 2014)

Introduction

Factors affecting skin wound healing have always been a central consideration in surgical practice. Breach in continuity of skin or mucous membrane exposes the deeper tissues to infections. In this regard, factors affecting healing of skin wound need to be understood thoroughly.

Stress and pollution are unavoidable phenomena affecting the body system at various levels. A large number of people are exposed to potentially hazardous noise levels in their daily modern life, such as noise from work environments, urban traffic, household appliances etc. Numerous studies have been carried out to study the effect of loud noise stress on various systems. Loud noise causes hearing loss.¹ In utero exposure to loud noise results in altered immune response in postnatal life.² There is a high prevalence of oropharynx infections of viral, bacterial and fungal origins in persons exposed to high pressure amplitude and low frequency noise.³ Exposure to loud noise causes raised systolic blood

pressure (SBP).⁴ Loud noise causes changes in nocturnal sleep architecture and heart rate (HR).⁵ A chronic noise-induced stress accelerates the ageing of the myocardium and increases the risk of myocardial infarction (MI).⁶ Noise exposure contributes towards community annoyance.⁷

Stress related alterations in wound repair could have important clinical implications, particularly for surgical recovery. The objective of the study was to determine the impact of loud noise stress on the biological process of wound healing. Understanding the effects of physical environmental stimuli would allow us to design healthcare environments that generate potential health benefits.

Subjects and Methods

The randomised control trial was conducted at the Department of Anatomy, College of Physicians and Surgeons Pakistan (CPSP) Regional Centre, Islamabad, from September 2007 to September 2008. Animals used in the study were obtained from the National Institute of Health (NIH), Islamabad. There were 240 male Sprague Dawley rats weighing between 250-300g. They were randomised into control group A and experimental group B. Each group comprised 120 animals. Important histological changes take place during 1, 3, 5 and 7 days after surgery,⁸ and therefore main groups were divided according to day of sacrifice (DOS) of animals into four

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subgroups i.e. (A1 to A4 and B1 to B4); each subgroup had 30 animals. The animals were kept in cages numbered 1 to 10. The animals were housed in cages in a temperature-controlled room (28°C-31°C). Twelve-hour light-dark cycle was maintained with lights on at 6am and off at 6pm. In order to minimise all undesired stressors, such as handling, habitat, etc., the animals were acclimatised to environment for at least one week in the animal house at the institution's Department of Anatomy. Twenty animals were used at one time (10 exposed group and 10 controls). On the day of surgery, a cage was selected randomly from a pool of all the 10 cages having animals fulfilling the inclusion criteria. The repetitive coin toss would bisect the set of cages until a selection was made. Heads would exclude animals in the lower half of the set, and tails would exclude animals in the upper half. For example, if the first toss was heads, then only cages between 6 and 10 were selected. If the second toss was tails, then only the cages between 6 and 8 were selected. If the third toss was tails again, then 6 and 7 were selected. If the fourth toss was heads, then cage 6 was selected.

In the first randomisation, cage 6 was selected and removed, the animals were removed from the cage and given their permanent designation i.e., 6a, 6b. Subsequent cages were selected in the same way after a repeat numerical designation. The number was noted on the cage card. All the 20 animals removed from different cages received the same anaesthesia and surgical procedure. Anaesthesia was induced by intramuscular injection Ketamine 5ml (50mg) + 0.5ml (50mg) Xylazine. Rat dose was 0.1ml/100g body weight.⁹ Skin on the right side of dorsum of rat was shaved and cleaned. A linear 2cm full-thickness incision, extending through epidermis and dermis to the panniculus carnosus, para vertebral to thoracic spine was made. The incision was surgically closed with metallic clips with disposable skin stapler. The animals were randomised to exposure by coin toss, heads = exposure to loud noise stimulus; tails = non-exposure.

Loud noise stimulus (recorded noise of aeroplanes and machine-guns) was played in an auto reverse stereo cassette player. Loud noise was produced by two loud-speakers (50 watts each) placed at distance of 40cm from the cages. A precision sound level meter was used to set the intensity of sound ranging from 97dB(A) to 102 dB(A) in cages. The level of noise was chosen as comparable with the noise frequently detected at industrial workplaces.¹⁰ Ten animals were exposed at one time. Control animals were exposed to only the usual 30dB(A) to 60 dB(A) noise of the animal care facility. All the animals of control group A and experimental group B were decapitated with guillotine on the scheduled day.

