Histomorphometry and detection of glycosaminoglycans in the endocervical epithelium of pregnant rats after local administration of hyaluronidase

Histomorfometria i wykrywanie glikozaminoglikanów w nabłonku szyjkowym ciężarnych szczurów po miejscowym podaniu hialuronidazy

Monica Regina Lourenço Luz[1], Edward Araujo Júnior[1], Luiz Camano[1], Manuel de Jesus Simões[2], Eduardo de Souza[1]

[1] Department of Obstetrics, Federal University of São Paulo (UNIFESP), São Paulo, SP, Brazil.
[2] Department of Morphology, Federal University of São Paulo (UNIFESP), São Paulo, SP, Brazil.

Abstract

Objective: *The aim of the study was to detect the presence of glycosaminoglycans and to investigate histomorphometric aspects of the endocervical epithelium in pregnant rats after local administration of hyaluronidase.*

Materials and methods: *Ten pregnant rats were randomly distributed into two groups. On day 18 of pregnancy, 1mL of distilled water and 0.02 mL of hyaluronidase were administered to the control group (CG) and the study group (SG), respectively. On day 20 the rats were sacrificed, followed by dissection and removal of the uterine cervix, which was prepared for histomorphometry (endocervical epithelium thickness and leucocyte infiltration) and for immunohistochemistry with alcian blue reaction and its respective blockers. The paired Student t test was used to compare the groups.*

Results: *The SG was characterized by reduced epithelial thickness (mean: 291.01±71.1 vs. 764.30±50.94; p<0.0001) and a larger number of eosinophils (mean: 3.72±1.60 vs. 0.54±0.70; p<0.0001). Alcian blue staining (pH 0.5) indicated a very strong reaction (3+) for the CG. With pH 2.5, the staining was also very intense (4+) in the CG. With methylation, both groups showed negative reactions after alcian blue staining (pH 2.5). With the methylation reaction followed by saponification and with enzymatic digestion of the lamina, staining showed a weak reaction (1+) in both groups.*

Conclusion: *The SG presented with significant alterations related to the reduction of epithelial thickness and an increase in leucocyte infiltration. Furthermore, the use of hyaluronidase resulted in a significant decrease of the sulfated glycosaminoglycans.*

Key words: **cervical epithelium** / **glycosaminoglycans** / **hyaluronidase** / **pregnant rats** /

Streszczenie

Cel: *Celem pracy było zbadanie histomorfometrycznych aspektów i wykrycie glikozaminoglikanów w nabłonku szyjki macicy u ciężarnych szczurów po miejscowym podaniu hialuronidazy.*

Corresponding author:
Edward Araujo Júnior
Department of Obstetrics, Federal University of São Paulo (UNIFESP)
Rua Carlos Weber, 956 apt. 113 Visage
Vila Leopoldina, Sao Paulo – SP, Brazil, CEP 05303-000
tel./ fax: +55 11 37965944
e-mail: araujojred@terra.com.br

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Materiały i metody: *Dziesięć ciężarnych szczurów podzielono losowo na dwie grupy: 1 ml wody destylowanej podawano w grupie kontrolnej (CG) w 18 dniu ciąży, a 0,02 ml hialuronidazy podawano w grupie doświadczalnej (SG) w tym samym czasie. W 20 dniu ciąży szczury uśmiercano, następnie dokonywano wycięcia szyjki macicy, która została przygotowana do badania histomorfometrycznego (grubość nabłonka i infiltracja leukocytów), a także do histochemicznego badania reakcji alcianem niebieskim i jego odpowiednich blokerów. Test t-Studenta wykorzystano dla porównania obu grup.*

