

Expression of collagen in ovular membranes of pregnant smokers and non-smokers: a pilot study

Ekspresja kolagenu w błonach płodowych u ciężarnych palących i niepalących: badanie pilotażowe

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Abstract

Objective: Our study compared the amount of total collagen and type I collagen in ovular membranes of pregnant smokers and non-smokers.

Material and methods: The study group consisted of 14 pregnant smokers at 24-36 weeks of gestation; 39 pregnant non-smokers between 24-36 weeks of gestation comprised the control group. The expressions of total collagen and type I collagen were analyzed using two histological sections of the fetal membranes. The assessment of total collagen was performed using the Picro-Cirius red stain, and type I collagen expression was determined by means of immunohistochemistry. The Mann-Whitney test was applied to verify possible differences between the groups.

Result: The average area covered by total collagen was lower in smokers (20630.45 μm^2) as compared to non-smokers (24058.61 μm^2), although the difference was not statistically significant ($p = 0.454$). Comparison involving collagen type I deemed similar results (20001.33 μm^2 vs. 25328.29 μm^2 , $p = 0.158$).

Conclusion: The amount of total collagen and type I collagen was lower in ovular membranes of pregnant smokers as compared to non-smokers, although the difference was not statistically significant.

Key words: **ovular membranes / collagen / smoking / pregnancy /**

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Streszczenie

Cel pracy: W niniejszej pracy porównano ilość całkowitego kolagenu i kolagenu typu I w błonach płodowych ciężarnych palących i niepalących.

Materiał i metody: Badaniami objęto 14 palących ciężarnych w wieku między 24 a 36 t.c., a grupę kontrolną stanowiło 39 ciężarnych niepalących w tym samym wieku ciążowym. Ekspresję całkowitego kolagenu i kolagenu typu I analizowano za pomocą dwóch skrawków histologicznych z błon płodowych. Oceny całkowitego kolagenu przeprowadzono stosując barwienie Piero-Cirius czerwonego barwnika a typ I kolagenu ekspresji oznaczano za pomocą metody immunohistochemicznej. Test Manna-Whitney'a zastosowano do sprawdzenia ewentualnych różnic pomiędzy grupami.

Wyniki: Średnia powierzchnia dla całkowitego kolagenu była niższa u palaczy ($20.630,45 \mu\text{m}^2$) w porównaniu z niepalącymi ($24.058,61 \mu\text{m}^2$), mimo że nie była istotna statystycznie ($p = 0,454$). Podobne wyniki uzyskano dla udziałem kolagenu typu I ($20.001,33 \mu\text{m}^2$ kontra $25.328,29 \mu\text{m}^2$ $p = 0,158$).

Wniosek: Ilość całkowitego kolagenu i kolagenu I była niższa w błonach płodowych palących kobiet w ciąży w porównaniu do osób niepalących, chociaż nie była istotna statystycznie.

Słowa kluczowe: **błony płodowe / kolagen / palenie / ciąża /**

Introduction

Premature rupture of preterm ovular membranes affects 2% of all pregnancies, accounting for 40% of prematurity cases [1-3], and is associated with inherent complications like umbilical cord prolapse, chorioamnionitis, higher rate of Caesarean delivery, and endometritis [4-6].

The exact cause of membrane rupture often remains unknown or speculative. Until the 1960s, it was believed to be caused solely by mechanical agents and not normal actions, such as vigorous Braxton-Hicks. A study by Meudt and Hawrilenko [7], involving over a thousand ovular membranes, showed that the membranes remain intact outside pressures greater than those resulting from pregnancy-related contractions. These authors concluded that only weakened ovular membranes rupture when subjected to mechanical stress. On the basis of those findings, researchers turned their attention to factors capable of undermining the amnion and the chorion. Numerous studies have focused on collagen concentration in these membranes, considering that it constitutes the main component of the connective tissue [8]. Some authors reported reduced collagen content in membranes that ruptured prematurely [9-11].

