Separate Effects of Tobacco Extract and Nicotine on Wound Healing in Rats.

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ABSTRACT

The separate and combined effects of nicotine and nicotine free tobacco extract on the healing of skin wounds in rats were investigated. 56 female Wistar rats were divided into four groups exposed to: tobacco extract plus nicotine, tobacco extract, nicotine and vehicle. Nicotine was administered through a subcutaneously implanted osmotic pump and the tobacco extract was administered orally as an aqueous solution. After two weeks of exposure a 55 mm incisional skin wound was made parallel to the vertebral column on the left side of the rats and a cellulose sponge, divided in half and held together by a suture, was implanted subcutaneously on the right side. After 7 days of healing, the mechanical strength of the skin wounds and the newly formed granulation tissue connecting the two pieces of the sponge were measured. Also, the amount of newly formed collagen in the sponge was determined. No difference was found between the groups in the m echanical strength of the skin wounds or in the collagen content and mechanical strength of newly formed granulation tissue in the sponges. The serum nicotine levels observed in the rats were well above the levels found in human smokers. The wound healing and sponge models used are widely accepted and proved their value in this study.

INTRODUCTION

Smoking impairs the healing of skin wounds. In 1977, Mosely and Finseth described the detrimental effects of smoking on a healing hand wound (1). Since then, many studies have confirmed the harmful effects of smoking on healing wounds (2,3,4). Today, it is generally accepted that smoking impairs wound healing but the pathogenesis behind this effect is still not documented.

Cigarette smoke consists of a particulate and a volatile phase. The volatile phase accounts for about 95 % of the weight of cigarette smoke and consists of some 500 gaseous compounds. The particulate phase accounts for the other 5 % and is comprised of over 3500 compounds, one of which is nicotine (5). Besides from being an addictive stimulant, nicotine causes cutaneous vasoconstriction (6) and thereby reduces blood flow to the skin. This could explain the impaired wound healing seen in smokers but the effect may not be due to nicotine alone.

In a study on smoking and incisional wound infection, smoking cessation significantly decreased the rate of infection but with no difference between subjects using nicotine patches and those using placebo patches (7). A recent study on fracture healing showed than when separating nicotine from tobacco and exposing rats to nicotine and tobacco extract (nicotine free tobacco) separately, the nicotine alone had no negative effect, whereas the tobacco extract significantly impaired the mechanical strength of fracture healing in rats (8).

As the use of nicotine replacement therapy becomes more and more prevalent with smokers undergoing elective surgery, establishing the role of nicotine in the wound healing process is essential in order to ensure the best care for these patients.

The purpose of this study is to investigate the effect of nicotine versus that of the thousands of other chemicals included in cigarettes (tobacco extract) on wound healing. The experiment includes four groups of rats. A control group and three treatment groups exposed to nicotine, tobacco extract, a combination of nicotine and tobacco extract, respectively. The outcome measures are the mechanical strength of skin incisional wounds after 7 days of healing plus the amount of collagen formed and the mechanical strength of the granulation tissue formed in a subcutaneously implanted cellulose sponge after 7 days of healing. To the best of our knowledge, a similar study has not previously been performed on skin wounds.

Hypothesis

We anticipated that, compared with the control group, mechanical strength development and collagen formation would be most impaired in the groups exposed to tobacco extract and/or nicotine.

MATERIALS AND METHODS

Study groups and observation period

Animals

A total of 56 female Wistar rats were used. The rats had free access to either tap water or tobacco extract solution and pellet food and were kept in 12-hour light, 12-hour darkness cycles. All surgical procedures were performed under isoflurane (Baxter A/S, Denmark) anesthesia (50 mg/kg BW) and an overdose (0,1 ml i.p.) of pentobarbital (Mebumal SAD 50 mg/ml, Sygehus Apotekerne i Danmark) was used for sacrifice. The animals received analgesia (Temgesic® 0.3 mg/ml, Shering-Plough) at the end of each surgical procedure as an injection (0.1 ml sc.) and for the following three days through the drinking water (1 ml per 150 ml of water).

Study groups

The rats were randomized according to weight into four experimental groups receiving nicotine, tobacco extract, nicotine plus tobacco extract or vehicle (control group).
Administration of the designated substrate began 14 days prior to wound infliction / sponge implantation and continued until the rats were sacrificed after 7 days of healing.