Wyniki: *Zwężenie nabłonka szyjki i większą liczbę eozynofili zaobserwowano w SG. SG scharakteryzowała się zmniejszoną grubością nabłonka (średnia 291,01 ± 71,1 vs 764,30 ± 50,94, p <0,0001) i większą liczbą eozynofili (średnia 3,72 ± 1,60 vs 0,54 ± 0,70, p <0,0001). Bardzo silną reakcję (3+) zaobserwowano w CG przy barwieniu alcianem niebieskim (pH 0,5). Przy pH 2,5 zabarwienie było także bardzo silne (4+) w CG. W przypadku metylacji obie grupy wykazały ujemną reakcję po zabarwieniu alcianem niebieskim (pH 2,5). W reakcji metylacji, po której nastąpiła saponifikacja, oraz enzymatyczne trawienie w blaszce, barwienie wykazało słabą reakcję (1+) w obu grupach.*

Wnioski: *W SG odnotowano istotne różnice odnoszące się do redukcji grubości nabłonka i zwiększoną infiltrację leukocytów. Ponadto, istnieje wyraźna redukcja w ilości glikozaminoglikanów związana ze stosowaniem hialuronidazy.*

Słowa kluczowe: **nabłonek szyjki macicy** / **glikozaminoglikany** / **hialuronidaza** / / **ciężarne szczury** /

Introduction

Research on labor induction methods has resulted in greater knowledge about cervical maturation. Numerous methods of evaluating cervical maturation have been described and used over the past few years, but the search for an ideal agent to aid this process continues [1]. Currently used methods of preparation of the uterine cervix before labor induction are mechanical and/or pharmacological.

Among pharmacological methods, we point to the use of hyaluronidase, an endoglycosidase that acts by depolarizing hyaluronic acid present in the cervix in fragments of lower molecular weight [2]. Hyaluronidase was proposed to accelerate the process of cervical effacement and dilation, also in cases of excess in the connective tissue component of the cervix (sclerotic or inelastic cervix). The effect of hyaluronidase seems to be advantageous, since the uterine cervix under normal circumstances in 85% is composed of connective tissue and the medication can affect this tissue excess and solidity [3]. Use of hyaluronidase in cervical ripening of pregnant women has been reported since the 1850s, with doses varying between 200 and 1.000 units, producing varying results on cervical maturation and decrease in labor time [4]. More recently, studies with the administration of higher doses, between 1.000 to 20.000 units of intracervical hyaluronidase, reported better results [5,6].

Experimental confirmation of the intracervical action of hyaluronidase appeared at the beginning of the 1990s. Li [7] observed intense collagenolysis with different degrees of destruction of blood vessels and an increase of the interstitial space in groups of full-term rats who received hyaluronidase as compared controls. Another more recent experimental study by de Souza *et al*. [8], reported that the use of hyaluronidase promoted lamina propria, with looser connective tissue, rich in blood vessels and eosinophils, besides a smaller concentration of collagen fibers.

Cervical ripening is a dynamic and complex process, and modifications of the cervical stroma after a local injection of hyaluronidase have already been evidenced. Nevertheless, data on modifications that might occur in the endocervix induced by this enzyme are scarce. The endocervix possibly also undergoes structural and functional alterations, since it is a region with glandular epithelium and therefore, with secretory activity.

Objectives

The aim of the study was to evaluate structural modifications of the uterine endocervix of female albino rat determined by local administration of hyaluronidase at the end of pregnancy, related to histomorphometric aspects (thickness of the epithelium and leucocyte infiltration), and to detect the presence of glycosaminoglycans.

Materials and methods

Female albino rats (*Rattus norvegicus albinus, Rodentia, Mammalia*), about 90 days of age, virgins, weighing approximately 200g, of the EPM – Wistar lineage, from the *Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia* (CEDEME) were used. Each cage housed five animals maintained on *ad libitum* food (Nuvital CR-1 feed) and water for seven days, considered the adaptation period. Room temperature was approximately 22°C (±2°C), with artificial lighting, fluorescent bulbs/lamps (Philips brand, Light of Day 40-Watt model), with a photoperiod of 12 hours of light and 12 hours of darkness (light period from 7:00am to 7:00pm). The study was approved by the Research Ethics Committee of the *Universidade Federal de São Paulo* (UNIFESP).