Chorioamnionitis is the condition that more clearly highlights the importance of collagen degradation, with the consequent impairment of the ovular membranes. Deterioration of collagen during an infection is due to the release of toxins by pathogenic bacteria, what activates leukocytes with the consequent production of metalloproteinases, enzymes responsible for the degradation of the collagen matrix [12-14]. Smoking is yet another condition that seems to favor the embrittlement of ovular membranes. The fact that this habit increases the risk of premature rupture of the ovular membranes has been well-documented [15-17]. However, the underlying mechanisms remain to be fully elucidated.

Various studies conducted in different tissues have shown that harmful substances present in tobacco change collagen production both, directly or indirectly [18, 19]. Nicotine appears to inhibit the function of erythrocytes, macrophages and fibroblasts, in addition to the reduction of tissue perfusion, by stimulating the production of catecholamine vasoconstrictors.

Carbon monoxide binds to hemoglobin, hindering the release of oxygen to the tissues, while cadmium lowers the production of procollagen by fibroblasts [18, 19]. On the other hand, smoking reduces the plasma levels of copper and Ascorbic acid, important enzymatic cofactors involved in the production of collagen [15].

Yin et al. [20], studying the behavior of fibroblasts exposed to a tobacco extract, assessed the expression of metalloproteinase with specific inhibitors. The extract reduced the production of procollagen types I and III by 40%. Lahmann et al. [21], evaluated the effect of cigarette smoking on the production of metalloproteinase type 1 from skin samples of 14 smokers and 19 non-smokers exposed to ultraviolet radiation. Using PCR, they determined the amount of messenger RNA for the production of this enzyme to be significantly higher among smokers ($p=0.013$).

In a study involving cutaneous tissue biopsies of 98 volunteers, of whom 47 were smokers and 55 non-smokers, Knuutinen et al. [22], evaluated the amount of collagen and enzymes involved in skin metabolism. They found concentrations of type I procollagen to be 18% lower in smokers as compared to non-smokers (115.9 vs. $95.6 \mu\text{g L}^{-1}$, $p=0.08$), and levels of metalloproteinase type 8 to be about 50% higher in smokers as compared to non-smokers (16.4 vs. $7.9 \mu\text{g L}^{-1}$, $p<0.001$). The concentration of tissue metalloproteinase inhibitors was 14% lower in smokers than non-smokers (185.3 vs. $214.9 \mu\text{g L}^{-1}$, with $p<0.01$).

As far as the specific action of tobacco is concerned, ovular membranes exposed to cigarette smoke extract have increased apoptotic substances such as caspase 3, which are involved in many proteolytic processes [23]. In 2011, a new study from Menon et al. [24], demonstrated that when the egg membranes were exposed to cigarette smoke extract, major F2 prostanoid production occurred (242.8 pg/mL/mg vs. 131.5 pg/mL/mg, $p<0.0001$). This substance is, admittedly, an important marker of oxidative stress that is responsible for cell apoptosis, including in fibroblasts. This oxidative action of tobacco was once again proven by Menon et al. [25], who exposed term ovular membranes that had not suffered premature rupture to cigarette smoke extract and found it increased mitogen-activated protein kinase (MAPK), triggering enzyme p-38, another important marker of oxidative stress.

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To the best of our knowledge, the literature offers no reports about the deleterious action of harmful tobacco agents on collagen within the ovular membranes. Therefore, we opted to investigate the increased risk of premature ovular membrane rupture in pregnant smokers versus the reduced level of collagen in the amnion and the chorion.

Objectives

Considering that cigarettes can reduce the amount of collagen in numerous tissues, we hypothesized that the same effect would occur in fetal membranes. Our present study compared the expression of collagen type I and total collagen in ovular membranes of pregnant smokers vs. non-smokers.

Material and methods

We conducted a cross-sectional study involving ovular membranes of 53 pregnant women at the gestational age of 24-36 weeks, who gave birth in the maternity wards of the Irmandade da Santa Casa de São Paulo (ISCMSp), São Paulo-SP, Brazil. In light of the fact that the literature offers no studies with the same characteristics, we did not have the standard deviation to accurately determine the sample size. Therefore, we decided to conduct this study as a pilot, selecting all women who met the inclusion criteria during the recruitment period. All patients signed the informed consent, and the study was approved by the Local Ethics Committee. The trial is listed with the Brazilian Government research platform under the number of 33601014.7.0000.5479.