Preparation and administration of substrates

Nicotine

Nicotine bitartrate was obtained from Minneapolis Medical Research Foundation (Minnesota, USA). Osmotic minipumps (Alzet® 2ML4, Scanbur AB, Sweden) were loaded with the nicotine preparation containing a concentration of 12.5 mg/ml. The 2ML4 pumps deliver 2.5 µl/hour for four weeks, which yielded a dosage of 3.0 mg nicotine/kg/day. The pumps were implanted subcutaneously using aseptic conditions. All animals received a pump containing either saline (control group) or the nicotine solution.

Serum analysis

To determine the nicotine level, blood samples were taken from a tail vein (1 ml) after two weeks of exposure, and at sacrifice. Then serum was stored at –80º C until assayed by gas chromatography (Minneapolis Medical Research Foundation, Minnesota, USA) (9).

Tobacco extract

The tobacco extract solution was prepared every 10 days from nicotine-free Quest Cigarettes (Vector Tobacco Inc., Mebane, NC, USA), as described by Demady et al. (10). Tobacco from four cigarettes was ground with a mortar and pestle and suspended in a 50-ml plastic tube containing 40 ml water. The tubes were mixed overnight at room temperature using a tube tipper to extract the tobacco. The suspension was filtered through gauze and the filtrate was centrifuged at 3000 rpm for 30 min. The supernatant was then vacuum filtered using a 5 μm Whatman® filter paper and then filtered again using a 0.2 μm cellulose acetate filter (Sartorius, Germany). The tobacco extract was stored at 5°C and was diluted 1/30 in tap water immediately prior to administration as drinking water. The daily amount of tobacco extract consumed was recorded.

Surgical procedures (11)

Skin incisional wounds

The back of the rat was shaved. On the left side a 55 mm long incision was made through the epidermis, dermis and subcutaneous muscle parallel to the vertebral column. The incision was 20 mm from the midline and had the thoracolumbar transition as midpoint. The edges of the wound were adapted and fixed with wound closure strips and dressed with gauze and circular adhesive plaster. The wound was analyzed mechanically after 7 days of healing.

Cellulose sponge implantation

Before implantation the sponges (Polyvinyl Alcohol Foam, Unipoint Industries Inc., USA) were cut into 10 mm x 10 mm x 30 mm and boiled in isotonic saline for 2 x 20 minutes followed by sterilization in an autoclave. Then the dry weight of the sponges was determined. They were then soaked in sterile isotonic saline and divided into

halves, which were readapted by a Vicryl® 3/0 suture under sterile conditions. The sponges were implanted subcutaneously through a transverse lumbar incision. They were placed on the right side, parallel to the vertebral column in the infrascapular region. The incision was closed with skin staples. The sponges were excised after 7 days of implantation, wrapped air tight and stored at -20°C until analysis.

Outcome measures

Mechanical testing (11)

Skin wounds:

After the wound closure strips were removed, three 10 mm wide and 22 mm long strips were punched out perpendicular to the incision line. All subcutaneous muscle was removed with the exception of the area directly beneath the incision line. Skin thickness and specimen width corresponding to the incision line was measured using a sliding caliper. The specimen was clamped in a materials testing machine (Alwetron TCT5, Lorenzen & Wettre, Stockholm, Sweden) and stretched at a constant deformation rate (10 mm/min) until rupture. The test was performed at room temperature with the specimen soaked in isotonic saline. The uniaxial tension resulted in a load-deformation curve and the maximum load was obtained. By normalization with regard to cross-sectional area the maximum stress was calculated. The maximum load and maximum stress values for each rat were calculated as the average of the three specimens.

Cellulose sponges:

After excision of the sponge the newly formed fibrous capsule surrounding the sponge was removed by meticulous dissection using an extra fine razor blade. Then, the suture was removed making it possible to measure the mechanical strength of the granulation tissue holding the two halves together. The testing was performed as described for the wound strips with the exception that the sponges were not soaked in saline during the test.