After the adaptation period, the rats were mated with the proportion of three females to one male, between 7:00pm to 07:00am, and the onset of pregnancy was determined soon afterwards by using the Hamilton and Wolfe technique [9], represented by the confirmation of sperm in the rat vagina. This was considered as Day 1 of pregnancy.

Ten female rats were randomly distributed into two groups: the control group (CG) and the study group (SG). On day 18 of pregnancy, 1mL of distilled water and 0.02 mL of hyaluronidase, diluted in 0.98 mL of distilled water (1 mL of solution), were administered to the control group (CG) and the study group (SG), respectively, as a single dose, by intracervical route.

To prepare the enzyme (Hyalozima® in suspension form), the ampoule/flask of lyophilized powder was diluted in 4 mL of distilled water with rotating movements around its own axis to avoid formation of foam and to facilitate the dilution of the drug. The recommended dose for a female rat was proportional to that established for humans, equivalent to 20.000 TRU [10].

To apply the hyaluronidase and the distilled water, we used a disposable 1 mL syringe, sterilized with ethylene oxide, under anesthesia (Ketamine and Xylazine 0.1 mg/kg), with the help of a plastic cone-shaped guide, introduced along the entire extension of the rat vagina to guide the trajectory of the Whitacre (B-D) needle. The needle was introduced into the prolonger and the limit of insertion was defined by the sensation of a local barrier. Next, slight pressure was made on the needle at the location, covering 2 to 3 mm through the uterine cervix, where 1 mL of solution was injected, maintaining the needle in place for about 30 seconds to avoid reflux.

On day 20 of pregnancy, the rats were again anesthetized (Ketamine and Xylazine 0.1 mg/kg). After opening the abdomen by means of a median abdominal incision, disjunction of the pubic symphysis was performed in order to facilitate longitudinal opening of the vagina along its entire extension, with visualization of the cervical portion. After careful observation of the pelvic organs, we proceeded to the dissection and removal of the uterine cervix. The pieces were immediately immerged into a solution of 10% buffered formaldehyde for fixation, where they remained for 24 hours.

Next, the pieces were dehydrated in growing concentrations of ethyl alcohol, diaphanized by xylol, impregnated and included in liquid paraffin in a heating chamber regulated to a temperature of 59ºC, in order to obtain longitudinal slices of the uterine cervix for histological examination. Posteriorly, the slices were obtained in a Minot type (Leica, model RM-2035) microtome, adjusted for 5 μm, and collected on slides previously covered with Meyer albumin and maintained in a heating chamber at a temperature of 37º C for 24 hours for drying and collage/adhering.

The slides were stained by the hematoxylin-eosin technique for a morphometric description. We used a Zeiss microscope with an eye piece that magnifies 10 times and objective lenses with 4 to 100 magnifications. With these magnifications, the SG and the CG were evaluated and compared to histomorphometry (epithelial thickness and leukocyte infiltration). Epithelial thickness was measured with the help of Softium-lab computer coupled with a Zeiss microscope. The height of the epithelial cells (μm) was obtained from the digitized images. In order to do that, only slices perpendicular to the endocervical epithelium were considered. Ten measurements were made for each slice. On the lamina propria, leukocytes (eosinophils) per 1 mm^2 were counted, which corresponds to 10 microscope fields on each analyzed slice.

Alcian blue stain was used for the histochemical analysis of the uterine cervix of the rat. Glycosaminoglycans (GAG) are stained blue with alcian blue, where the carboxylated radicals (for example, hyaluronic acid) acquire color only at pH 2.5, while the sulfates are also stained at pH 0.5 [11]. We proceeded to the classic blocks of alcian blue, including techniques of methylation, saponification, and enzymatic digestion with hyaluronidase in order to better characterize the type of GAG in the analyzed tissues.

