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Effects of 17β-estradiol in the uterine cervix of ovariectomized rats with and without streptozotocin-induced diabetes

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Studies suggest that postmenopausal women may be at an increased risk of developing diabetes mellitus (DM), whereas DM has been reported to elevate the risk of cervical cancer in postmenopausal women. While estrogen replacement has been shown protective effects on the uterine cervix, it remains unclear whether DM influences estrogen uterine effects. This study evaluated the impact of estrogen replacement on the uterine cervix in ovariectomized (OVX) rats with and without streptozotocin (STZ)-induced DM. Twenty adult female rats were divided into four groups: OVX-control (GI), OVX-Estradiol (subcutaneously treated with 10 µg/kg/body weight of 17-β estradiol) (GII), OVX+DM induced by STZ (GIII), and OVX+DM+17-β estradiol (GIV). After 60 days, the animals were euthanized and their uterine cervices were processed for histological analysis and immunohistochemistry for Ki-67 and VEGF-A detection. GI exhibited the smallest glandular and vascular areas, along with fewer reddish birefringent collagen fibers and reduced GAGs content. Lower Ki-67 and VEGF-A immunopositivity in epithelial and stromal cells were observed in the uterine cervix of GI. These parameters were higher in the estradiol-treated groups, with no significant differences between GII and GIV. These results suggest that STZ-induced DM does not influence estrogenic effects on the rat uterine cervix.

Keywords: Estrogen deficiency, Diabetes Mellitus, Streptozotocin, Uterine cervix, Rats

The depletion of ovarian hormones, primarily estrogens, is the main cause of atrophy in the female genital system following menopause. This is often accompanied by other symptoms, including hot flashes, night sweats, depression, low libido, dry skin, and bone loss[1]. While estrogen replacement, with or without progestogens, has been considered the gold standard treatment to counteract these effects, it may lead to several adverse outcomes, including an increased risk of breast, ovarian, and endometrial cancers[2]. However, recent scientific literature suggests that the risks associated with hormone therapy are small, and its benefits generally outweigh the risks[3]. Despite this, the decision to undergo hormone replacement therapy becomes more complex when a menopausal woman has one or more chronic diseases, which may alter the risk-to-benefit balance[4]. One such chronic condition is diabetes mellitus[3].

Diabetes mellitus (DM) is a medical condition that encompasses a group of metabolic disorders characterised by hyperglycemia, which results from changes in insulin secretion and/or action[5]. The two most common types of DM are type 1 (DM1) and type 2 (DM2). DM1 results from the destruction of pancreatic beta cells, usually caused by an autoimmune process, leading to little or no insulin production[6]. In contrast, DM2 arises from a complex interaction between genetic factors and lifestyle choices such as poor diet, physical inactivity, and obesity[7]. This interaction disrupts the balance between insulin production and insulin sensitivity, resulting in insulin resistance and functional insulin deficiency[5]. Currently, around 425 million people are affected by DM worldwide, and this number is projected to rise to 629 million by 2045, imposing significant financial burdens on global healthcare systems[8].

Recent studies suggest that women in the menopausal or postmenopausal stages may have an increased risk of developing DM2, possibly due to metabolic changes that lead to insulin resistance[3,9]. On the other hand, the effects of estrogens on glucose metabolism remain a subject of debate. Some evidence suggests that exogenous estrogens may have a protective role, with reports indicating that estrogen replacement reduces the incidence of DM2 in

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postmenopausal women[10]. However, an association between elevated endogenous estrogen levels and a higher risk of developing DM2 has also been observed in postmenopausal women[11].

Studies have also shown that diabetic women are at a higher risk of developing cardiovascular diseases and certain types of cancer compared to non-diabetic populations[4,12]. For example, recent reports have linked both DM1 and DM2 to an increased risk of cervical cancer and reduced overall survival[13]. Additionally, postmenopausal women with DM2, as well as young women with DM1, are reported to have a higher risk of cervical cancer[14-16]. Meanwhile, it has been reported that estrogen replacement therapy during the menopausal and postmenopausal periods does not increase the risk of cervical cancer and may even reduce its incidence[17]. However, it remains unclear whether diabetes mellitus interferes with the effects of estrogen replacement on the cervix in postmenopausal women. Thus, our goal is to investigate the effects of estrogen replacement on the uterine cervix in ovariectomized (OVX) rats, both with and without STZ-induced DM.