For the comparative study, the women were separated into two groups. The study and the control groups included 14 and 39 women, respectively. Two researchers (MCC and ARSR) selected the groups and collected the samples. All data were analyzed by another researcher (RN), blinded to the treatment groups.

Both groups had the following exclusion criteria: age >35 years, maternal diseases, which involved amendment of collagen, fetal malformations, changes in the placenta, umbilical cord and amount of amniotic fluid, premature rupture of membranes, use of illicit drugs, medications or any clinical complications or emergencies that may promote degradation of vascularization of the fetus, placental preeclampsia, heart disease, diabetes, anemia, multiple pregnancy, cohabitation with smokers during pregnancy, and low weight considering the body mass index (BMI) [26]. We included only pregnant women who reported daily consumption of six or more cigarettes a day.

Initially, the placenta and ovular membranes were placed in plastic containers containing formaldehyde solution at a concentration of 10%. The tissues were then sent to the Pathology Department of the Central Hospital of ISCMSp, where the membranes were separated from the placenta. Next, a ribbon-shaped fragment (3 cm x 1 cm) of the extension point located approximately 5 cm below the placental membrane reflex threshold of each case was taken. Each tape was wrapped, constituting the so-called *jelly roll*. After preservation in formalin, these fragments were wrapped in paraffin. Each block of paraffin contained two histological cuts (3 μ m in thickness), using a rotary microtome. One of the cuts, which was earmarked for evaluation of total collagen, was stained by Picro-Cirius red, which stains collagen fibers [27]. The other cut, used to evaluate the expression of type I collagen, was stained by immunohistochemistry using streptavidin-biotin tertiary antibody-peroxidase (Dako LSAB Kit

Systems), which stains type I collagen fibers. Reagent preparation was done immediately before use to avoid dilution bias.

The examination of the cuts stained with Picro-Cirius red was conducted using an optical microscope Axioscope model 40 (Carl Zeiss of Brazil), with 10x eyepiece and 20x objective. The microscope was coupled to MRc AxioCam camera (Zeiss) for obtaining digital images of the material using Axiovision 5.1, whereby three images of random points were obtained at 200x.

The images were analyzed using Image Pro Plus 4.5[®] for the quantification of collagen. For calibration purposes, the caliper of the program was carried out in areas measures square micrometers (μ m²) on the images of each case, using Axiovision 5.1. Thus, it was possible to determine the correspondence between areas in the pictures and measures in the program areas. Then, a color calibration was made so the program would recognize the tonal gradation of red and consequently facilitate the quantification of collagen fibers. Regions with tonality of greater intensity contained greater concentrations of collagen fibers, while the stained in red corresponded to regions with lower concentration of these fibers.

Areas flushed in deep red were interpreted as depositary of collagen (**Figure 1**). Each of the three images was used to determine the amount of collagen. Two types of measurements were performed: one based on the estimates by measuring the length and the width, and another from the marking of an entire outline of the region to be analyzed; we opted for the second to be more precise. Collagen concentration was considered the average of collagens of all photos from that case.

The choice of using immunohistochemistry to quantify the expression of collagen type I was based on the results obtained previously by Keynon et al. [8]. The type of microscope employed in this evaluation was the same as the one used in the analysis of the stained cuts using the Picro-Cirius red stain. The areas showing expression of type I collagen were initially selected and increased 200 times, being photographed in the sequel. The obtained photographic images were analyzed with Image Pro Plus 4.5[®], using the same abovementioned calibration.

The statistical package IBM SPSS (*Statistical Package for Social Sciences*), version 21.0, was used for result analysis. The Mann-Whitney test was used to verify possible differences between the groups. The comparison between the measurements of total collagen and collagen I only was considered as statistically significant at p-value of <0.05.