The process of methylation was performed by treating the slides with methyl alcohol containing 0.4 mL of concentrated hydrochloric acid for every mL of methyl alcohol for 5 hours, heated to 60°C. Next, the preparation was submitted to alcian blue staining at pH 2.5. After methylation, the saponification technique was applied, washing the slides in absolute alcohol and 80% alcohol. Next, the slides were exposed to a solution of 1% potassium hydroxide in 80% alcohol. Finally, the preparation was again exposed to alcian blue staining at pH 2.5. The structures that were positive with alcian blue staining became negative to the same staining after methylation, and went back to being positive after saponification, when the reactions were due to carboxylated radicals and remained negative when they were due to sulfated radicals [12]. We used subjective evaluations (1+ to 4+), indicating a variation of the reaction and the symbol (-) for its negativity to better represent the intensity of the histochemical reactions.

Data were tabulated on an Excel 2007 spreadsheet (Microsoft Corp., Redmond, WA, USA). Paired Student's *t* test was used with a significance level of $p < 0.05$ for comparison among the groups.

Results

1. Histologic analysis

In the CG we noted that the endocervix was formed with stratified mucous epithelium. This epithelium was composed of 5 to 8 layers of cells containing a large quantity of mucus in their interior. The lower cells do not display mucus. In some sites, we noted the presence of leukocytes, especially in the most superficial portion. In the SG we noted that the endocervix was covered by stratified mucous epithelium containing 3 to 5 layers of cells.

In some sites, we identified the presence of mucus cysts. In some areas we noted leucocyte infiltration in this epithelium, especially in mucus cysts and on the surface (Figure 1).

The SG group presented with greater thinning of the endocervical epithelium (mean of 291.01±71.11 *vs*. 764.30±50.94; $p<0.0001$) and a greater number of eosinophils (mean of 3.72±1.60 *vs*. 0.54±0.70; $p<0.0001$) (Tables I and II, respectively).

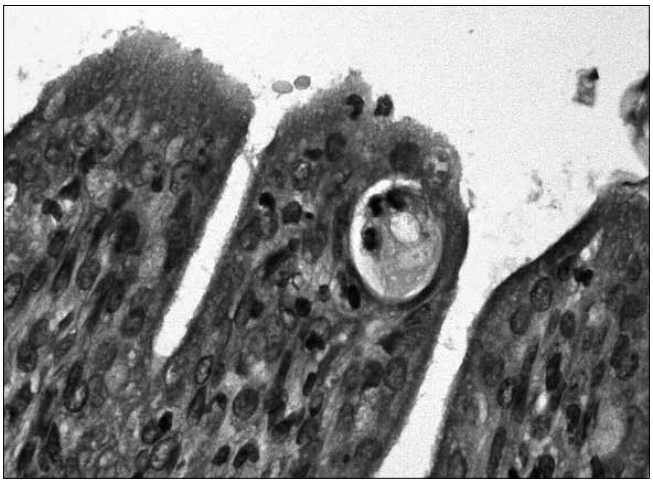

Figure 1. Photomicrography showing endocervical epithelium of a rat from the study group. Note the presence of a cyst containing leukocytes in its interior (400 X).

Table I. Measurement values of thickness of the endocervical epithelium (μM) of the rats on day 20 of pregnancy in controls and the study group.

Epithelial thickness	Groups	
	Control group	Study group
1	759.10	356.50
2	610.24	234.57
3	850.30	258.00
4	750.10	319.00
5	680.20	322.00
6	780.20	300.50
7	810.24	245.67
8	785.35	234.50
9	679.20	278.00
10	750.20	358.90
11	820.30	245.67
12	789.00	235.90
13	658.24	450.23
14	756.12	245.00
15	768.80	349.60
16	712.35	254.89
17	812.45	253.00
18	780.00	450.00
19	760.12	359.00
20	789.30	238.00
21	810.24	240.00
22	760.12	317.00
23	756.12	320.00
24	679.20	305.50
25	759.10	240.67
26	812.45	230.50
27	750.20	270.00
28	759.10	340.90
29	780.00	230.67
30	610.24	220.90
31	812.45	400.23
32	759.10	201.50
33	780.00	343.60
34	789.00	230.89
35	768.80	241.50
36	750.20	450.00
37	820.30	359.00
38	759.10	232.50
39	750.10	222.90
40	770.00	402.23
41	822.30	201.50
42	783.00	343.60
43	785.00	230.89
44	817.00	241.50
45	805.00	450.00
46	798.00	359.00
47	783.00	232.50
48	692.20	233.25
49	795.00	234.15
50	797.24	235.10
Mean	**764.30**	**291.01*/†**
Standard deviation	**50.94**	**71.11**

*Paired Student's *t* test; †p<0.0001

Table II. Values of eosinophil count present in the endocervical epithelium of rats on day 20 of pregnancy in controls and the study group, of a field of view at 40x.