Materials and Methods

Animals and experimental procedures

The protocol for this study was approved by the Ethics Commission for Animal Use (CEUA) at the Federal University of São Paulo – Paulista School of Medicine (UNIFESP-EPM), Brazil (CEUA #304675/**15**). The experimental procedures adhered to the recommendations of the ARRIVE guidelines, the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Additionally, good laboratory practices and quality assurance protocols were followed.

A total of twenty virgin female Wistar rats (*Rattus norvegicus albinus*), aged 90 days and weighing between 230 g and 250 g, were purchased from the Center for the Development of Animal Models for Biology and Medicine (CEDEME) at the Federal University of São Paulo – Paulista School of Medicine (UNIFESP-EPM). The rats were housed in an animal facility with controlled temperature (23 ± 2°C), humidity (55 ± 10%), and a standard 12-hour light/dark cycle, with free access to water and standard chow (Nuvilab CR1, Paraná, Brazil). After a one-week adaptation period, the animals underwent bilateral ovariectomy (OVX), followed by a two-week rest period to ensure estrogen depletion, as previously described[18] Anestrous condition in the OVX rats was confirmed through daily vaginal smear assessments over five consecutive days.

After confirming permanent anestrous, the animals were subjected to a 6-hour fasting period. Ten of the twenty animals received a 60 mg/kg body weight (b.w.) dose of streptozotocin (STZ) [Sigma-Aldrich Co. LLC, Brazil], administered intraperitoneally (i.p.) and diluted in a vehicle solution of 0.1 M citrate buffer at pH 5.5 to induce diabetes mellitus (DM), as previously described[19]. The remaining ten rats received only the vehicle solution, injected i.p. To prevent STZ-induced hypoglycemia, the rats were provided with a 10% sucrose solution in drinking water for 48 hours. Seventy-two hours after STZ or vehicle administration, the animals underwent another 6-hour fasting period, after which blood glucose levels (BGL) were measured from blood obtained via tail vein using a digital glucometer (Accu-Chek® Active, Roche Diagnostics; Jaguaré, Brazil). Rats that received STZ were considered diabetic when two consecutive BGL measurements exceeded 250 mg/dL, as previously reported[20]. The animals used in this study were the same as those in a previous study conducted by our group, in which significant estrogen depletion was confirmed in OVX rats and a diabetic state was confirmed by BGLs exceeding 500 mg/dL[21].

The animals were subsequently divided into the following groups (n = 5): Group I (OVX-C), which received a subcutaneous injection of corn oil vehicle solution; Group II (OVX + Estradiol), which was treated subcutaneously with 17β-estradiol (10 μg/kg body weight) [Sigma-Aldrich Co. LLC, Brazil] for 60 consecutive days; Group III (OVX + DM), which received a subcutaneous injection of corn oil vehicle solution; and Group IV (OVX + DM + Estradiol), which was treated with 17β-estradiol as described above. After 60 days of treatment with estradiol or vehicle solutions, the animals were euthanized using an anesthetic overdose of ketamine and xylazine.

Sample processing and histological analyses

After euthanasia, uterine cervix samples were fixed in 4% formaldehyde in phosphate-buffered saline (0.1 M, pH 7.2), freshly prepared from paraformaldehyde, for 24 hours at room temperature (RT). The specimens were then dehydrated in ascending concentrations of ethanol,

cleared in xylene, and embedded in paraffin. Sections (4 μm thick) were stained with Masson's trichrome for histomorphometric analysis, which was performed on three non-serial sections per animal (with at least 100 μm between sections). Using a light microscope (Axio Lab.A1, Carl Zeiss) attached to a high-resolution camera (AxioCam ICc5, Carl Zeiss, Germany), five fields per section were randomly captured at 200x magnification. In each field, the glandular and vascular areas were measured and estimated in mm² using image analysis software (Axiovision 4.8 REL, Carl Zeiss, Germany).

To assess the structure and organization of collagen fibers, three non-serial sections per animal, with at least 100 μm between sections, were subjected to the Picrosirius red-polarization method, as previously described[22,23]. Images were obtained and analyzed at 400x magnification using the same microscope employed for histomorphometric analysis, with the addition of polarized light. Other sections were subjected to the Alcian Blue (AB) method at pH 0.5 to detect sulfated glycosaminoglycans (GAGs), or to the AB method at pH 2.5 to detect carboxylated GAGs, as previously described[24]. GAG content was evaluated in three non-serial sections per animal, with at least 100 μm between sections. In these sections, the staining intensity (expressed as blue) of sulfated and carboxylated GAGs was graded as negative (-), weak (+), moderate (++), or strong (+++). The evaluations of collagen fibers and GAG content were conducted by two researchers who were blinded to the study.