Results

Mean age in the study group was 27.5 years and mean gestational age was 31.93 weeks. The study group included 1 pregnant woman at 24 weeks, 1 at 25 weeks, 1 at 26 weeks, 2 at 30 weeks, 6 at 34 weeks, and 3 at 36 weeks of gestation. Mean age in the control group was 26.56 years and mean gestational age was 32.13 weeks. The control group included 2 pregnant women at 26 weeks, 1 at 27 weeks, 3 at 28 weeks, 1 at 29 weeks, 3 at 30 weeks, 5 at 31 weeks, 2 at 32 weeks, 2 at 33 weeks, 6 at 34 weeks, 7 at 35 weeks and 7 at 36 weeks of gestation. The statistical analysis revealed homogeneity between the groups, as shown in **Table I**. There was no sample loss. Data collection was scheduled, for financial and administrative reasons, for periods of four and a half months, from June 2014 to October 2014. No harm was expected because there was no clinical intervention.

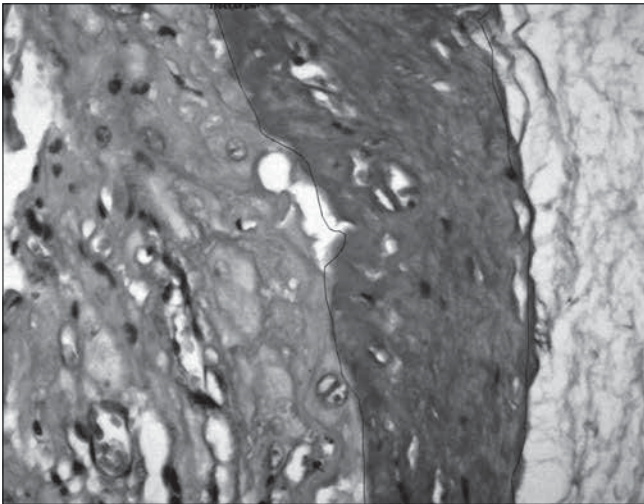
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Figure 1. Picture of collagen staining using Picro-Cirius red. 200x magnification.

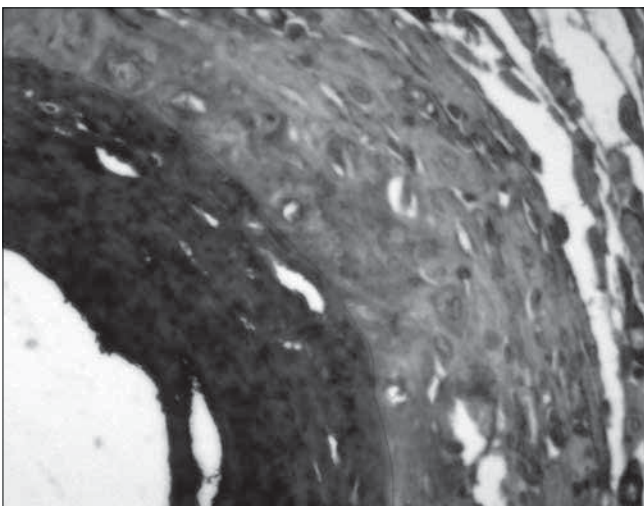


Figure 2. Picture of collagen type I staining using immunohistochemistry. 200x magnification.

The results (**Table II and Figure 3**) demonstrated that mean area occupied by total collagen was 20630.45 μm^2 in the smoking group and 23058.61 μm^2 in the control group, which shows greater difference in favor of the study group, although not statistically significant ($p=0.545$).

In terms of type I collagen (**Table III and Figure 4**), the difference was even greater, with smaller areas of collagen in the study group as compared to controls, with regard to the means of the areas of all the photos (20001.33 μm^2 for the study group and 25328.29 μm^2 for the controls, with $p=0.158$), in which the means of the areas of each of the images were analyzed separately. That difference also lacked statistical significance.