Subgroup B1 was exposed to loud noise 8 hours for 1 day. Subgroup B1 and A1 were decapitated 1 day after surgery. Subgroup B2 was exposed to loud noise 8 hours daily for 3 days. Subgroup B2 and A2 were decapitated 3 days after surgery. Subgroup B3 was exposed to loud noise 8 hours daily for 5 days. Subgroup B3 and A3 were decapitated 5 days after surgery. Subgroup B4 was exposed to loud noise 8 hours daily for 7 days. Subgroup B4 and A4 were decapitated 7 days after surgery. Skin wound was harvested with sharp scissors along with a rim of healthy tissue (approximately 5mm). Skin tissue was fixed in 10% formalin. Skin tissue was processed and sectioned at approximately 6µm thickness. Tissue sections were stained with haematoxylin and eosin (H&E) for the study of cell morphology and epithelialisation of skin tissue. The connective tissue elements of skin tissue were studied after staining with Mallory's trichrome stain.

The number of neutrophils, macrophages, fibroblasts and blood vessels were recognised in the incisional space under oil immersion lens in a pre-calibrated unit area in eye piece graticule. Regeneration of epithelium was assessed as whether the incisional space had been bridged by the migrating edge of the growing epithelium from the wound edge or not. The observation was labelled as complete or incomplete bridging of the incisional space by the newly-formed epithelium.

Statistical analysis of the data was done with SPSS 10. Quantitative data regarding the number of neutrophils, macrophages, fibroblasts and blood vessels was analysed by Student's t test for the detection of any significant differences between the mean number in the experimental and control groups. All the quantitative data was expressed as mean±SD. $P \leq 0.05$ was considered statistically significant. Content of collagen fibres and regeneration of epidermis was analysed qualitatively.

Results

In subgroups A1 and B1, 1 day after surgery the epithelial cells from the wound edges migrated across the incisional space, but did not bridge the incisional space. Subgroup A2 and B2, 3 days after surgery demonstrated that the incisional space was bridged with two to three layers of epithelium. A complete re-epithelialisation of the surface of the incisional space was observed in subgroups A3 and B3, 5 days after surgery. Subgroups A4 and B4, 7 days after surgery showed all the layers of epithelium.

Quantitative analysis in subgroups A1 and B1, A2 and B2, showed that the difference in the mean number of neutrophils between the experimental and control subgroups was insignificant (Table-1). In subgroups A3 and B3, polymorphonuclear leukocytes (PMNL)

Table-1: Mean number of neutrophils, macrophages and fibroblasts in the incisional space of the wound in control (A) and experimental (B) group per unit area.

Sub group	Neutrophil Mean±SD	Pvalue	Macrophage Mean±SD	Pvalue	Fibroblast Mean±SD	Pvalue	Blood vessels Mean±SD	Pvalue
A1 (DOS 1)	19.82± 1.43	0.47	ZERO		ZERO		0.78 ± 0.33	0.63
B1 (DOS 1)	19.39 ± 1.83						0.81 ± 0.34	
A2 (DOS 3)	3.11 ± 1.58	0.4	1.61 ± 0.80	*0.01	5.95 ± 1.55	* 0.04	1.11±0 .56	0.1
B2 (DOS 3)	2.74 ± 1.82		2.18± 0.84		6.95 ± 1.94		1.41 ± 0 .38	
A3 (DOS 5)	0.75 ± 0 .95	0.22	1.95± 0.36	0.007*	16.18± 3.98	0.19	3.16 ± 1.24	0.1
B3 (DOS 5)	0.41± 0.38		1.70 ± 0.50		14.61 ± 2.88		2.66 ± 1.29	
A4 (DOS 7)	0.12 ± 0. 33	0.53	0.99 ± 0.59	0.06	10.25 ± 3.47	*0.00	1.86 ± 0.98	*0.00
B4 (DOS 7)	0.16 ± 0.36		0.69±0.47		6.75 ± 2.35		1.13 ± 0.42	

*Significant= p? 0.05.

DOS= Day of sacrifice.

Table-2: Number of specimens (percentage) and frequency showing different staining intensity of collagen fibres in the incisional space in the control (A) and experimental (B) group.

