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Effect of nicotine on bone healing in rats - A histological study

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Abstract: *Background & Objectives:* Nicotine is the major alkaloid in tobacco products (*Nicotiona tabacum*) and a psychoactive ingredient responsible for the Central Nervous System (CNS) effects and tobacco addiction. It's been reported to have effects directly on the small blood vessels in producing vasoconstriction and increased vascular resistance that exerts on the microvasculature inhibiting the angioblastic response during re-vascularization and limits the recruitment of cytokines, Bone Morphogenic Proteins (BMPs), Transforming Growth Factor – β (TGF – β), Platelet Derived Growth Factor (PDGF) and the basic Fibroblast Growth Factor (FGF). This leads to inhibition of re-epithelialization, osteogenesis and cellular healing. This study intends to demonstrate histologically the effect of nicotine on bone healing and the healing of bone defects incorporated with autogenous bone graft in an animal model. *Methods:* 60 female Wistar rats were used in the study. Nicotine hemisulfate at a dose of 3mg/kg body weight of the animal given twice daily for 4 weeks prior to creation of a bone defect. The defect on the ramus of the mandible and the healing in the defect was evaluated at weekly intervals for four weeks for both the quality and quantity of new bone formation by histological and histomorphometric analysis. *Results:* **S**ignificant impairment of healing of bone both in the early and late stages due to the influence of Nicotine was seen. *Conclusion:* In our study, incorporation of autogenous bone did significantly improve the bone healing process in the end stages of healing while nicotine significantly impaired the healing of bone in early stages.

Keywords: Nicotine Hemi-sulfate; Bone Defect; Bone Healing; Autogenous bone graft; Histomorphometry

Introduction

The ill effects of tobacco use in any format resulting in mortality and morbidity has been well known and documented. It has been implicated in the development of osteoporosis and increased fracture risk, impaired healing of fractures, failure of spinal fusion, periodontal disease and development of oral cancers [1].

Nicotine is the major alkaloid in tobacco products (*Nicotiona tabacum*) and is the most psychoactive ingredient responsible for the CNS effects and tobacco addiction. Nicotine has been reported to have effects directly on the small blood vessels in producing vasoconstriction and increased vascular resistance. This intense vasoconstriction that nicotine exerts on the microvasculature inhibits the angioblastic response during re-vascularization in the healing area and limits the recruitment of cytokines, BMPs, TGF-β, PDGF and the basic FGF. These events eventually lead to inhibition of re-epithelialization, osteogenesis and cellular healing. The negative effects of nicotine have been attributed to be detrimental to healing of extraction sockets, fracture healing, healing post-orthognathic surgery, healing during distraction osteogenesis, healing of periodontal tissues and osteointegration of implants etc [1].

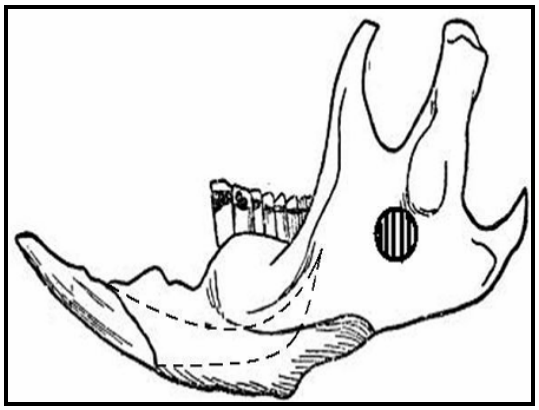
This study was performed to demonstrate histologically the effect of nicotine on bone healing and the healing of bone defects incorporated with autogenous bone graft in an animal model. This can further be used as a basis to study the deleterious effects of nicotine on bone healing that can occur in humans.

Material and Methods

Sixty healthy female wistar rats aged 3-4 months with a body weight of 150-200grams were used for the studies which were housed in the animal research facility.

The rats were randomly divided into three groups (A, B and C) consisting of 20 rats in each group. Group A and B were taken as experimental groups and group C as the control. Nicotine hemisulphate (Sigma-Aldrich Chemicals Pvt Ltd) in a 40% w/v diluted with sterile saline solution to a concentration of 1mg per ml. An amount of 3mg/Kg body weight of the animal was injected subcutaneously twice daily at 12^{th} hourly intervals in the experimental groups A and B for 4 weeks before surgery and till day of necropsy post surgically. The surgery was performed under chloroform inhalation anesthesia. Through Stab incisions and blunt dissection, the lateral surface ramus of mandible was approached with a 2.5mm trocar to act as a protective sleeve; a 2 mm drill bit was introduced through a trocar to create a bony defect [Fig-1].