Discussion

Numerous studies have demonstrated the damaging effects of cigarette components on the formation and development of a fetus [28-30]. Recent studies have shown that elevated risk of premature rupture of the ovular membranes is another serious obstetric complication resulting from smoking [15-17]. Notably,

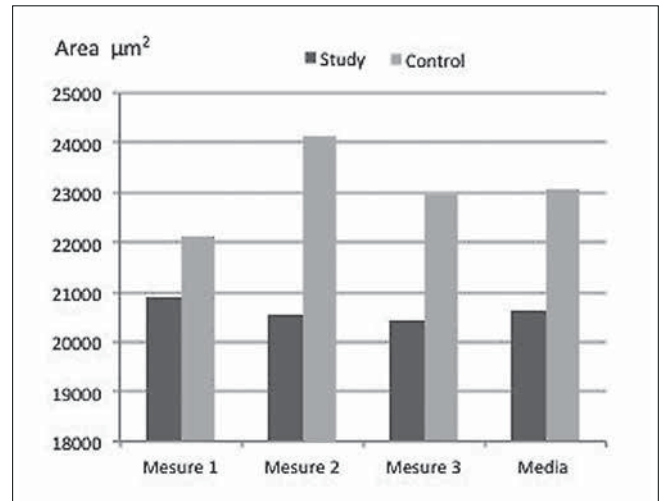


Figure 3. Comparison of means of total collagens in ovular membranes from pregnant smokers and non-smokers.

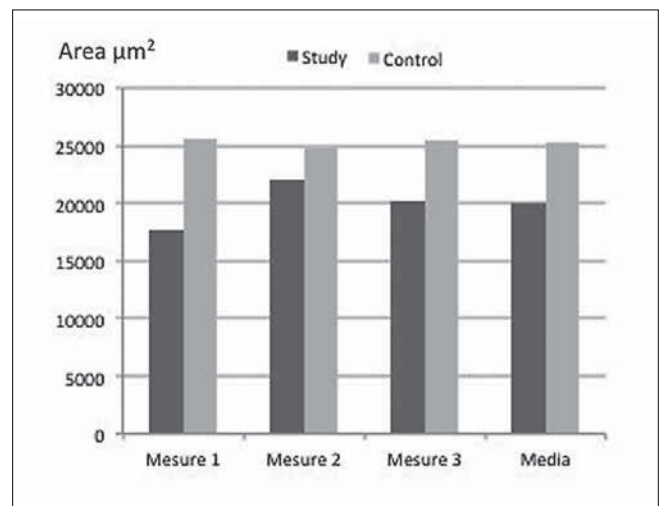


Figure 4. Comparison of means of areas covered by type I collagen in ovular membranes from pregnant smokers and non-smokers.

this event is the main cause of pre-term labor, affecting 30-40% of all pregnancies globally, and the incidence rates continue to increase [31-34]. Collagen deficiency is most likely the fundamental factor for the impairment of the amnion and chorion, with consequent risk of premature rupture. Given the lack of studies related to collagen expression in ovular membranes from pre-term smokers, our present research represents an important step towards understanding the mechanisms of one of the major processes responsible for premature birth.

Our decision to analyze only membranes from pregnant women at the gestational age of 24-36 weeks was based on the importance of membrane rupture in the context of prematurity. It is likely that collagen expression in membranes modify during the gestation period until term. However, this does not have the same implications as the fetus has already acquired sufficient maturity [35].

Importantly, changes in the amount and distribution of collagen in the ovular membranes observed by Skinner et al. [10], are not consistent with our findings. Firstly, because homogeneous

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Table I. Comparison of age and gestational age between the study and control groups.

Variable	Group	n	Average	Standard deviation	Minimum	Maximum	25 th Percentile	50 th Percentile	75 th Percentile	(p)*
Age	Study	14	27.50	7.53	18.00	42.00	21.75	25.50	32.50	0.544
	Control	39	26.56	7.36	17.00	41.00	20.00	24.00	33.00	
	Total	53	26.81	7.34	17.00	42.00	20.50	24.00	32.50	
Gestation- al Age	Study	14	31.93	4.20	24.00	36.00	29.00	34.00	34.50	0.887
	Control	39	32.13	3.47	24.00	36.00	30.00	33.00	35.00	
	Total	53	32.08	3.63	24.00	36.00	30.00	34.00	35.00	

*Mann-Whitney test

Table II. Comparison of means of total collagens in ovular membranes from pregnant smokers and non-smokers.

