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Gene Expression Analysis of Extracellular Matrix and Cytokines after Uterine Artery Embolization

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Abstract

Arterial embolization of myomas (AEM) is an established option for the conservative treatment of uterine leio-myomas; it treats all present uterine nodules at once, is less invasive than other procedures and effective in controlling symptoms, and does not require long term hospitalizations. Nevertheless, the potential impact on endometrial morphological and functional outcomes after the procedure is still controversial based on reports of endometritis or eventual transient ischemia. This study evaluated endometrial reorganization in uterine leiomyoma patients, before and after AEM, through gene expression analyses of extracellular matrix and cytokines genes in the endometrial tissue. Eight patients with leiomyomas were evaluated before AEM and 6 months after. The examinations included transvaginal pelvic ultrasonography, dosing of the follicle-stimulating hormone, and endometrial biopsy during the second phase of the menstrual cycle. RNA was extracted from endometrial samples, cDNA was synthesized, and applied on PCR array™ plates to evaluate the expression of extracellular matrix (ECM) genes and cytokines and their receptors' genes (CYT). The ECM overexpressed genes were MMP (1, 3, 10, 11, and 14), CTGF1, ICAM1, TBHS1, ITGA2, ITGA3, ITGB3, COL7A1, COL12A, SPP1, and TNC; ADAMTS8 was underexpressed. The CYT overexpressed genes were SPP1, BCL6, CXCL12, IL-8, and CEBPB; CXCL13 and CCL21 were underexpressed. The ECM results showed overexpression of proteases that are responsible for dysfunctions in the ECM, and of genes responsible for adhesion and membrane components. The CYT results showed overexpression of chemokines responsible for endometrial repair, and underex-

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pression of cytokines involved in inflammatory processes in the endometrial tissue. AEM treatment did not negatively affect the endometrial function at 6 months after embolization. This study broadens the knowledge about using a procedure that is relevant to the treatment of leiomyomas and contributes to the establishment of future guidelines for the decision making process for physicians and patients.

Keywords

Component, Myoma, Arterial Embolization of Myomas, Extracellular Matrix, Cytokines

1. Introduction

AEM is an established option for the conservative treatment of uterine leiomyomas because it treats all present uterine nodules at once, is less invasive than other procedures, is effective in controlling symptoms, does not require long term hospitalizations, and allows for fast patient recovery. Nevertheless, the potential impact on endometrial morphological and functional outcomes after the procedure is still controversial based on reports of endometritis or eventual transient ischemia. Moreover, changes in blood flow resulting from vascular insufficiency, potential release of local vasoactive substances, and changes in the extracellular matrix and cytokines can occur [1].

Despite the many studies reporting normal and uncomplicated pregnancies in patients who received this treatment [2]-[4], the main discussion around the use of AEM is about its possible effects on the endometrium that could lead to infertility. These controversies weights heavy on the decision making process that doctors and patients have to walk through; candidates for the procedure must be carefully evaluated, couples must be informed of risks, and informed voluntary consent statements must be signed before a decision is made to proceed to treatment.

Many of the endometrial molecular processes occur through interactions between components of the extracellular matrix (ECM) and cytokines (CYT) during the menstrual cycle, especially in the luteal phase. Possible changes in the endometrial flow in women who had undergone EAM can affect the gene expression in the ECM and of CYT genes, and promote changes in the reproductive function of this tissue. Some genes have been associated with endometrial reorganization such as from the ECM (metalloproteinases, integrins, and collagen) and CYT genes (CCL, SPP1, and CEBPB) [5]-[7]. The molecular analysis of the endometrium allows the assessment of morphology and functionality; however, these on site factors have only been studied in the last two decades, and molecular studies correlating results with clinical post-AEM outcomes in the endometrium are still scarce.

In this study, we evaluated the endometrial reorganization in patients with uterine leiomyomas, before and after AEM, in prospective and experimental cases, through molecular analyses of gene expressions in the ECM and of CYT genes in the endometrial tissue.

2. Material and Methods

2.1. Patients

Eight women were selected among outpatients attending the Leiomyoma Clinic between 6/8/2006 and 7/31/2008 at the Gynecology Department of the Paulista School of Medicine, Federal University of São Paulo. The study protocol was approved by the Ethics and Research Committee from the Federal University of São Paulo (Protocol number 794/2000); information about treatment options and procedures in the study were provided to the medical staff involved and patients. All patients voluntarily signed an informed consent form before study start.