Collagen content in incisional space	3 DOS GROUP II SUBGROUP		5 DOS GROUP III SUBGROUP		7 DOS GROUP IV SUBGROUP	
	A2 (n=30)	B2 (n=30)	A3 (n=30)	B3 (n=30)	A4 (n=30)	B4 (n=30)
Mild (%age)	100 (n=30)	90 (n=27)	-	-	-	-
Moderate (%age)	-	-	76 (n=23)	83 (n=25)	7 (n=2)	17 (n=5)
Intense (%age)	-	-	23 (n=7)	17 (n=5)	93 (n=28)	83 (n=25)
Absent(%age)	-	10 (3=n)	-	-	-	-

n= number of specimens. DOS= Day of sacrifice of animals.

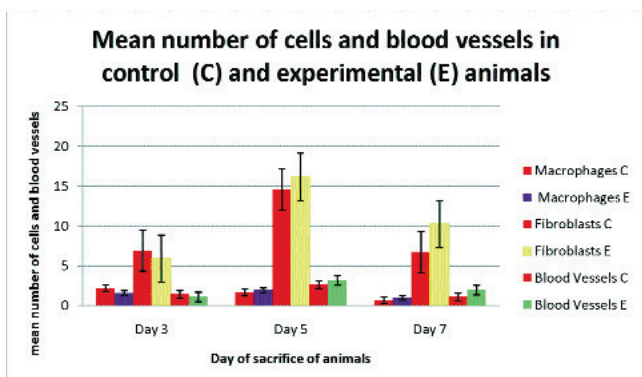
represented only the minor fraction of the cellular infiltrate. The difference in the mean number of PMNL in the subgroup B3, and its control subgroup was insignificant ($p>0.05$). In subgroups A4 and B4 the cells of acute inflammation i.e. neutrophils, became almost extinct. The statistical difference between the mean number of PMNL in the subgroup B4 and its control subgroup A4 was insignificant ($p>0.05$).

In subgroups A1 and B1 the mean number of blood

vessels between the experimental and control subgroup was insignificant ($p>0.05$). Similarly, mean number of blood vessels in the experimental subgroup B2 was not significant compared to control subgroup A2 ($p>0.05$). Also the mean blood vessels in the subgroup B3 and its control subgroup A3 were insignificant ($p>0.05$). However, in subgroups B4 mean number of blood vessels were present in significantly increased number compared to its match control subgroup A4 ($p<0.05$).

Fibroblasts did not appear in subgroups A1 and B1. Fibroblasts were significantly decreased in the experimental subgroup B2 compared to control subgroup A2 ($p<0.05$). However, the difference in the mean number of fibroblasts in the subgroup B3 and its control subgroup A3 was insignificant ($p>0.05$). Fibroblasts were present in significantly increased number in the incisional space of the wound in the subgroup B4 compared to subgroup A4 ($p<0.05$).

Macrophages were not seen in subgroups A1 and B1. Macrophages were significantly decreased in the subgroup B2 compared to its control subgroup A2 ($p<0.05$). The mean number of macrophages between subgroup A3 and subgroup B3 was statistically significant ($p<0.05$). Macrophages were significantly increased in

**Figure:** Cells and blood vessels in the incisional space of wound in different post wounding days.

wound in the experimental subgroup B4 compared to control subgroup A4 ($p < 0.05$).

Regarding the extra-cellular matrix components, no collagen fibres were seen in subgroups A1 and B1. In subgroups A2 and B2 collagen was more near the periphery of the incisional space. There was a mild staining reaction. In experimental subgroup B2 90% revealed the presence of collagen fibres whereas control subgroup A2 exhibited collagenisation of incisional space in 100% of specimens (Table-2). In subgroups A3 and B3 there was a moderately intense staining reaction when compared to day 3 specimens. Collagen was arranged in fibre bundles. Bundles of collagen fibres were running between blood vessels and other cells constituting the granulation tissue. There was a moderate staining reaction in 83% of specimens and intense reaction in 17% of specimens in subgroup B3 and 76% moderate and 24% intense in subgroup A3. In subgroups A4 and B4 collagen fibres were arranged in irregular manner compared to organised pattern seen in the adjacent unwounded dermis. In some of the specimens, collagen fibres were seen running parallel to the epidermis signifying the beginning of the organisation of the wound. Collagen fibres gave an intense staining reaction in both subgroups B4 and A4.