Number of fields	Groups	
	Control group	Study group
1	0	5
2	2	3
3	1	2
4	0	3
5	0	2
6	0	1
7	1	6
8	2	4
9	1	5
10	0	6
11	0	4
12	0	5
13	0	6
14	1	3
15	1	4
16	0	1
17	1	2
18	0	4
19	0	2
20	0	4
21	0	5
22	2	3
23	1	2
24	0	3
25	0	2
26	0	1
27	1	6
28	2	4
29	1	5
30	0	6
31	0	4
32	0	5
33	0	6
34	1	3
35	1	4
36	0	1
37	1	2
38	0	4
39	0	2
40	0	4
41	0	2
42	2	1
43	1	6
44	0	4
45	0	5
46	0	6
47	1	4
48	2	5
49	1	6
50	0	3
Mean	**0.54**	**3.72*/†**
Standard deviation	**0.7**	**1.6**

*Paired Student's *t* test; †p<0.0001

2. Histochemical Analysis

2.1 alcian blue reaction at pH 0.5

Only sulfated GAG stained blue in this reaction. In the endocervical epithelium, a strongly positive reaction (3+) was noted in CG and weakly positive in SG (1+), as is illustrated in Figure 2.

2.2 alcian blue reaction at pH 2.5

With this stain, both sulfated and carboxylated GAG stained blue. In the endocervical epithelium, a strongly positive stain (4+) was noted in CG and a weakly positive reaction (2+) in SG, as is illustrated in Figure 3.

2.3 Methylation followed by alcian blue reaction with pH 2.5

The methylation process impeded staining, both in sulfated and carboxylated GAG; with the alcian blue reaction at pH 2.5, negativity of the reaction in CG and SG was observed.

2.4 Methylation and saponification followed by alcian blue reaction at pH 2.5

In this reaction, only carboxylated GAG stained blue. A weakly positive reaction (1+) was noted in the endocervical epithelium in CG and SG.

2.5 Enzymatic digestion with hyaluronidase

After enzymatic digestion with hyaluronidase, carboxylated GAG did not stain blue with alcian blue reaction at pH 2.5. A weakly positive reaction (1+) was noted in CG and SG.

Discussion

The importance of cervical modifications anteceding and accompanying the birth process has been amply discussed in recent reports. Alterations of the cellular structure of this organ have been studied with great detail, especially the region of its stroma. The uterine cervix has a complex and heterogeneous structure, constituting predominantly of fibrous connective tissue composed of cellular component formed by smooth muscle (5 to 10%), fibroblasts, epithelium, blood vessels, and extracellular matrix [13,14]. Use of rats in this type of experiment is accepted because of functional similarities between human and rat cervical structures, as has already been highlighted by some authors [15, 16].

The cervical mucus is primarily secreted by the cervical glands, with a small portion derived from the cervical interstitial tissue. Therefore, mucus components probably reflect the biochemical changes of the cervical tissue. Ogawa *et al*. [17], in their study of cervical mucus in pregnant women, demonstrated higher levels of hyaluronic acid in those with the diagnosis of inhibited pre-term labor as compared to the control group.