Immunohistochemical analysis of Ki-67 and VEGF-A

Deparaffinized and rehydrated sections were immersed in 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. After washing with PBS, the sections were immersed in 10 mM sodium citrate buffer (pH 6.0) and heated at 90–94°C in a steamer for 1 hour for antigen retrieval. After cooling to room temperature, the slides were washed in PBS and incubated with 4% bovine serum albumin at room temperature for 20 minutes. The sections were then incubated overnight at 4°C with either a rabbit monoclonal primary antibody against Ki-67 (Spring Bioscience, CA) diluted 1:200 or a rabbit polyclonal primary antibody against VEGF-A (Spring Bioscience, CA) diluted 1:800. After washing in PBS, the sections were incubated for 30 minutes at room temperature with a biotinylated secondary antibody. The sections were then washed in PBS and incubated with Streptavidin-HRP for 30 minutes using the Universal LSAB+ System-HRP Kit (Dako North America, Inc., USA). Peroxidase activity was revealed with 0.06% DAB (Sigma-Aldrich Chemie) in PBS, and the sections were counterstained with Carazzi's hematoxylin. For negative controls, the primary antibody was replaced with non-immune serum.

Immunohistochemical analysis was performed on three non-serial sections per animal (with at least 100 μm between sections). Five fields per section of the uterine cervix, each with a total area of 0.04 mm², were randomly analyzed at 400x magnification using the same microscope used for histomorphometric analysis. A staining intensity score, graded as negative (0), weak (1), moderate (2), or strong (3), was used to assess the intensity of immunoreactivity in the glandular epithelium and stromal area separately. The immunohistochemical analysis was conducted by two researchers who were blinded to the study.

Results

Histomorphometric analyses

The glandular area was significantly greater (P<0.05) GII compared to all other groups. Additionally, the glandular area in GIV was significantly higher than in both GI and GII, while no significant difference was observed between GI and GIII. Meanwhile, the vascular area was significantly higher (P<0.05) in GII and GIV compared to both GI and GIII (Fig. 1).

Evaluation of collagen fibers and glycosaminoglycans (GAGs) content

The Picrosirius red-polarization method revealed a predominance of collagen fibers with a greenish birefringence pattern in GI and GIII, while GII and GIV groups exhibited an orange/reddish birefringence pattern. Additionally, a higher presence of sulfated and carboxylated glycosaminoglycans (GAGs) was observed in the GII and GIV groups compared to the other groups. No significant differences were found between GI and GIII (Fig. 2).

Immunohistochemical detection of VEGF-A and Ki-67 in the uterine cervix

A high VEGF-A immunoreactivity score was observed in both GII and GIV, while it was lower in GI and GIII in both epithelial and stromal cells. Additionally, similar VEGF-A immunoreactivity scores