After mechanical testing, the cross-sectional area of the adapted surfaces of the sponges was determined by making an imprint of both surfaces using black ink. The imprints were then scanned into a computer and the edges traced using ImageJ

(NIH). The exact area was then calculated and used to determine maximum stress. For sponges, the maximum load was further normalized for the collagen content in the sponge.

Histological studies of the scar tissue

From the skin wounds, transverse sections of the wounds were prepared and stained with H.E.

Determination of collagen (11)

After mechanical testing, the two pieces of sponge were dried, defatted in acetone for 48 hours +24 hours, freeze-dried and weighed (DDW). Then, the hydroxyproline content was measured as previously described (12). Briefly, after hydrolysis in 6 N HCl for 16 hours at 100º C, the hydrolysates were neutralized with NaOH. 1 ml of freshly prepared chloramines-T diluted with methyl cellosolve (0.35% w/v) was added to 2 ml of the hydrolysate, then mixed and incubated for 20 minutes. Then, 1 ml 19% (v/v) perchloric acid was added, and after 5 minutes 1 ml 5 % (v/v) p-dimethylaminobenzaldehyde was added. The samples were then incubated for 20 minutes at 60º C and after cooling to room temperature the amount of hydroxyproline was determined photometrically at 561 nm according to included hydroxyproline standards. The measurement was made in duplicate. The amount of hydroxyproline was then converted to collagen by multiplying the amount of hydroxyproline by 7.46 (13).

Statistical methods

Results were expressed as group means ± SEM. Effects of the following exposures were evaluated: tobacco extract, nicotine, tobacco extract plus nicotine, and vehicle. Statistical analysis was performed using Stata 9 and SigmaStat 3.1. Data were tested for normal distribution and homogeneity of variances. Differences between groups were established by one-way analysis of variances (ANOVA) and Student's t-test. In case of heterogeneity of variances non-parametric analyses (Kruskall-Wallis test and Mann-Whitney *U*-test) were performed. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Of the 56 rats operated a total of 44 rats were included in the study. 9 animals were terminated because of open or partially open wounds and 3 died during surgery.
At termination the number of animals in each group was: nicotine (n = 10), tobacco extract (n = 11), nicotine plus tobacco extract (n = 11), and vehicle (saline) (n = 12).

Body weight

The animals were weighed at baseline, on the day of wound surgery / sponge implantation (day 14), and at termination (day 21). The weights are shown in table 1. There was no significant difference in weight between groups ($p > 0.46$) on either of these days.
All groups had a small but insignificant weight loss between baseline and termination ($p > 0.1$).

Table 1: Bodyweight by group at baseline, day 14, and day 21.

Weight (g)	Tobacco extract plus nicotine	Tobacco extract	Nicotine	Vehicle
Baseline	244±6	245±7	247±7	248±6
Day 14 *Wound surgery, Sponge implantation*	234±11	249±11	235±9	243±11
Day 21 *Termination*	229±7	237±8	230±6	242±6

Mean±SEM

Nicotine

The serum nicotine and serum cotinine levels are listed in table 2. Serum nicotine concentrations in the nicotine group and in the tobacco extract plus nicotine group were 60-83ng/ml after 14 days of exposure and 70 ng/ml after 21 days. The corresponding cotinine levels were 537-734 ng/ml and 586-668 ng/ml, respectively. There was no significant difference in the nicotine levels but the cotinine levels were significantly higher in the group receiving tobacco extract plus nicotine compared with the group receiving nicotine alone, both on day 14 ($p = 0.005$) and day 21 ($p = 0.015$) of the study.

Table 2: Serum nicotine and cotinine levels by group on day 14, and day 21.

	Tobacco extract plus nicotine	Tobacco extract	Nicotine	Vehicle
Nicotine (ng/ml)				
Wound surgery, Sponge implantation (2 weeks of exposure)	83±9	0±0	60±9	1±2
Termination (3 weeks of exposure)	70±6	0±0	70±4	0±0
Cotinine (ng/ml)				
Wound surgery Sponge implantation (2 weeks of exposure)	734±33	0±0	537±55	0±0
Termination (3 weeks of exposure)	668±18	0±0	586±26	0±0

Mean±SEM.

Tobacco extract

The average daily intake of tobacco extract is listed in table 3. A significant difference in the average daily intake of tobacco extract between the tobacco extract plus nicotine group and the tobacco extract group was found ($p < 0.005$).

