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Original Article

NICOTINE EFFECTS IN PROLIFERATION OF MYOFIBROBLASTS AND BLOOD VESSELS ON ABDOMINAL WALL SCAR TISSUE OF SUCKLING RATS: IMMUNOHISTOCHEMICAL STUDY

Influência da nicotina na proliferação de miofibroblastos e de vasos sanguíneos no tecido cicatricial da parede abdominal de ratos lactentes: estudo imunoistoquímico

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ABSTRACT – ***Background*** - Smoking during pregnancy is detrimental to the intrauterine growth of the child. As in pregnancy, its effects on lactation and breast-fed child is the sum of adverse infant development. ***Aim*** - To analyze the effects of nicotine on the proliferation of myofibroblasts and of blood vessels, on the abdominal scar of suckling rats born of mothers that received nicotine, during pregnancy and lactation. ***Methods*** - Pregnant Wistar rats were randomly divided into two groups, the nicotine group (NG), in which nicotine was administered subcutaneous at a dose of 2 mg/kg/day, during pregnancy and lactation, and the control group (CG), that received subcutaneous isovolumetric saline solution at 0,9%, in the same period of time. Sixty baby rats, divided into two groups, were weaned at 21 days and a transverse laparotomy was performed. Each group was divided into two subgroups, each one containing 15 baby rats, in accordance with the date when the surgical scar was analyzed: on the 7th post-operative day, control subgroup C7 and nicotine subgroup N7, or on the 21st post-operative day, control subgroup C21 and nicotine subgroup N21. The surgical scar in the healing area was evaluated by immunohistochemistry study for identification of myofibroblasts and of blood vessels. The statistical analysis was based on the Anova model, at a significance level of 5%. ***Results*** - The subgroup N7 presented lower number of myofibroblasts (9,93 ± 3,06 vs. 21,87 ± 9,07, p=0,007) and lower number of blood vessels (8,33 ± 4,43 vs. 13,4 ± 5,33, p=0,031) when compared to C7. The subgroup N21 presented no significant difference on the number of myofibroblasts (7,47 ± 3,96 vs. 12,00 ± 7,21, p=0,121) and on the number of blood vessels (9,47 ± 2,42 vs. 12,93 ± 4,35, p=0,090) when compared to C21. ***Conclusion***: Adverse effect of nicotine, administrated in pregnancy and lactation period, was observed on the proliferation of myofibroblasts and of blood vessels on the abdominal wall wound healing of suckling rats.

HEADINGS – Wound healing. Laparotomy. Nicotine. Immunohistochemistry.

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RESUMO – ***Racional*** - O tabagismo na gestação acarreta prejuízos para o crescimento intra-uterino da criança. Assim como na gestação, as suas consequências sobre a lactação e a criança amamentada constituem somatório de efeitos prejudiciais ao desenvolvimento do lactente. ***Objetivo*** - Avaliar a influência da nicotina na proliferação de miofibroblastos e de vasos sanguíneos no tecido cicatricial da parede abdominal de ratos lactentes nascidos e amamentados por ratas que receberam nicotina durante a gestação e lactação. ***Método*** - Ratas Wistar prenhas foram divididas aleatoriamente em dois grupos; um grupo recebeu nicotina (GN) na dose diária de 2 mg/kg de peso durante a gestação e lactação; o grupo controle (GC) recebeu solução fisiológica a 0,9%, no mesmo período. Foram utilizados 60 ratos lactentes Wistar, divididos igualmente em dois grupos. Após o término do período de lactação (21 dias), todos foram submetidos à laparotomia transversa e ao fechamento da camada músculo-aponeurótica. Cada grupo foi dividido em dois subgrupos, com 15 animais cada, de acordo com o intervalo de tempo utilizado para a avaliação. As aferições foram realizadas no 7.º dia (subgrupos C7 e N7) e no 21.º dia (subgrupos C21 e N21) de pós-operatório. Foram retirados os retalhos da parede abdominal contendo a linha de sutura, os quais foram estudados pelo método imunoistoquímico para identificação de miofibroblastos e de vasos sanguíneos. A estatística foi baseada no modelo ANOVA, ao nível de significância de 5%. ***Resultados*** - No subgrupo N7 houve redução do número de miofibroblastos (9,93 ± 3,06 vs. 21,87 ± 9,07, p=0,007) e do número de vasos sanguíneos (8,33 ± 4,43 vs. 13,4 ± 5,33, p=0,031) em comparação ao C7. No subgrupo N21, não houve diferença significativa do número de miofibroblastos (7,47 ± 3,96 vs. 12,00 ± 7,21, p=0,121) e do número de vasos sanguíneos (9,47 ± 2,42 vs. 12,93 ± 4,35, p=0,090) em comparação ao C21. ***Conclusão*** - Observou-se o efeito deletério da nicotina no período de gestação e lactação sobre a proliferação de miofibroblastos e de vasos sanguíneos na cicatrização dos filhotes.