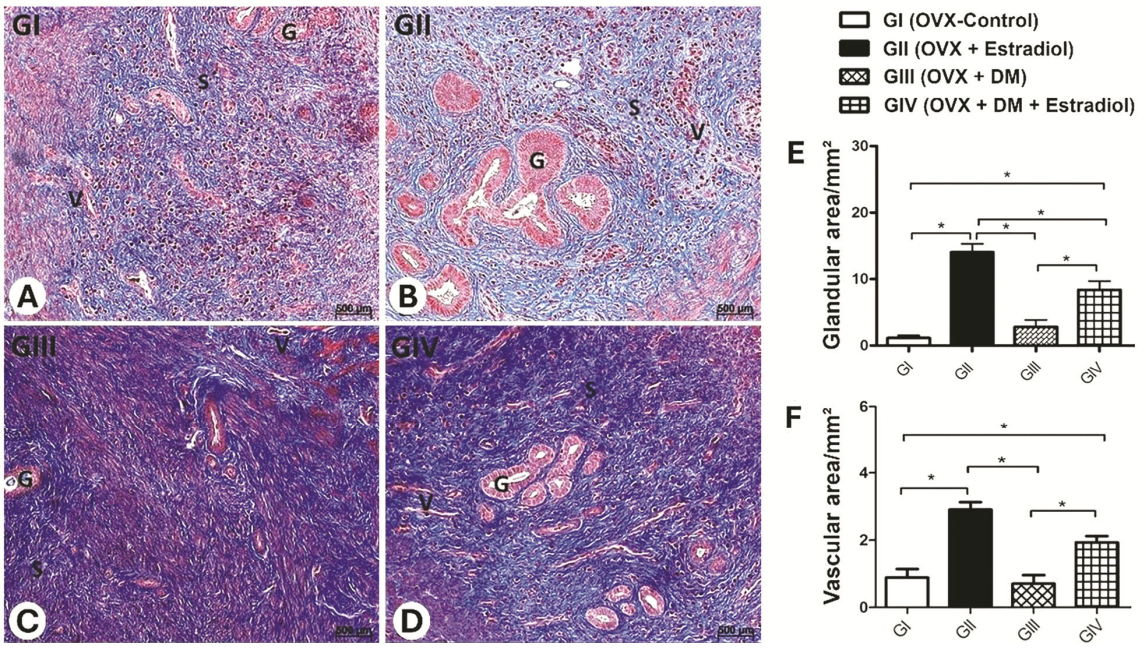

Fig. 1 — (A-D) Photomicrographs of histological sections from regions of the uterine cervix of rats, stained with Masson's trichrome. Note the higher presence of uterine glands (G) and blood vessels (V) in GII (B) and GIV (D), compared to their sparse presence in GI (A) and GIII (C). (E-F) Graphs showing data for glandular (E) and vascular (F) areas. Glandular area: GII > GI, GIII, and GIV; GIV > GI and GIII (**P*< 0.05). Vascular area: GII > GI and GIII; GIV > GIII (**p*< 0.05).