Fig-1: Site of Bone Defect



For experimental group B the autogenous bone graft was obtained from the contra-lateral ramus. Through Stab incisions and blunt dissection a core of bone graft was obtained using custom modified 2.5mm hollow tapered implant osteotomy drill bit. The bone graft was introduced into the bone defect through the trocar sleeve. The wounds were closed with 4-0 silk sutures. For experimental group the nicotine was administered post-operatively at 12hour intervals until the day of necropsy. The 5 rats from each group were sacrificed at intervals of 1, 2, 3 and 4 weeks and blocks of bone with osteotomy site were retrieved for histological examination.

Histological Slide Preparation: The bone retrieved en bloc was washed and fixed in 10% formalin for two days before preparation of the slide. Demineralization of the fixed bone specimen was carried out by 5% Nitric acid for a period of one hour. Tissue was then processed and embedded in paraffin wax. Semiserial sections of 6 μm thickness were obtained and stained with Eosin and Hematoxylin for Histological and Histometric analysis.

Histological and Histomorphometric analysis: Histological sections were analyzed for the presence of: Granulation tissue, Blood clot, Osteogenic activity, Areas of resorption, New formed bone and Fulfillment of medullary space Histometric analysis was done by defining an area of interest in the histologic field and obtaining a photomicrograph using a digital camera (Nikon P2700) attached to the binocular microscope (Lawrence and Mayo). The image obtained was analyzed using an Image Analysis software (Sigmascan Pro 5.0) using the point count method for obtaining the trabecular volume as a percentage of the volume of the area of interest.

Results

Bone healing in all three groups was evaluated at 1^{st}week, 2^{nd} week, 3^{rd} week and 4^{th} week in five rats sacrificed from each group at weekly intervals. Histologic evaluation for the quality of bone formation and Histomorphometric measurements for the quantity of bone formation was performed and recorded using standardized nomenclature[2]. The percentage value of bone formation obtained through Histomorphometry was the parameter subjected to Descriptive statistics, One way ANOVA, Repeated measure ANOVA and Independent Samples t-test for intergroup comparison using SPSS software (version 16.0).

At 7 days, Group C showed defect filled with well vascularized neoformed connective tissue rich in fibroblast at the periphery of the defect. Immature trabeculae of bone was seen budding from the periphery of the defect lined by numerous osteoblasts suggestive of osteogenic activity [Fig-2]. In Group A the connective tissue was immature and less trabaculae compared to controls with poor osteoblast lining was seen [Fig-3]. Group B showed lamellar bone islands with osteoclast lining showing active remodeling of the bone graft with some osteogenic activity at the periphery of the defect [Fig-4].

Fig-2: Healing after 1 week (Group C)

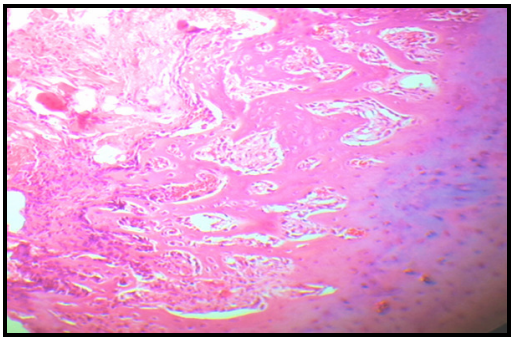


Fig-3: Healing after 1 week (Group A)

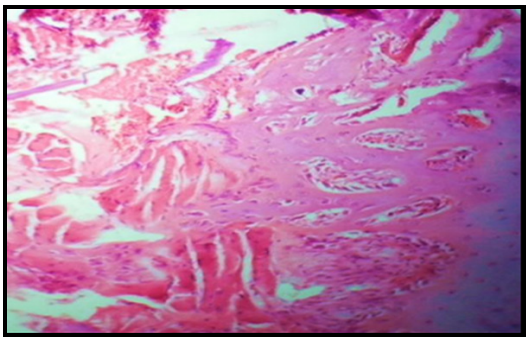
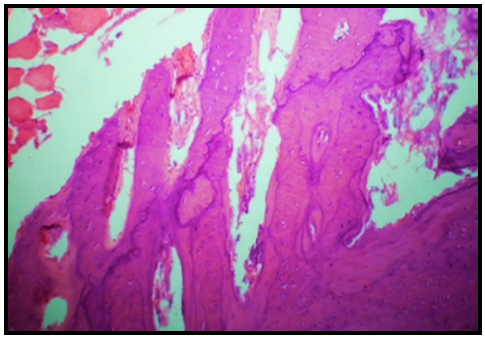