Table 3: Average daily intake of tobacco extract by group.

	Tobacco extract plus nicotine	Tobacco extract	Nicotine	Vehicle
Tobacco extract (ml)	21.1±0.8	24.5±0.7	0±0	0±0

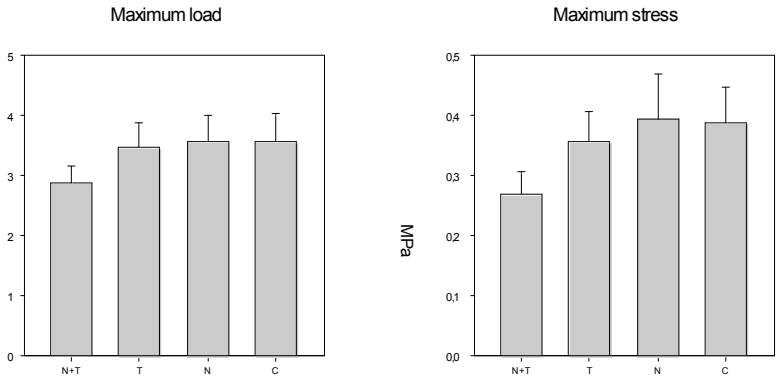
Means±SEM. The extract consumed per day corresponds to water-soluble materials in 39-44 mg dry tobacco.

Mechanical testing

Skin wounds:

The results of the mechanical testing of the wounds after 7 days of healing are given in figure 1. No significant difference between the groups was found for maximum load ($p = 0.53$) or for maximum stress ($p = 0.36$).

Figure 1: Mechanical properties of skin wounds

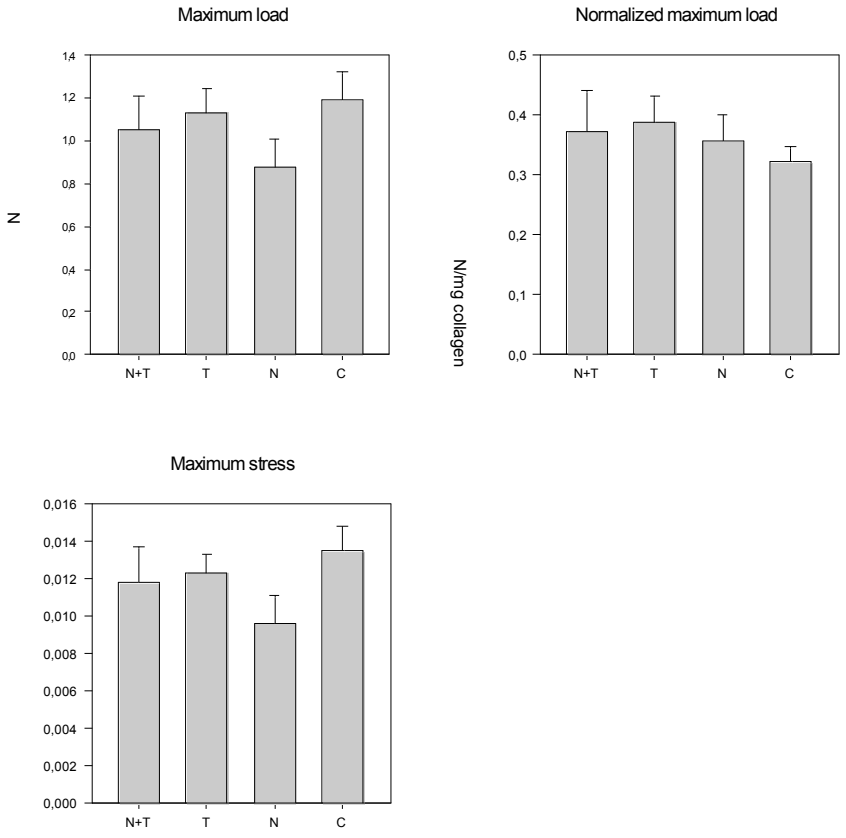


Cellulose sponges:

The results of the mechanical testing of the subcutaneously implanted sponges after 7 days of healing are shown in figure 2.

Again there was no significant difference in maximum load and maximum stress, or in normalized maximum load between the groups ($p > 0.2$).