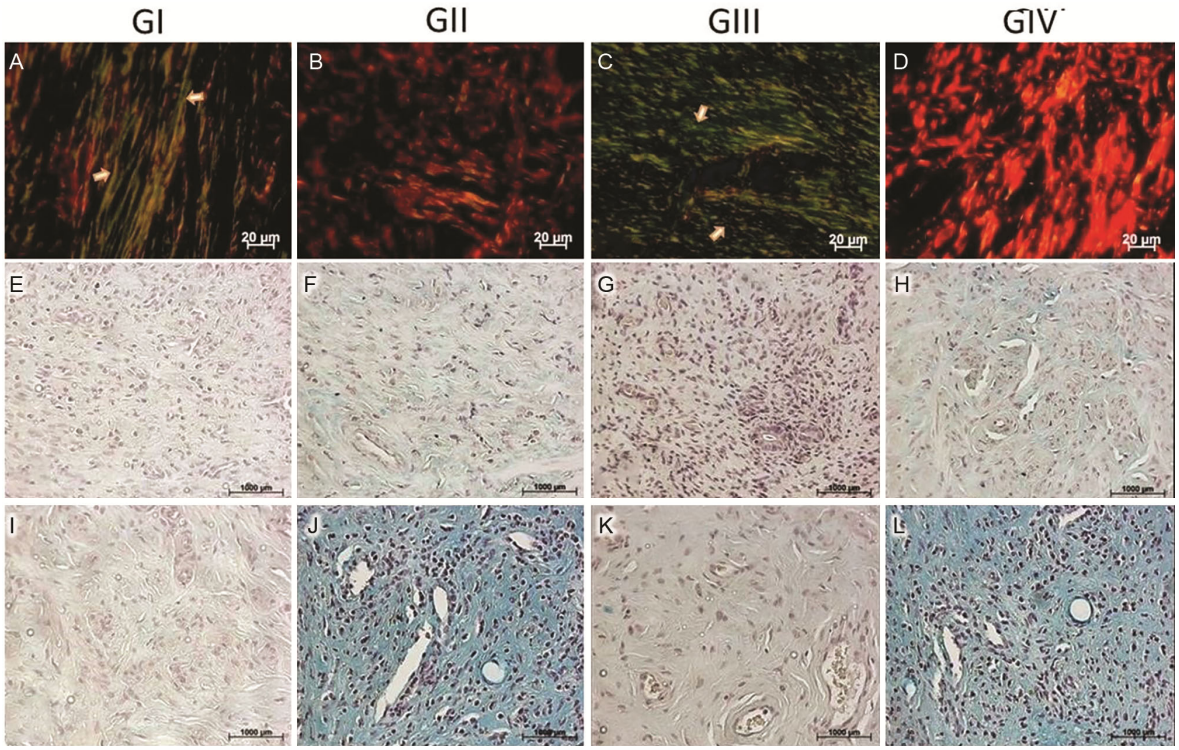

Fig. 2 — (A-L) Photomicrographs of histological sections from uterine cervix regions subjected to the Picrosirius Red-polarization method (A-D), or the Alcian Blue method at pH 0.5 (E-H) and pH 2.5 (I-L). Note the predominance of collagen fibers with an orange-reddish birefringence pattern in Group II (GII) (B) and Group IV (GIV) (D), as well as a greenish birefringence pattern (arrows) in Group I (GI) (A) and Group III (GIII) (C). Also, observe the higher presence of sulfated and carboxylated glycosaminoglycans (GAGs), stained in blue, in the GII (F, J) and GIV (H, L).

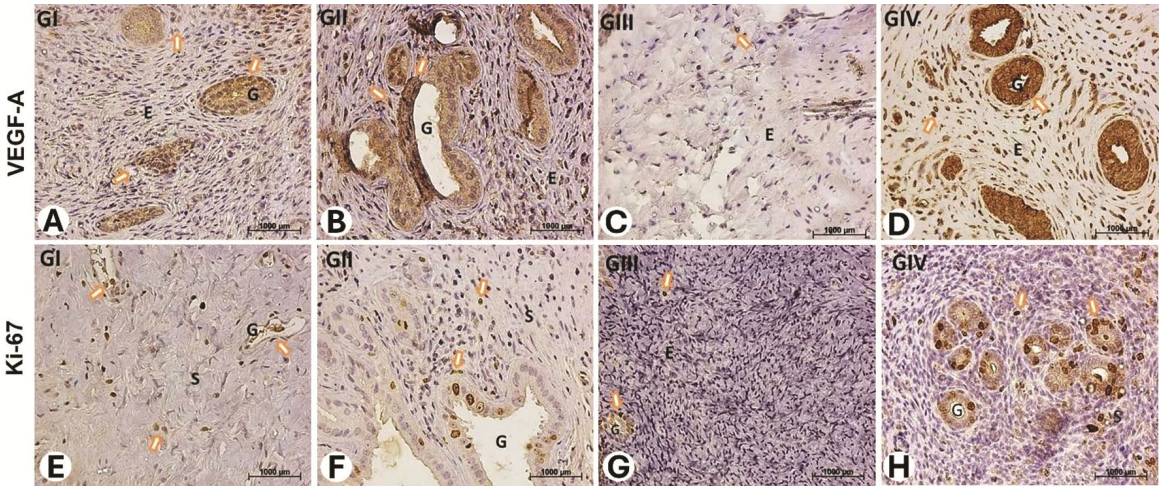

Fig. 3 — (A-H) Photomicrographs of histological sections from uterine cervix regions subjected to immunohistochemistry for the detection of VEGF-A (A-D) and Ki-67 (E-H), and counterstained with Carazzi's hematoxylin. Increased VEGF-A immunoreactivity (arrows) is observed in GII) (B) and GIV (D), while lower immunoreactivity is seen in GI (A) and GIII (C), in both epithelial and stromal cells. A higher presence of Ki-67-immunopositive cells is also evident in GII (F) and GIV (H). G: glands; S: stroma.

Table 1 — Immunoreactivity scores of VEGF-A and Ki-67 in epithelial (Ep.) and stromal cells, as well as collagen birefringence patterns and glycosaminoglycan content in the uterine cervix of rats