Significant changes occurred in the mean number of macrophages, fibroblasts and blood vessels in the experimental subgroups in different post-wounding days (Figure).

Discussion

The results showed re-epithelialisation of the wound surfaced 3 days after surgery invariably in all the experimental and control animals. The process of re-epithelialisation is facilitated by factors released by the activated fibroblasts and macrophages in the wound space.¹¹ Although macrophages and fibroblasts decreased 3 days after surgery in the present study in the experimental group, but it can be assumed that the factors released by the activated fibroblasts and macrophages might be in enough concentration to contribute to the re-epithelialisation process.

Although neutrophil function is impaired by stress and distressed individuals faced additional risks from infection after wounding¹² but in the present study the difference in the mean number of neutrophils between the experimental and their control subgroups was not significant. It can be presumed that their function might be compromised which subsequently affected the other cells taking part in the wound-healing process. In the present study the macrophages were significantly

reduced in the experimental rats compared to their control subgroups 3 days after surgery. Our results correspond with the previous study that states that the innate non-specific immune response, as well as the specific immune response, is delayed by stress.¹³ Also, elevated adrenal glucocorticoids in acute stress conditions or major depression attenuate the differentiation and function of monocyte.¹⁴ Macrophages were still decreased in number in the stressed subgroup 5 days after surgery in the present study. That shows that macrophages are more sensitive to stress compared to neutrophils.

The results of the present study regarding the number of fibroblasts in the area of wound showed a significant decreased fibroblast population in the experimental subgroup 3 days after surgery. Decreased fibroblast in this study correlate with the observations made by earlier study¹⁵ that levels of plasma cortisol were elevated in post-operative period and the peaks were seen 1 and 3 days after surgery. Cell movement into tightly woven extra-cellular matrix may require an active proteolytic system, including collagenases, gelatinase A and stromelysin that can cleave a path for cell migration.¹⁶ Stress impairs the synthesis of collagenases, elastase and tissue plasminogen activator by macrophages.¹⁷ It probably retards the fibroblast migration in the wound space as seen in this study.

Fibroblasts seen in experimental subgroup 5 days after surgery was not significantly different from its normal control subgroup. Recruitment of macrophages in the wound and their subsequent activation in the wound space might cause increased proliferation of fibroblasts in the wound. Also, the activated keratinocytes initiate growth factors that cause the proliferation of fibroblasts in the wound.¹⁸ Fibroblasts were seen in a significantly increased number in experimental subgroup than in their control counterparts 7 days after surgery. One possible explanation could be their delayed migration in the wound space under the effect of stress.

Macrophages and wound hypoxia are the two predominant factors controlling the wound angiogenesis.¹⁹ Wound macrophages, but not unstimulated monocytes or macrophages, are capable of inducing angiogenesis.¹⁹ Activated macrophages in the wound secrete the pro-angiogenic cytokines.²⁰ There was no recruitment of macrophages in the incisional wound space after 1 day of surgery. So the number of vessels in the experimental subgroup after 1 day of surgery was not significantly different from its control subgroup. Blood vessel formation in the area of wound was not

significantly different in our study 3 days after surgery in the experimental animals compared to their controls. The most probable cause of the decreased vascularity of wound could be due to decreased macrophage function in the wound i.e. pro-angiogenic cytokines released by the activated macrophages. The blood vessels in the incisional space of the wound in experimental subgroup 5 days after surgery were not statistically significant compared to its matched control subgroup. Hypoxia is one of the factors to drive the angiogenic response by up-regulating angiogenic cytokines observed during wound-healing. Hypoxia peaked in the granulation tissue stage at day 4 due to increased cellularity and cellular proliferation.²¹ There was significantly increased number of vessels in the stressed subgroup than their control subgroup 7 days after surgery. The increased vessels could be a part of normal phenomenon of wound-healing. Little evidence for the induction of this process exists, but the reduced demands on oxygen supply might lead to regression of the neo-vessels by endothelial cell apoptosis.¹⁶ Thus persistence of vessels in the granulation tissue could be due to the presence of fibroblasts in the wound synthesising the extra-cellular matrix. The nutrients to the active cells in the wound space were being provided by these vessels.