Fig-4: Healing after 1 week (Group B)



Histomorphometric analysis showed that the mean percentage volume for Group A was 21.3300 ± 1.10923, Group B was 29.7380 ± 8.45233 and for Group C was 24.6400 + 3.38351. When the data was subjected to ANOVA test, F value was found to be non significant at 0.077 level (F = 3.199; p>0.05) (Table-1).

Table-1: Intergroup comparison of the mean values between various time intervals in the three groups using Descriptive statistics and One way ANOVA

		Mean	SD	F	p
Percentage volume 1st week	Group A	21.3300	1.10923	3.199	.077
	Group B	29.7380	8.45233		
	Group C	24.6400	3.38351		
	Total	25.2360	6.07031		
Percentage volume 2nd week	Group A	29.6180	4.35740	13.350	.001*
	Group B	42.2020	5.52832		
	Group C	42.7440	3.52490		
	Total	38.1880	7.55677		
Percentage volume 3rd week	Group A	59.4180	7.90877	2.450	.128
	Group B	70.8940	9.28690		
	Group C	66.3140	7.45257		
	Total	65.5420	9.06745		
Percentage volume 4th week	Group A	77.3620	5.59579	6.896	.010*
	Group B	89.0660	4.26935		
	Group C	80.0860	5.66101		
	Total	82.1713	7.07825		

At 14 days, Group C showed significant amount of woven bone with osteocytes extending from the periphery of the defect with some amount of connective tissue interspersed within the defect. Group A showed similar type of bone but with poor osteogenic activity and poorly organized connective tissue. Group B showed neoformed woven bone extending into the

defect with interspersed connective tissue and island of trabaculae of the bone graft. Histomorphometric analysis showed that the mean percentage volume for Group A was 29.6180 ± 4.35740, Group B was 42.2020 ± 5.52832 and for Group C was 42.7440 + 3.52490. When the data was subjected to ANOVA test, F value was found to be significant at 0.001 level ($F = 13.350$; $p<0.05$) (Table-1).

At 21 days, Group C showed extensive new bone formation with numerous osteoblast surrounding the trabaculae and associated with smooth connective tissue. Mature bone with reversal lines were seen at the periphery of the defect. Group A showed similar features as that of control. Group B showed active remodeling of the bony trabeculae. Grafted bone trabaculae were indistinguishable from that of new formed bone. Histomorphometric analysis showed that the mean percentage volume for Group A was 59.4180 ± 7.90877, Group B was 70.8940 ± 9.28690 and for Group C was 66.3140 + 7.45257. When the data was subjected to One way ANOVA test, F value was found to be non significant at 0.128 level ($F = 2.450$; $p>0.05$).

At 28 days, Group C showed mature trabecular bone filling most of the defect with formation of haversian system within mature bone tissue.[Fig-5] Scant areas of connective tissue were seen. Group A [Fig-6] and B [Fig-7] showed similar features. Histomorphometric analysis showed that the mean percentage volume for Group A was 77.3620 ± 5.59579, Group B was 89.0660 ± 4.26 and for Group C was 80.0860 + 5.66101. When the data was subjected to One way ANOVA test, F value was found to be non significant at 0.010 level ($F = 3.199$; $p>0.05$).

Fig-5: Healing after 4 weeks (Group C)

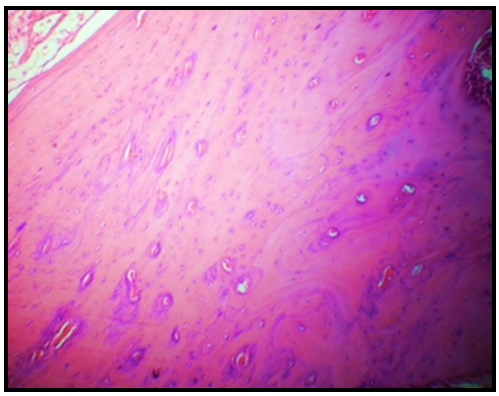


Fig-6: Healing after 4 weeks (Group A)