	Parameter	GI	GII	GIII	GIV
VEGF-A	Positive cells score	Ep.cells: 1 Stroma: 1	Ep.cells: 3 Stroma: 3	Ep.cells: 0 Stroma: 1	Ep.cells: 3 Stroma: 3
	Staining intensity	Ep.cells: weak Stroma: weak	Ep.cells: strong Stroma: strong	Ep.cells: weak Stroma: weak	Ep.cells: strong Stroma: strong
	Total score	Ep.cells: 2 Stroma: 2	Ep.cells: 6 Stroma: 5	Ep.cells: 1 Stroma: 1	Ep.cells: 6 Stroma: 5
	Expression status	Ep.cells: low Stroma: low	Ep.cells: high Stroma: high	Ep.cells: low Stroma: low	Ep.cells: high Stroma: high
Ki-67	Positive cells score	Ep.cells: 1 Stroma: 1	Ep.cells: 2 Stroma: 2	Ep.cells: 1 Stroma: 1	Ep.cells: 3 Stroma: 2
	Staining intensity	Ep.cells: weak Stroma: weak	Ep.cells: moderate Stroma: moderate	Ep.cells: weak Stroma: weak	Ep.cells: strong Stroma: moderate
	Total score	Ep.cells: 2 Stroma: 2	Ep.cells: 4 Stroma: 4	Ep.cells: 2 Stroma: 2	Ep.cells: 6 Stroma: 4
	Expression status	Ep.cells: low Stroma: low	Ep.cells: moderate Stroma: moderate	Ep.cells: low Stroma: low	Ep.cells: high Stroma: moderate
Collagen	Colour of collagen birefringence pattern	Most green	Most orange-red	Most green	Most orange-red
GAGs	Sulfated GAGs	+	++	+	++
	Carboxylated GAGs	+	+++	+	+++

[The percentage of positive cells for VEGF-A and Ki-67 was scored as follows: "0" (≤ 5%), "1" (6-25%), "2" (26-50%), and "3" (51-100%). Staining intensity was graded as negative (0), weak (1+), moderate (2+), or strong (3+). The total score was determined by adding the positive cell score and the staining intensity score. Expression status was categorized as no staining (total score = 0), low (total score = 1-3), moderate (total score = 4), or high (total score = 5-6). The content of sulfated and carboxylated glycosaminoglycans (GAGs) was graded as negative (-), weak (+), moderate (++), or strong (+++)]

were found between GII and GIV, as well as between GI and GIII. A low Ki-67 immunoreactivity score was noted in GI and GIII, whereas it was moderate in GII, in both epithelial and stromal cells. In contrast, GIV showed a high Ki-67 immunoreactivity score in epithelial cells, while the score was moderate in stromal cells (Fig. 3; Table 1).

Discussion

In this study, we aimed to investigate the effects of estrogen replacement on the uterine cervix in OVX rats, both with and without STZ-induced diabetes mellitus. We found that the effects of estrogen on the uterine cervix were similar in both diabetic and non-diabetic OVX rats.

The ovariectomized rat is a well-established animal model of post-menopause[25,26]. In the present study, we used the same animals from a previous investigation conducted by our group, where significant estrogen depletion was confirmed in OVX rats, while the estrogen-treated groups exhibited significantly increased estradiol levels[21]. Additionally, all animals were in permanent anestrus, as confirmed by vaginal smear collection over five consecutive days. These results validate the effects of estrogen depletion induced by OVX and the effects of estrogen replacement. Furthermore, our previous study demonstrated that the STZ-induced diabetic rats used in the present investigation had serum glucose levels reaching up to 500 mg/dL, which exceeds the threshold considered diagnostic for diabetes mellitus in this animal model, as reported by our group and other researchers[19,21,27-29].

Atrophy of the female genital tract is a common condition following menopause, resulting from the depletion of ovarian hormones, primarily estrogens, in both women and animal models[30-32]. In our study, estrogen withdrawal induced by ovariectomy led to a significant reduction in the glandular area of the uterine cervix. This reduction may be associated with a decline in the proliferation of glandular epithelial cells, as evidenced by a significant decrease in Ki-67 immunopositivity, a nuclear marker of cell proliferation[33]. Furthermore, we observed a significant reduction in the vascular area, along with weak VEGF-A immunostaining in both epithelial and stromal cells in the ovariectomized control group (GI), suggesting a decrease in angiogenesis within the uterine cervix. VEGF-A is a critical marker of angiogenesis and plays an essential role in sustaining cell proliferation[34]. Therefore, it is possible that the reduced cell proliferation observed in the ovariectomized control group is linked to the decline in angiogenesis.

Conversely, we observed that estrogen replacement counteracted the decrease in cell proliferation and angiogenesis. The estrogen-treated groups exhibited larger glandular and vascular areas, as well as higher Ki-67 and VEGF-A immunostaining in both epithelial and stromal cells. The trophic effects of estrogen on the female genital tract are well-documented in the literature[30,31]. A previous study by our group also showed a reduction in VEGF-A immunoreactivity, accompanied by atrophy of the uterine cervix in OVX rats, whereas estrogen replacement resulted in trophic effects and increased VEGF-A immunoreactivity[35]. Similarly, increases in VEGF-A expression and uterotrophic effects have been reported in ovariectomized rats treated with 17β-estradiol[36]. Thus, our study supports a potential association between the increased VEGF-A expression and enhanced cell proliferation in the uterine cervix of OVX rats following estrogen replacement.