Our results revealed that the endocervical epithelium, as is true in the stroma, undergoes significant modifications determined by local use of hyaluronidase. Morphologically, the epithelium underwent transformation in which its thickness decreased significantly and leukocyte infiltration (eosinophils),

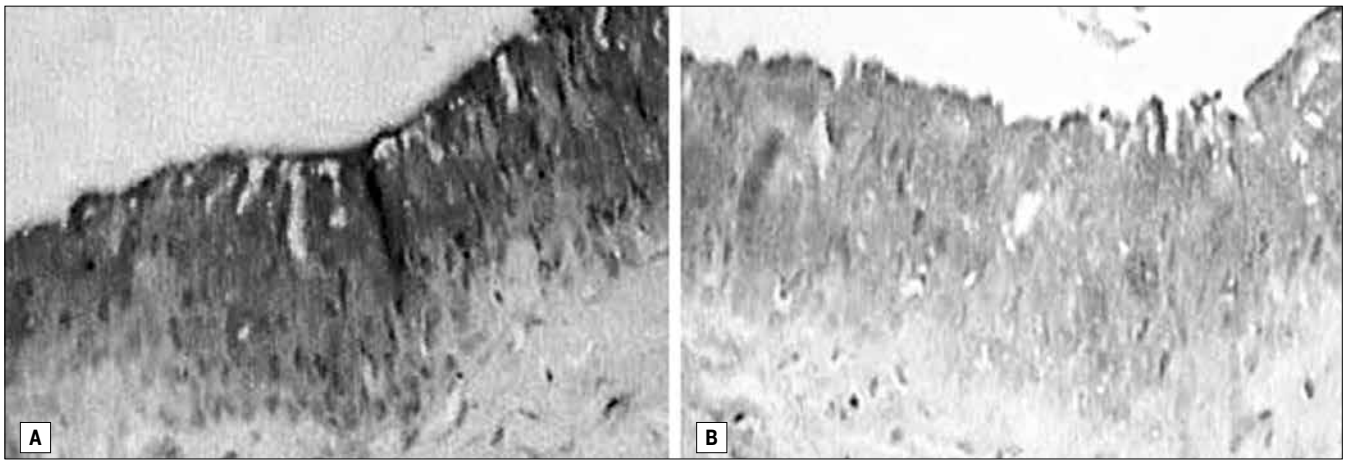

Figure 2. Staining of the endocervical epithelium of the cervix of a rat from the control group (**A**) and from the study group (**B**) with alcian blue reaction at pH 0.5 (400X).

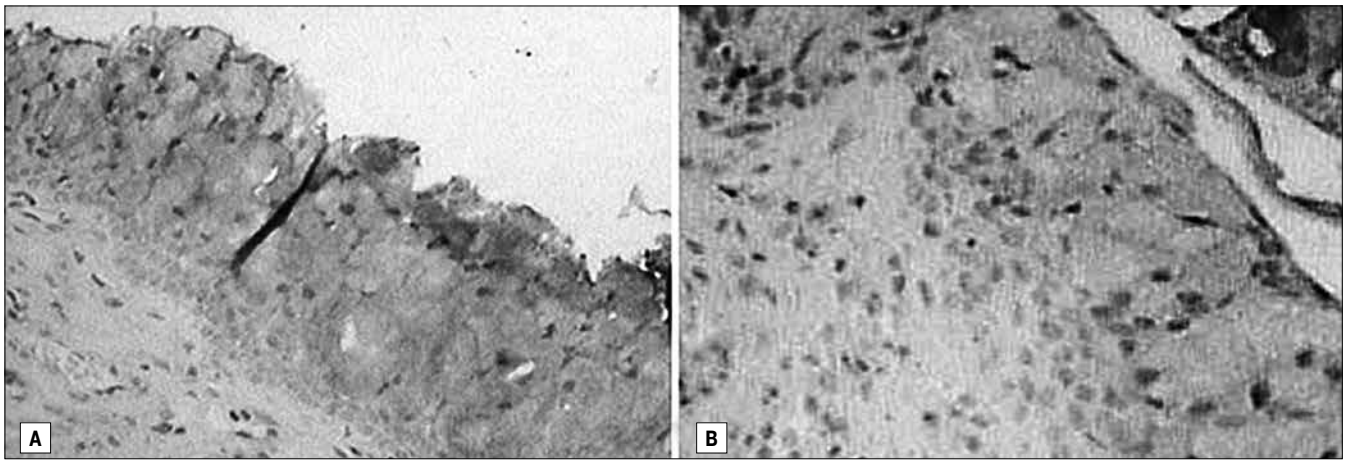

Figure 3. Staining of the endocervical epithelium of the cervix of a rat from the control group (**A**) and the study group (**B**), with alcian blue reaction at pH 2.5 (400X).