DESCRITORES - Cicatrização de feridas. Laparotomia. Nicotina. Imunoistoquímica.

INTRODUCTION

In the 90s, the National Cancer Institute (INCA) has estimated that one third of Brazilian adults smoke, and approximately 11.2 million of them were women. Ninety percent of them became dependent at a young age, and the incidence rate was higher in smokers aged 20 to 49 years[5].

Fortunately, thanks to public policies on smoking prevention, according to the survey results for Surveillance of Risk Factors for Chronic Diseases and Protection through telephone (Vigitel Brazil 2008), decreased the percentage of smokers to 17.2% in total population[4].

Smoking during pregnancy is detrimental, recognized and reported, for the intrauterine growth of the child. In the '90s, the population of pregnant women, 33.5% were smokers[15]. Just as in pregnancy, the consequences of maternal smoking on lactation and breast-fed child is the sum of adverse infant development[21].

Among the various components of tobacco that affect the outcome of pregnancy there is the action of nicotine and carbon monoxide[18]. Nicotine is the principal vasoactive component in tobacco smoke, odorless and colorless, and when inhaled or injected can release catecholamines and result in vasoconstriction and decreased tissue perfusion[13].

Several studies in the literature investigated the effects of nicotine on the healing process[3]. In 1977, Mosely and Finseth were the authors who initially reported the adverse effects of nicotine on the healing tissue, observing delayed wound healing in the hand of a smoker[20].

Several studies have reported inhibition of fibroblast proliferation by direct action of nicotine, as well as reducing the production of collagen[8,13] and angiogenesis[8].

The influence of maternal smoking during pregnancy and lactation on the healing of wounds in infants, was the subject of a previous study that showed worsening tensiometric patterns of tension, maximum strength and breaking strength of the scar tissue of pups exposed to nicotine[1]. However, further studies were needed to clarify which structures were related to the reduction of scar tissue strength in the tensiometer.

The aim of this study is to evaluate experimentally the influence of nicotine during gestation and lactation on the healing of the abdominal wall of rats and infants breastfed rats that received nicotine in relation to proliferation of myofibroblasts and blood vessels in scar tissue.

METHOD

Were used 90 adults Wistar rats, with 90 days of life, being 45 males and 45 females, forming couples randomly mated in a 1:1 ratio. Of 45 females, all nulliparous with an average weight of 260 g, 35 of them became pregnant, belonging 19 rats to the nicotine group (NG) and 16 in the control group (CG). Were selected, at random, four rats of NG group with 43 puppies in total, and four other rats in CG, totaling 44 pups. The animals received standard balanced diet and water ad libitum.

In NG animals nicotine was administered subcutaneously at a dose of 2 mg per kg day in two divided doses of 1 mg/kg of body weight each (12/12 hours), diluted in 0.3 ml of saline solution at 0,9%, adjusted to pH 7.4. In animals of CG solution at 0.9% was applied, on volume of 0.3 ml twice daily (12/12 h) subcutaneously and being adjusted to pH 7.4. Were administered both nicotine (NG) and saline (CG) from day 2 of pregnancy throughout the pregnancy and up to 21 days of nursing the puppies. Both doses were adjusted according to weight, when necessary, until immediately after delivery and the end of lactation.

The newborns were breastfed by their mothers, weaned at 21 day of life and underwent surgery immediately after weaning. They were randomly selected in a total of 60 pups.

The surgical technique consisted, in all groups, in a supra-umbilical transverse incision involving all levels of the abdominal wall with 30 mm in length, starting on the right and crossing the midline.

The plans dieresis - cuticular, aponeurotic, muscle and peritoneal - started with incision of 5 mm done with a scalpel, held at the lateral edge of transverse laparotomy. There was, then, the expansion of the incision using an iris scissors in the same direction of skin incision, opening a total of 30 mm in all planes of the abdominal wall.

Continuous non-anchored suture was performed, en-block, interesting the aponeurosis and musculature, using multifilament polyglactin 4-0. In the skin was used anchored non-continuous subcuticular suture with multifilament polyglactin 7-0. The surgical wound was maintained without dressings.

Groups CG and NG were randomly divided into four subgroups, each containing 15 animals, according to the solution applied in the mothers during pregnancy and breastfeeding: animals at 7th day postoperatively, being divided into C7 and N7, and animals dead on 21 days postoperatively, being divided into C21 and N21.