Conclusion

Consistent with the evidence that stress delays wound-healing, the results of the study suggested that loud noise was another important environmental stressor that affects the wound-healing cascade.

References

1. Akhter NH. Noise induced hearing loss in traffic constables. *J Coll Physicians Surg Pak* 1996; 6: 265-8.
2. Sobrian SK, Vaughn VT, Ashe WK, Markovic B, Djuric V, Jankovic BD, et al. Gestational exposure to loud noise alters the development of humoral and cellular components of the immune system in offspring. *Environ Res* 1997; 73: 227-41.
3. Castelo B. The clinical stages of vibroacoustic disease. *Aviat Space Envi* 1999; 70: 32-9.
4. Mahmood R, Khan GJ, Alam S, Safi AJ, Salahuddin, Amin- ul-Haq. Effect of 90 decibel noise of 4000 hertz on blood pressure in young adults. *J Ayub Med Coll Abbottabad* 2004; 16: 30-3.
5. Gitanjali B, Ananth R. Effect of acute exposure to loud occupational noise during daytime on the nocturnal sleep architecture, heart rate, and cortisol secretion in healthy volunteers. *J Occup Health* 2003; 45: 146-52.
6. Ising H, Babisch W, Kruppa B. Noise-Induced Endocrine Effects and Cardiovascular Risk. *Noise Health* 1999; 1: 37-48.
7. Lim C, Kim J, Hong J, Lee S. Effect of background noise levels on community annoyance from aircraft noise. *J Acoust Soc Am* 2008; 123: 766-71.
8. Nukumi K, Masuda M, Obata A, Yumoto E. Differences in Expression of Basic Fibroblast Growth Factor during Wound Healing between Oral Mucosa and Skin. *Wound Repair and Regen* 2004; 12: 7-71.
9. Kohn DS. Ed's. *Anesthesia and Analgesia in Laboratory Animals*. Inc. 2nd ed. New York: Academic Press Inc; 1997.
10. Cohen S, Krantz DS, Evans G, Stokols D. Cardiovascular and behavioral effects of community noise. *Am Sci* 1981; 69: 528-35.
11. Braun S, Keller UD, Steiling H, Werner S. Fibroblast growth factors in epithelial repair and cytoprotection. *Phil Trans R Soc Lond B* 2004; 359: 753-7.
12. Shurin MR, Kusnecov A, Hamill E. Stress-induced alteration of polymorphonuclear leukocyte function in rats. *Brain Behav Immun* 1994; 8: 163-9.
13. Kusnecov AW, Rabin BS. Stressor induced alterations of immune function: mechanisms and issues. *Int Arch Allergy Immunol* 1994; 105: 107-21.
14. Werb Z, Foley R, Munck A. Interaction of glucocorticoids with macrophages. Identification of glucocorticoid receptors in monocytes and macrophages. *J Exp Med* 1978; 78: 608-12.
15. Saito T, Tazawa K, Yokoyama Y, Saito M. Surgical stress inhibits the growth of fibroblasts through the elevation of plasma catecholamine and cortisol concentrations. *Surg Today* 1997; 27: 627-31.
16. Thomas DW, O'Neill ID, Harding KG, Shepherd JP. Cutaneous wound healing: A current perspective. *J Oral Maxillofacial Surg* 1995; 53: 442-7.
17. Werb Z. Biochemical actions of glucocorticoids on macrophages in culture. Specific inhibition of elastase, collagenases and plasminogen activator secretion and effects on other metabolic functions. *J Exp Med* 1978; 147: 1695-12.
18. Werner S, Kreig T, Smola H. Keratinocyte-fibroblast interaction in wound healing. *J Invest Dermatol* 2007; 127: 998-1008.
19. Koch AE, Polverini PJ, Leibovich SJ. Induction of neovascularization by activated human monocytes. *J Leukoc Biol* 1986; 39: 233-8.
20. Crowther M, Brown NJ, Bishop ET, Lewis CE. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *J Leukoc Biol* 2001; 70: 478-90.
21. Haroon ZA, Raleigh JA, Greenberg CS, Dewhirst MW. Early wound healing exhibits cytokine surge cytokine surge without evidence of hypoxia. *Ann Surg* 2000; 231: 137-47.