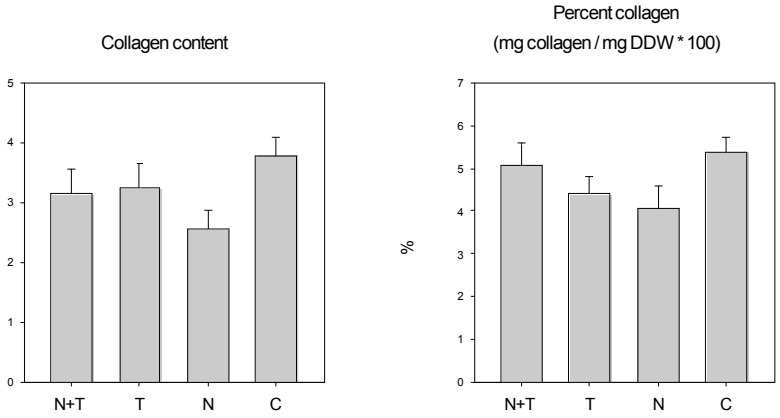
Figure 2: Mechanical properties of sponges



Collagen determination

The amounts of collagen formed in the implanted cellulose sponges and percent collagen (100 * mg collagen / mg dry defatted weight) are given in figure 3.
No significant difference was found between groups in the amount of newly formed collagen or percent collagen after 7 days of healing ($p > 0.15$).

Figure 3: Collagen content in sponges



DISCUSSION

Our study shows no significant impairment of wound healing or granulation tissue formation after a two-week exposure to tobacco extract, nicotine or a combination of these. The results do, however, show a tendency towards an impaired healing (impaired mechanical properties and inferior collagen content) of the wound in the tobacco extract group and the tobacco extract plus nicotine group.
A recent study, using the same exposure groups (8), found an impaired fracture healing in the group exposed to tobacco extract. The dosages of nicotine and tobacco extract administered to the animals correspond to the dosages used in our study. In the fracture study the animals were exposed to nicotine and tobacco extract one week prior to and three weeks after fracture. In light of this, the discrepancies between the outcomes of the two studies are most likely to be due to differences between bone fracture healing processes and skin wound healing.

Administration of substrates

The rats were exposed to nicotine and tobacco extract for two weeks prior to the wound surgery and sponge implantation in order to equilibrate the plasma and tissue concentrations of nicotine and tobacco extract.

Nicotine

Nicotine was administered through osmotic pumps, a method which is a widely used and accepted. The nicotine levels detected (60-83 ng/ml) in the exposed rats correspond to those found in previous studies using the same type of osmotic pump (14) and are slightly above the levels detected in humans smokers (5,15). Therefore, there is no reason to believe that the absent effect of nicotine on wound healing is due to insufficient nicotine exposure. As mentioned earlier the two-week exposure period prior to wound infliction was in order to reach equilibrium. As there was no significant difference in nicotine and cotinine levels on day 14 compared with day 21 this was achieved. However, this study does not take into consideration, the long-term effects of nicotine exposure.

Tobacco extract

The tobacco extract was administered orally in an aqueous solution of non-combusted tobacco. In this way, the tobacco extract is absorbed through the oral and gastro-intestinal mucosa where the absorption may last for some time after drinking. A cigarette smoker is exposed to tobacco smoke through the airway mucosa including the lungs, which may give a different absorption, and as is well known, at least concerning the nicotine, a fast absorption. In addition, the tobacco extract administration as applied does not expose the rats to the tar fraction and other components, which are chemically modified in the combusted tobacco smoke.

In a previous study by Skott et al. a pilot study was performed to examine the absorption of tobacco extract by measuring the urine content of anatabine, a minor tobacco alkaloid present in the tobacco extract. The rats were kept in metabolic cages and anatabine was measured in 24-hour urine samples. They found an overall mean of 15 ng/ml, which corresponds to the urine anatabine levels detected in Caucasians, who smoke 20 cigarettes per day (16).