Variable	Group	n	Average	Standard deviation	Minimum	Maximum	25 th Percentile	50 th Percentile	75 th Percentile	(p)*
PA	Study	14	20909.11	10449.13	4007.34	37670.10	13420.98	19212.97	29120.42	0.809
	Control	39	22110.75	11843.39	2519.52	58837.56	13148.06	21764.90	27713.39	
	Total	53	21793.33	11405.44	2519.52	58837.56	13653.37	21224.42	28348.62	
PB	Study	14	20565.35	11274.36	3576.34	39561.78	13000.56	19098.30	29824.38	0.506
	Control	39	24130.54	13394.37	4333.32	58218.90	13530.66	22093.73	31496.22	
	Total	53	23188.79	12860.89	3576.34	58218.90	14116.72	20079.53	30366.93	
PC	Study	14	20416.87	13666.02	2618.97	51673.92	11393.39	17686.20	30090.89	0.375
	Control	39	22934.55	12164.86	1614.68	58179.26	12078.21	23928.17	30552.82	
	Total	53	22269.50	12493.51	1614.68	58179.26	12362.74	21476.65	30248.73	
med P	Study	14	20630.45	9921.30	3604.70	37670.10	13233.51	17427.09	29417.27	0.545
	Control	39	23058.61	11790.61	2916.60	58411.91	15314.33	23105.05	30552.82	
	Total	53	22417.21	11285.68	2916.60	58411.91	14657.68	22733.44	29505.26	

PA, PB and PC: first, second and third photos of stained ovular membranes by Picro-Cirius red. med P: mean of the areas of all photos of slides stained with Picro-Cirius red. *Mann-Whitney test.

distribution patterns occur during each gravid period. Secondly, because the analysis is constant, and thirdly, due to the fact that the study and control groups are similar in terms of mean gestational age. Therefore, even though the arrangement of collagen change was predictable with the advancement of gestation, this fact also occurs among the studied groups due to homogeneity. For the same reason, it can be considered that mean time of exposition to cigarette smoke and the amount of collagen of membranes were similar between the study and the control groups. However, our sample size was small and we did not include patients in the first trimester of pregnancy. It is possible that these changes could occur in the ovular membranes in the first trimester. New prospective studies with larger samples and including pregnant women in the first trimester are necessary to confirm our findings.

Reports by Artal et al. [9], Skinner et al. [10], and Kanayama et al. [11], in which the ovular membranes rupture prematurely, feature decreased collagen content, which has led us to the new hypothesis that tobacco is responsible for its degradation. To reinforce this theory, the literature shows that smokers have significant changes in collagen synthesis within different tissues [15, 18, 22]. There is also an increase in substances responsible for apoptosis and proteolysis in ovular membranes subjected to

cigarette extract [23-25]. For such reasons, the role of collagen in the ovular membranes should be considered as important in the genesis of weakening and, consequently, of premature rupture of membranes.

In our study, the exclusion criteria adopted for both groups were rigorous in order to avoid possible bias in the results, e.g. low birth weight, since there are indications that malnutrition can reduce collagen deposition in some tissues [36, 37]. We included this criterion when we found pregnant women with poor nutrition. Also, the presence of studies that reveal the damaging effects of second-hand smoke has led us to also add cohabitation with smokers (i.e. passive smoking) as a criterion for deletion [38, 39]. Inclusion of pregnant women who consumed up to the minimum of six cigarettes a day was based on the study by Prescott et al. [40], which involved 12149 individual smokers. Certainly, the rigor of the exclusion criteria and a small sample size of pre-term births among smokers in our study suggests the need for greater numbers of participants to confirm our findings.