Were removed the abdominal surgical scars and block, and divided into fragments containing the scar with 5 mm long and 30 mm wide, immersed in 10% buffered formalin and sent for histology.

Immunohistochemical study used the technique described by Hsu, et al.[16]. They applied the primary antibodies anti-factor VIII at a dilution of 1:200 and anti-α-smooth muscle actin in separate slides.

Positive results of immunohistochemical staining was identified in the areas of brownish pigmentation. Positive and negative controls were

used. The slides were analyzed without identifying the groups of animals.

The identification of myofibroblasts was performed by counting the cells positively stained by immunohistochemical staining with monoclonal anti-α-smooth muscle actin in high-power field (40X objective) in three different fields randomly along the suture of the abdominal wall corresponding to an area of 17.726 μm^2 expressed as arithmetic means. The digital images were captured and analyzed by specific software, through an objective tool previously calibrated to 40 times. There was used a system consisting of an optical microscope coupled with a videocamera connected to computer and high-resolution color monitor.

The identification of blood vessels was performed by counting the number of circular structures positively stained by polyclonal anti-factor VIII, which shows endothelial cells of the intima of the vessels. The counting was done in the area of the suture of the abdominal wall in an increase of 40 times in three different fields randomly into 17.726 μm^2 area, expressed as arithmetic means.

Statistical analysis was performed in 8 rats, 4 rats in CG and other 4 rats NG, and 60 pups were randomly selected from the CG and NG, with 30 pups belonging to the GC, subdivided into groups C7 and C21 group, and others 30 pups belonging to the NG, subdivided into groups N7 and N21.

Statistical analysis was performed with the Fisher-Snedecor test. It was compared the change in weight during lactation and collate the data of immunohistochemistry. Considering the hierarchical structure of data, selection of mothers and after selection of pups of each mother, with measurements over time (longitudinal data) was more appropriate to use the model the Conditional Hierarchical Linear Models (Anova), at the level of 5% significance.

RESULTS

The average initial weight of pregnant rats of CG was similar to the average initial weight of rats in NG (259.50 ± 21.46 g vs. 265.50 ± 8.70 g, p=0.6228). The groups completed the gestational period with similar average final weight (376.50 ± 20.87 g vs. 368.00 ± 10.42 g, p=0.4937).

In lactation, the rats of NG had similar average final weight of the rats in CG (310.50 ± 18.57 g vs. 317.50 ± 26.74 g, p=0.6822). Comparing the average weight gain also were similar between groups (33.50 ± 17.29 g vs. 38.50 ± 15.15 g, p=0.6788).

The comparison of the average weight during lactation, of the 30 CG and another 30 pups NG showed no significant difference at any examined time: at birth (p=0.4544), at 7 days (p=0.3447), 14 days (p=0.6056), at weaning (p=0.4264) and weight gain in the total period of lactation (0.4475).

The immunohistochemical results are summarized in Table 1 and Figures 1 and 2.

For the group of animals sacrificed at 7th day postoperatively, it was observed that in the NG the average number of myofibroblasts was less in comparison to CG (9.93 ± 3.06 vs. 21.87 ± 9 07, p=0.007). For the group of animals killed at 21 days after surgery, the number of myofibroblasts in the NG was similar in comparison to controls (7.47 ± 3.96 vs. 12.00 ± 7 , 21, p=0.121). (Table 1 and Figure 1).

Even in animals sacrificed at 7th day postoperatively, the NG had a lower mean number of blood vessels compared to CG (8.33 ± 4.43 vs. 13.4 ± 5.33, p=0.031) . In animals sacrificed at 21 days after surgery, the NG had a mean number of blood vessels similar in comparison to CG (9.47 ± 2.42 vs. 12.93 ± 4.35, p=0.090). (Table 1 and Figure 2).