This verifies that the aqueous tobacco extract solution can be absorbed when administered orally. However, nicotine is continuously infused in the present study and the tobacco extract may be absorbed gradually, whereas smoking is intermittently and implies high peak values of both nicotine and other tobacco compounds, in order to obtain a similar 24-hour urine level (17). Of course, anatabine is only one of the compounds that make up tobacco. With over 3500 compounds in the particulate phase of cigarette smoke it is unlikely that all of these compounds are water soluble, and thus present in the aqueous solution. We assess, however, that an oral exposure of the aqueous solution mimics that of a person ingesting smokeless tobacco such as snuff or chewing tobacco. The pH in the mouth is fairly neutral, thus, the water soluble compounds extracted from the tobacco and included in the tobacco extract solution are likely to be the same compounds that would be absorbed from the oral cavity when smoking or chewing tobacco.

To further investigate this issue a pilot study was performed in which the water solubility was examined. To do so we prepared a single batch of tobacco extract as described earlier, freeze-dried the solution, and determined the dry weight of the substance left in the tube. All tobacco remnants left in the gauze, precipitate and

filters were freeze-dried. This dry material was re-extracted in 99% ethanol in the same manner, and the filtered ethanol extract was evaporated to dryness to obtain the dry weight. The water extract compiled about 38% of the tobacco dry weight and the ethanol extract about 3%. Thus, the ethanol extracted material only represents a small fraction of the tobacco, but the toxic effect of this extract is unknown.

The concentration of tobacco extract to be provided in drinking water has been determined in previous experiments by first extracting tobacco from cigarettes containing nicotine (Quest Low Nicotine) and then calculating how much of this tobacco the rats will need to drink daily to ingest 3 mg/kg of nicotine (1.0 ml). A 1:30 dilution of the tobacco extract provided in place of drinking water for 16 h/day caused an approximately 3 mg/kg dose of nicotine, as the rats drank an average of 30 ml of the solution during the 16 hr exposure period. The same procedure was then used to produce a similar dilution of tobacco extract from nicotine free cigarettes (Quest No Nicotine).
As the rats in our experiment drank slightly less than described in the previous experiment they were supplied with the tobacco extract solution 24 hours a day to ensure sufficient intake.

Even though many steps can be made to ensure and examine the absorption of the tobacco extract solution, this type of exposure may still differ significantly from that of cigarette smoking. Therefore, an interesting future experiment would be to expose the animals to cigarette smoke through a smoke chamber instead of the tobacco extract solution in order to ensure that they receive all the compounds that exist in combusted tobacco. This oral absorption of tobacco used in this study does, however, mimic that of person using smokeless tobacco products.

Wound healing models

Skin incisional wounds

The main problem with this model is to avoid disturbance of the mechanical properties of the wound when preparing it for testing and mounting the strip in the clamps. The hardest part is removing the adhesive plaster and the wound closure strips. The skin was soaked in saline to facilitate removal of the strips, which was done very cautiously. Also, punching out the strips and measuring the cross sectional

areas of the wounds were carefully done by hand. As uniform measurements are crucial to the outcome, the same person did the procedures to avoid inter-individual variation.

Cellulose sponges

Strength development in a wound is based primarily on collagen formation. After subcutaneous implantation of a cellulose sponge, granulation tissue, containing collagen, will form in the sponge, without admixture of old, pre-existing collagen. The sponge can then be excised and collagen deposition can be estimated by measuring the content of hydroxyproline in the sponge. Hydroxyproline is an amino acid quantitatively found almost exclusively in collagen and thus, it can be used for estimation of collagen deposition (11).

The sponge model used is widely accepted as a means in describing wound healing with correlation between collagen deposition and mechanical strength development. The granulation tissue formation has been shown to parallel the development of mechanical strength in skin incisional wounds in the early proliferative stage of healing (11, 18). One of the problems with the model is the fibrous capsule that forms around the sponge during healing. Dissecting away this granulation tissue before testing is necessary and must be done very carefully in order to avoid loss of sponge and thereby ingrown granulation tissue.

During mechanical testing it was often apparent that granulation tissue in-growth was unequally bridging the sutured pieces of sponge. Thus, when most pronounced, the granulation tissue lacked in one side of the sponge and could be due to hemorrhage, serous exudation or the absence of blood vessels available for angiogenesis in the sponge. However, the unequal bridging was observed in the majority of the rats but since the problem was uniform in all groups, none of the sponges were excluded from the study.

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