Although it has not been possible to quantify the entire membrane area covered by collagen, staining with Picro-Cirius red, followed by the analysis of the images, provided adequate knowledge for the quantification of total collagen. The immunohisto-

Romulo Negrini et al. *Expression of collagen in ovular membranes of pregnant smokers and non-smokers: a pilot study.***Table III.** Comparison of means of areas covered by type I collagen in ovular membranes from pregnant smokers and non-smokers.

Variable	Group	n	Average	Standard deviation	Minimum	Maximum	25 th Percentile	50 th Percentile	75 th Percentile	(p)*
IA	Study	14	17730.45	12598.16	2810.13	47789.25	7777.92	15304.57	26025.24	0.074
	Control	39	25665.65	14254.96	4382.18	57406.25	13989.77	24460.38	35123.22	
	Total	53	23569.56	14165.04	2810.13	57406.25	11072.20	23689.37	32223.66	
IB	Study	14	22004.95	15320.10	2394.07	50059.44	7930.29	19852.66	34552.94	0.364
	Control	39	24799.48	13858.59	4398.03	58930.93	16128.76	21858.28	28165.28	
	Total	53	24061.30	14162.46	2394.07	58930.93	14835.40	20840.66	30017.21	
IC	Study	14	20268.60	11826.32	3136.77	49540.05	11742.94	19260.58	26897.55	0.193
	Control	39	25519.73	13075.78	5292.51	57406.25	18028.59	22831.97	30258.08	
	Total	53	24132.64	12859.72	3136.77	57406.25	14215.71	22726.76	29843.41	
med I	Study	14	20001.33	10325.12	2780.32	42496.75	11923.77	19800.16	27898.96	0.158
	Control	39	25328.29	12803.23	4979.89	57406.25	16969.47	23670.18	31507.58	
	Total	53	23921.17	12331.40	2780.32	57406.25	15664.30	22351.70	29285.41	

IA, IB and IC: first, second and third photos of ovular membranes prepared by immunohistochemistry for type I collagen. med I: average of the areas of all photos of slides prepared by immunohistochemistry.

*Mann-Whitney test.

chemical technique presents some challenge in quantification, as it is a qualitative method in most cases. However, with the use of software it was possible to calculate the areas occupied by collagen marked in order to estimate the quantity in ovular membranes. Such facts reinforce the certainty that the results were reliable.

As for type I collagen, chosen because it is the most abundant and most densely concentrated in the egg membranes [41], immunohistochemical staining showed a high degree of effectiveness, as had been earlier demonstrated by studies already carried out in different locations of the body [42], e.g. a research about the content of this type of collagen in the parametrium of women with prolapsed uterus. Our study showed that the levels of total collagen and type I collagen were lower in the membranes of pregnant smokers as compared to non-smokers, although the difference was not statistically significant. These findings reinforce the hypothesis that the elements resulting from smoking cause degradation of collagen and inhibition of enzymes that participate in its production, similarly to other tissues of the body [21, 22].

In view of the absence of planned studies depending on the methodology used in this research, it is important to await further investigations based on more thorough studies. That includes studies that can stratify women for gestational age and the number of consumed cigarettes.

Our results are an example of a red flag as far as the deleterious action resulting from smoking during the gravid-puerperal cycle is concerned, which is especially relevant since the rate of pregnant smokers is still relatively high. Agaku et al. [43], found that in 2008 the rate of pregnant smokers who continued smoking during the last three months of pregnancy was 12.8% [43].

Conclusion

In summary, we observed that the amount of total collagen and type I collagen was lower in ovular membranes of pregnant smokers as compared to non-smokers, although the difference was not statistically significant.

Authors' contribution:

1. Romulo Negrini – concept, assumptions.
2. Edward Araujo Júnior – article draft, corresponding author.
3. Sebastião Piato – study design.
4. Milca Cezar Chade – acquisition of data.
5. Adriana Ribeiro Santos Rios – acquisition of data.
6. Maria Antonieta Galvão Silva – analysis and interpretation of data.
7. José Mendes Aldrighi – revised article critically.

Romulo Negrini et al. *Expression of collagen in ovular membranes of pregnant smokers and non-smokers: a pilot study.***Authors' statement**

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