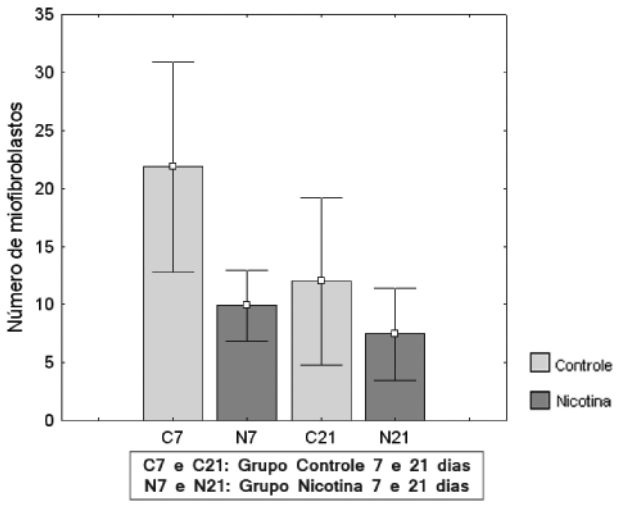

FIGURe 1 - Mean ± SE number of myofibroblasts subgroups C7, N7, N21 and C21

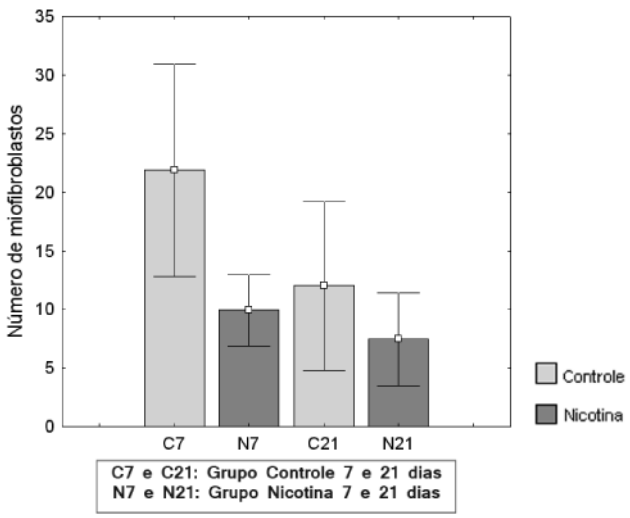

FIGURA 2 - Mean ± SE number of blood vessels of the subgroups C7, N7, N21 and C21

TABLE 1 – Mean number of myofibroblasts and blood vessels of the subgroups C7, N7, N21 and C21

			Measure	
Variable	Grup	N	Mean ± SE	P
Number of myofibroblasts	C7	15	21,87 ± 1,75	0,007
	N7	15	9,93 ± 1,75	
	C21	15	12,00 ± 1,50	0,121
	N21	15	7,47 ± 1,50	
Number of blood vessels	C7	15	13,4 ± 1,27	0,031
	N7	15	8,33 ± 1,27	
	C21	15	12,93 ± 0,91	0,090
	N21	15	9,47 ± 0,91	

NOTE: SE = Standard error, C7 and C21=Control 7 and 21 days, N7 and N21=Nicotine 7 and 21 days. The p value indicates the rejection of the null hypothesis at significance level of 5%. Used the Fisher-Snedecor test

A decrease of the average number of myofibroblasts from C7 to C21 (21.87 ± 9.07 vs. 12.00 ± 7.21, p=0.013) was observed. However, no difference existed in the number of myofibroblasts from N7 to N21 (9.93 ± 3.06 vs. 7.47 ± 3.96, p=0.345).

There was no difference in the average number of blood vessels from C7 to C21 (13.4 ± 5.33 vs. 12.93 ± 4.35, p=0.780) and the N7 to N21 (8.33 ± 4,43 vs. 9.47 ± 2.42, p = 0.507).

DISCUSSION

Nicotine use during pregnancy and lactation was associated with decreased formation of the number of blood vessels in the abdominal wall scar tissue in animals sacrificed at 7 day postoperatively (p=0.031). For animals killed at 21 day of postoperative vascular neoformation was similar between groups. Also in this range between 7 and 21st days postoperatively, no difference in angiogenesis was seen in animals of NG and CG groups.

Angiogenesis is the formation of new blood vessels through a process whereby capillary sprouts are formed in response to external chemical stimuli. This occurs during embryogenesis, the growth of solid tumors and in wound healing. The development of new vessels is essential in healing and is related to local factors, chemical mediators, extracellular matrix, metabolic gradients and physical strength[2,23].

According to Zimmerman and McGeachie, using electron microscopy, nicotine causes changes in the lumen of blood vessels, causing vacuolation, mitochondrial swelling and subendothelial edem[33]. Riebel, et al.[26] concluded that nicotine causes a decrease in the order of 50% growth in vascular grafts. Pinto, et al.[24] observed that nicotine decreases the formation of fibroblasts and blood vessels in the ossification of dental alveoli, and concluded that nicotine inhibits angiogenesis. Chibata observed that nicotine reduces angiogenesis in the abdominal wall on the 7th day 7 and Skinovsky reported reduced proliferation of blood vessels in intestinal anastomoses of rats under the effect of nicotine in 28 day postoperatively[30].