became more pronounced. De Souza *et al*. [8], when evaluating morphological and morphometric alterations of the uterine cervix, observed that the use of hyaluronidase at the end of pregnancy also determined a significant increase of eosinophils in the connective tissue. Simões *et al*. [18], reported a growing supply of eosinophils to the uterine cervix of female albino rats at the beginning of labor due to high levels of estrogen present at the end of pregnancy. These facts seem to be implicated in the initial process of dissociation of collagen bands, thus provoking dispersion of collagen fibers and leading to cervical maturation.

In our histochemical evaluation of the cervix in the study group, we observed a clear decrease in sulfated GAG in the epithelium of that group. The cervical tissue of the immature cervix has a high proportion of sulfated GAG relative to carboxylated GAG, contrary to what occurs in matured cervices [19,20]. This situation was well demonstrated when we applied alcian blue staining at pH 0.5. In this reaction, only sulfated GAG stained blue. We noted a large quantity of sulfated GAG in the control group, typical of an immature cervix, whereas in the study group the reaction was weakly positive. It seems that high concentrations of sulfated GAG may be a defense against the process of cervical maturation. A study by Obara *et al*. [21], demonstrated that in pregnant women with inhibited pre-term labor, the levels of chondroitin sulfate in the endocervical mucus were elevated, in comparison with pregnant women in pre-term and at full-term. They further noted that its exogenous use inhibited the activity of endogenous hyaluronidase in the endocervical mucus, impeding depolymerization of the hyaluronic acid, thus avoiding the onset of the cascade of catabolic events in the extracellular matrix.

With alcian blue staining at pH 2.5, in which both sulfated and carboxylated GAG were stained blue, there was intense staining in the control group, probably owing to high concentrations of sulfated GAG in the immature uterine cervix [19,20]. On the other hand, in the study group a negative reaction was expected, reflecting the action of hyaluronidase on hyaluronic acid, but a weakly positive stain was observed regardless. That finding may be explained by the fact that hyaluronidase depolymerizes the long chain of hyaluronic acid, which is found at high concentrations in the cervical tissue of full-term gestations, in molecules of low molecular weight of hyaluronic acid. Therefore, in a matured cervix, there is also a high concentration of hyaluronic acid, albeit with low molecular weight, which justifies the positive staining with alcian blue reaction at pH 2.5 in the study group. Obara *et al*. [21], observed a significant increase in the concentration of hyaluronic acid and in the activity of endogenous hyaluronidase. They further observed a significant decrease of molecular weight of hyaluronic acid in the endocervical mucus of pregnant women during the first stage of labor, in comparison with their pre-term and full-term pregnant peers.

Among our results, the quantity of sulfated GAG seems to be the most relevant. That finding allows us to conclude that sulfated GAG may truly possess a protective role against cervical maturation and consequently, prematurity. We believe that future research and experimentation should better analyze this important role of sulfated GAG on the endocervical epithelium and address question how detection of low quantities of sulfated GAG in endocervical mucus may be used in clinical practice as an indicator of risk of a pre-term delivery, or held determine the ideal quantity of GAG in the endocervical mucus during gestation.

We believe that specific histological studies and interpretation of cervical mucus will help us bridge the innumerable gaps in our knowledge about determining pre-term delivery.

Conclusion

In summary, our study allows us to conclude that modifications evidenced in the endocervical epithelium support the concept that hyaluronidase injected locally promoted alterations consistent with cervical maturation.

Authors' Contribution

1. Monica Regina Lourenco Luz – acquisition of data.
2. Edward Araujo Junior – article draft, corresponding author.
3. Luiz Camano – revised article critically.
4. Manuel de Jesus Simoes – analysis and interpretation of data.
5. Eduardo de Souza – concept, study design.

Authors' statement

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