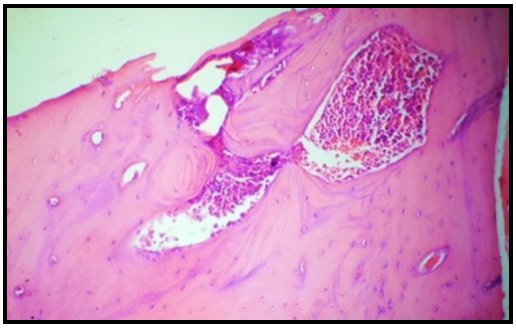
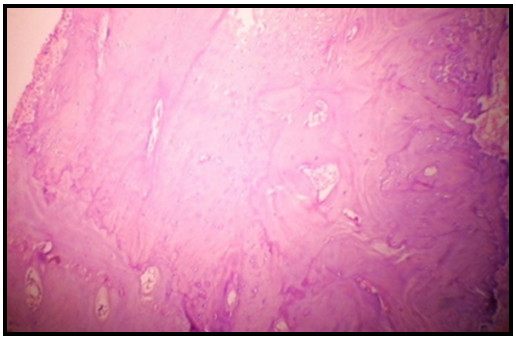


Fig-7: Healing after 4 weeks (Group B)



Discussion

Nicotine is the main chemical component which has been found to be of highest importance in more than 4000 of potentially toxic substances in tobacco products [3]. Heavy smokers normally have a plasma nicotine level in the range of 10-70ng/ml, while light smokers have nicotine levels less than 10ng/ml [4-5]. The influence of nicotine on tissue healing has been explored by various delivery systems on different animal models and the results remain conflicting [6-8]. Use of animal models circumvent some of the limitations of human studies since confounding variables (i.e., nutrient intake, body weight and alcohol consumption) which can be minimized or eliminated [4]. Nicotine concentration of 3mg/kg body weight of the animal at twice daily intervals were given which was in accordance to literature review [9]. The set concentration was sufficient to provide serum nicotine levels that correlated with human smokers who consume an average of 10-20 cigarettes per day. A subcutaneous injection was chosen for the drug delivery since this systemic route increases the bioavailability of the drug compared to its oral administration [4].

A twice daily dose also mimicked the diurnal variation of serum nicotine levels seen in human smokers. When comparing the effect of nicotine on bone healing with that of the controls, our results showed a statistically significant amount of bone fill in the defect of the control group at the second and fourth week of healing. This result was in contrast to the study done by Hollinger J.O et al [10] who postulated that bone healing was more severely affected in the later stages than at early stages of bone healing since significant endogenous cells and signaling factors promote the healing cascade irrespective of the effect of nicotine. Pinto J.R et al [11] stated that nicotine induced vasoconstriction predisposed to thrombotic microvascular occlusion and consequent tissue ischemia. In chronic smokers, nicotine causes reduced production of prostacycline which may potentiate this vasoconstrictor effect. Hapidin H et al [3] state that nicotine exposure induces an oxidative stress which produces free radicals which have been found to be involved in inducing osteoclastogenesis and inhibition of osteoblast differentiation. Both of these studies explain the possible reasons for the decreased bone healing in the nicotine group seen during the early stages.

Our study further evaluated the effect of nicotine on healing of bone defect that was grafted with an autogenous bone graft as compared to that of the controls and showed that there was a statistically significant increase in bone volume in the 4th week in the grafted group as compared to the controls. It has been widely discussed in literature that autogenous bone grafts show faster revascularization and their trabeculae are more easily reabsorbed thus allowing earlier bone substitution compared to that of other types of graft materials [9,12]. This explains the probable reason for the absence of deleterious effects of nicotine in healing of bone defects grafted with autogenous bone. Autogenous bone graft therefore had a promotional effect on the bone healing in a compromised setting. These findings were in accordance to studies conducted by Wong K.K et aland Silva R.V et al [13]. Although similar studies such as that by BanfanteS et al [12] showed that nicotine did jeopardize the early healing process of autogenous bone grafts in rats, but as pointed out in our study, it may have aided in healing of the bone in later stages.