The histochemical methods using AB staining revealed a higher presence of sulfated and carboxylated GAGs in the estrogen-treated groups (GII and GIV). Meanwhile, the Picrosirius red-polarization method showed that the GII and GIV groups also exhibited a higher presence of collagen fibers with a reddish birefringence pattern. In contrast, the ovariectomized groups that did not receive estrogen (GI and GIII) displayed a lower presence of sulfated and carboxylated GAGs, as well as a predominance of collagen fibers with a greenish birefringence pattern. It is known that the concentration of collagen fibers influences the colour pattern of their birefringence in tissue sections[23]. Specifically, at lower concentrations in the extracellular matrix, these fibers exhibit a greenish birefringence pattern, while at higher concentrations, they display a reddish birefringence pattern when visualized under polarized light[18,37]. Therefore, our results suggest that estrogen treatment promoted trophic effects in the constituents of the extracellular matrix. It is likely that estrogen stimulated the increased synthesis of collagen fibers and GAGs, presumably by the fibroblasts in the uterine cervix stromal region, as a result of increased fibroblast activity and proliferation. This hypothesis is supported by the observation of a higher increase in the number of Ki-67 positive stromal cells following estrogen treatment.

It has been reported that estrogen replacement therapy in women during the peri- and postmenopausal periods does not increase the risk of cervical cancer and may even reduce the risk of its incidence[17,38]. On the other hand, studies have shown that postmenopausal women with type 2 diabetes mellitus have a higher risk of developing cervical cancer compared to the same population without

diabetes[14]. Additionally, type 1 diabetes mellitus has been associated with a higher risk of cervical cancer in young women[15]. However, it remains unclear whether diabetes mellitus interferes with the effects of estrogen replacement on the cervix in postmenopausal women. In our study, no significant differences were found in the glandular and vascular areas, collagen fiber content, GAGs, or the immunoexpression of Ki-67 and VEGF-A in the cervix of ovariectomized rats treated with estradiol, with or without diabetes mellitus. Furthermore, it has been shown that estrogen replacement has beneficial effects on vascular function in both diabetic and non-diabetic ovariectomized rats through calcium regulation and anti-oxidation, with no effect of estrogen therapy on blood glucose levels[39]. These authors reported that uterine weight was similar in both diabetic and non-diabetic ovariectomized rats receiving estrogen replacement, suggesting that the diabetic condition in ovariectomized rats does not influence estrogen's action in the uterus. These findings are consistent with our results, as we observed that the trophic effect of estrogen on the uterine cervix was similar in both ovariectomized diabetic and non-diabetic rats.

However, our study has some limitations. First, although the rodent model of streptozotocin-induced diabetes mellitus is widely used[40,41], it more closely mimics the pathophysiological characteristics of type 1 diabetes mellitus rather than type 2 diabetes mellitus[41]. Therefore, we cannot extrapolate our results to postmenopausal women with type 2 diabetes mellitus who are receiving estrogen replacement therapy. Second, our analyses were based primarily on histomorphometric, histochemical, and immune histochemical approaches. Although molecular techniques (such as quantitative PCR or western blot) could provide additional insights into the regulation of extracellular matrix components and angiogenic markers, these analyses could not be performed within the scope of the present study. Nonetheless, this limitation does not compromise the reliability of our current findings, which remain consistent and biologically meaningful. Future investigations specifically designed to explore these molecular pathways will be essential to complement and expand our present observations.

Conclusion

Taken together, our results suggest that streptozotocin-induced diabetes mellitus does not interfere with the effects of estrogen on the cervix of ovariectomized rats. The findings of this study may contribute to the improved management of diabetic women receiving estrogen therapy during the postmenopausal period.

Ethical statement

The protocol for this study was approved by the Ethics Commission for Animal Use (CEUA) at the Federal University of São Paulo – Paulista School of Medicine (UNIFESP-EPM), Brazil (CEUA #304675/15). All experimental procedures were performed following national and international Guidelines Principles for the Care and Use of Animals for scientific research.

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Conflict of interest

The authors declare no conflict of interest to disclosure.

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