Also observed that nicotine use during pregnancy and lactation influenced the proliferation of myofibroblasts in the scar tissue of pups sacrificed on the 7th day after surgery. There was a lower number of myofibroblasts in the animals of NG (p=0.007). For animals killed at 21th day postoperatively, there was no difference between control and nicotine on the number of myofibroblasts. In the interval between 7 and 21 days postoperatively, the NG did not differ in the number of myofibroblasts in the scar, while the CG had a decrease (p=0.013).

Several studies related nicotine as inhibitor of the proliferation of fibroblasts, which, when stimulated, undergo modification of their phenotype. This change may be caused by the action of nicotine in other cells and/or chemical mediators involved in the proliferation of fibroblasts, lymphocytes and possibly macrophages[7,31].

According to Tipton and Dabbous Chamson[31] and Frey and Hivert[6] myofibroblasts are cells that produce collagen, predominantly type I, and their loss could be related to reduced synthesis of this protein.

Chamson, et al.[7] in vitro study, concluded using electron microscope that the ultrastructure of fibroblasts is altered by smoking, characterizing cell toxicity and growth inhibition of these cells .

Angiogenesis is related to the release of growth factors produced by various cells involved in wound healing. The fibroplasia and collagen synthesis begin in the first 24 hours after injury, while the following 48 to 72 hours is the migration of the endothelium. From the fourth day, the macrophages produce growth factors that stimulate both fibroblast proliferation and neovascularization[22].

The tensile strength is related to the proliferation and maturation of fibroblasts. Harvey[14] said it is necessary period of about four days for the maturation of fibroblasts. The intervals used to evaluate angiogenesis and the formation of myofibroblasts were 7 and 21 days after the surgical procedure. Myofibroblasts are found in the scar tissue between the fourth and sixth day after injury, with greater proliferation between 8 and 30 day 8 of the healing process[9].

In a study of angiogenesis in wound healing, Ruiter et al.[27] used as immunohistochemistry antigen the PAL-E, which is highly specific for the endothelium of blood vessel; but the factor VIII and CD 34 are more sensitive and are also expressed in arterioles 26. In this study the count of blood vessels was performed by immunohistochemistry using factor VIII antigen, which was very appropriate.

Immunohistochemistry was also used in this study for myofibroblasts using α-smooth muscle actin (anti-α-sm) as antibody, first studied by Skalli, et al.[29], which demonstrated positivity for identifying such cells.

The use of animals of both sexes in the study, with maximum time of 42 days of life, was based on

the study of Hughes and Tanner, who compared the morphometric variables from birth to adulthood, and found no differences related to sex before 50 days of life in the variable weight[17].

The use of the abdominal wall was chosen to study the importance of closing this segment in laparotomies, especially because they are transverse incision type most used in newborns and children. It is a type of anatomic incision that follows the lines of force skin.

Regarding the technique used in suture, continuous or not, it was decided in this study to use continuous[28]. Another important factor is the type of material to be used because it interferes on the healing process. The ideal is that one which produces the smallest possible biological reactivity. There is evidence that the monofilament nonabsorbable cause less tissue reaction and, consequently, less interference in the healing process[32]. Was chosen the multifilament poliglatina for being the kind of material which is usually done in the closure of laparotomies in humans.

The daily subcutaneous injections of nicotine proved to be well tolerated by animals and rapid drug absorption, plasma levels detected at five minutes[11]. This form of subcutaneous administration of nicotine has been described by several authors[8,30].

The dose used in this study (2 mg per kg in two daily doses) was based on plasma samples from human chronic smokers. According to Richardson, et al.[25]. Is equivalent to the consumption of 60 cigarettes a day .

Forrest, Pang and Lindsay[12] concluded that smoking affects the healing process when it does not stop for at least two weeks before the operation.

The effect of maternal exposure to nicotine subcutaneously during gestation and lactation, was evaluated for lung development in the neonatal period, and concluded that in pups whose mothers were exposed to nicotine, there was a decrease in the number of capillaries septa[19].

Regarding the duration of drug exposure, to measure with complete safety, was used the average gestation time of the mice, from the observation of pregnancy and throughout the lactation period (21 days), so that the pups were exposed via placenta and lactation at continuous similar doses of nicotine.

CONCLUSION

Nicotine use in rats at a dose of 2 mg/kg a day during pregnancy and lactation affects the abdominal wall healing in their respective offspring, when the surgical procedure is performed soon after weaning, by decreasing on 7 day after surgery, the proliferation of the number of myofibroblasts and angiogenesis in the healing phase. Such changes are probably involved in the reduction of tensiometric parameters of the wounds.

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