There are innumerable evidences in the literature where smoking has been attributed to higher risk of fracture and bone loss in older men and post-menopausal women [1,4, 13]. Although smoking is well established as an important risk factor for development of these complications, it is not clear as to the mechanism by which smoking affects bone physiology. There is also evidence of a higher incidence of bone loss and implant failure rates reported in the dental literature [14]. Since nicotine is the major alkaloid in tobacco products the factors causing these effects on bone has been attributed to nicotine itself. Numerous research have been conducted to evaluate the effect of nicotine on bone healing in animal models such studies will eventually form the basis for formulating strategies to reverse the effects of smoking on bone healing in humans [10, 15-17].

Conclusion

This study concluded that Nicotine has a significant qualitative and quantitative effect of bone healing at late stages of the healing cascade mainly owing to its inhibitory effects on neovascularization and osteoblast differentiation. Although when healing was aided with the incorporation of bone graft there was a significant improvement of bone healing both in the early and late stage owing to the ability of autogenous bone grafts to provide the necessary induction and conduction properties during healing of bone. Although nicotine is the major constituent in tobacco products there are numerous other constituents that need to be studied to know the deleterious effect of tobacco products on bone healing. Further research is being contemplated to study the effect of other ingredients of tobacco in healing of bone in animal models and it would lead to the development of clinical remedies in order to reverse the detrimental effects of nicotine in patients requiring osseous surgery in the maxillofacial region.

References

1. Fung YK, Iwaniec UT, Cullen DM, Akhter MP, Haven MC, Timmins P. Long-term effects of nicotine on bone and calciotropic hormones in adult female rats. *PharmacolToxicol* 1999; 85:181-187.
2. Parfitt AM. Bone Histomorphometry. Standardization of Nomenclature, Symbols and Units. *Journal of Bone and Mineral Researh* 1987; 2 (6): 595-610
3. Hapidin H et al. Negative effects of nicotine on bone-resorbing cytokines and bone histomorphometric parameters in male rats. *J Bone Miner Metab* 2007; 25:93-98.
4. Iwaniec UT, Fung YK, Akhter MP, Haven MC, Haynatzki GR, Cullen DM. Effects of nicotine on bone and calciotropic hormones in aged ovariectomized rats. *J Musculoskel Neuron Interact* 2002; 2(5):469-478.
5. Glowacki J, Schulten AJM, Perrott D, Kaban LB. Nicotine impairs distraction osteogenesis in the rat mandible. *Int J Oral Maxillofac.Surg*. 2008;37:156-161
6. Saldanha JB et al. Histologic evaluation of the effect of nicotine administration on bone regeneration.A study in dogs. *Braz Oral Res* 2004; 18(4):345-9.
7. Carvalho MD et al. Effect of Cigarette Smoke Inhalation and Estrogen Deficiency on Bone Healing Around Titanium Implants: A Histometric Study in Rats. *J Periodontol* 2006; 77: 599-605.
8. Skott M et al. Tobacco Extract but Not Nicotine Impairs the Mechanical Strength of Fracture Healing in Rats. *J Orthop Res* 2006; 24:1472-1479.
9. Silva RV, Camilli JA, Bertran CA, Moreira NH. The use of hydroxyapatite and autogenous cancellous bone grafts to repair bone defects in rats. *Int. J Oral Maxillofac. Surg*. 2005; 34:178-184.
10. Revell PA. Histomorphometry of Bone. *J ClinPathol* 1983; 36:1323-1331.
11. Pinto JR et al. Effect of Nicotine on the healing of extraction sockets in rats. A histological study. *Brazilian Dent J* 2002; 13(1): 3-9.
12. Bonfante S et al. Influence of Nicotine on Healing Process of Autogenous Bone Block Grafts in the Mandible: A Histomorphometric Study in Rats. *Int J Oral Maxillofac Implants* 2008; 23:437-444.
13. Wong PKK, Christie JJ, Wark JD. The effects of smoking one bone health. *Clinical Science* 2007; 113: 233-241.
14. Balaji SM. Tobacco smoking and surgical healing of oral tissues: A review. *Indian J Dent Res,* 2008; 19(4): 344-348.
15. Carvalho TLL, Bombonata KF, Brentegani LG. Histometric Analysis of Rat Alveolar Wound Healing. *Braz Dent J* 1997; 8(1):9-12.
16. Sharp RE. The Laboratory Rat. *CRC Press* 1998 101-113.
17. Hollinger JO, Schmitt JM, Soleyamani P, Buck. Impact of nicotine on bone healing. *J Biomed Mater Res,* 1999; 45: 294-301.

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