2.2. Inclusion Criteria

The inclusion criteria were the presence of menstrual disorders, dysmenorrhea or pelvic pain, urinary discomfort, deep dyspareunia, asymptomatic patients with bulky leiomyomas, and patients with the desire to maintain reproductive capacity but with no possibilities for other conservative treatments.

2.3. Exclusion Criteria

The exclusion criteria were genital neoplasms, use of hormonal contraceptive methods, use of hormones to control menstrual cycle, pregnancy, acute pelvic inflammatory disease, coagulopathy or vascular disorders, and prior pelvic irradiation.

2.4. Transvaginal Pelvic Ultrasonography (TVPUS)

The study participants underwent transvaginal pelvic ultrasonography (TVPUS) examination performed by a certified professional and using the same equipment for all events. Patients with the following conditions were not included: subserosal nodules with intramural component less than 30%, submucosal nodules with intramural component less than 30%, nodules of imprecise limits in expansive process, and a confirmation of adenomyosis. In situations with bulky tumors, suspicion of adenomyosis, and tumor growth after AEM pelvis, contrast-enhanced magnetic resonance imaging was performed to complement the diagnosis after the TVPUS examination because it provides better resolution of myoma lesions in the differential diagnosis with adenomyosis [8].

2.5. Dosing of the Follicle-Stimulating Hormone (FSH)

The follicle-stimulating hormone (FSH) was dosed in the early follicular phase (third day of menstrual cycle); patients were directed to suspend the use of any hormonal contraception or hormonal regulators thirty days before this dosing to avoid interference in the measurements. Patients with dosage ≥ 12 IU/ml were not included in the study.

2.6. Endometrial Biopsy

Endometrial biopsy was performed using a modified Novak curette during the second or secretory menstrual phase, between the 19th and 22nd days of the menstrual cycle, based on the date of the last menstruation and average of the last menstrual cycles for each patient. Each patient and respective samples were uniquely identified with a number from 1 to 30; the letter P identified samples obtained after AEM. The collected material was dry packed in foil, appropriately labeled, and stored at −80˚C.

2.7. Arterial Embolization of Myomas

The AEM procedure was performed using spherical and non-spherical gelatinous micro-particles (embospheres) with diameters ranging from 500 µm to 900 µm. The procedure's cut off was at the decrease of the contrast injection flow in the uterine arteries characterized by the "end point" image. Special attention was given to the possibility of embolization of ovarian arteries in which the prevalent blood flow sometimes originates in uterine arteries.

2.8. Reevaluation Post-AEM

The thirty study participants were evaluated six months after AEM through anamnesis, physical examination, re-examination by TVPUS, FSH dosing, and endometrial biopsy.

2.9. DNA Extraction

RNA was extracted from endometrial samples obtained pre- and post-AEM using the RNeasy Micro Kit (QIAGEN). Pre-AEM samples were used as control for the post-AEM samples. All pre samples were pooled to provide the control sample while all post samples were pooled to provide the test sample. Pooling was necessary because individual samples did not provide sufficient amounts of RNA.

2.10. cDNA Synthesis

cDNA was synthesized from these two groups using 350 ng of RNA through the RT2 First Strand Kit (C-03) (SA Biosciences) and according to the manufacturer's specifications.

2.11. PCR Array Reactions

Gene expression was evaluated in the cDNA samples applied on PCR array™ plates prepared to evaluate genes of the extracellular matrix and adhesion molecules (RT2 ProfilerTM PCR array—Human Extracellular Matrix and Adhesion Molecules, SA Biosciences) and cytokines genes (RT2 ProfilerTM PCR array Human Inflammatory Cytokines & Receptors SA Biosciences). Each plate was analyzed in duplicate. Real-time amplification reactions were performed according to the manufacturer's specifications in the real time PCR System MX3000p (Stratagene),

2.12. Statistical Analysis

Results were analyzed using the Mxpro QPCR Software (Stratagene). The differential gene expression between post-AEM and pre-AEM samples was calculated through the expression Fold Change (FC) = $2 - \Delta\Delta C$ [20]. The GNCProTM Gene Network Central system (SA Biosciences) was used to evaluate the function and interaction between over or underexpressed genes. Pre- and post-AEM results were matched using the software from the kit supplied by the manufacturer. Post-AEM/pre-AEM ratios ≥ 3 were considered as gene overexpression and ratios ≤ -3 were considered as gene underexpression. Values of $p \leq 0.05$ based on the Student's t-test were considered statistically significant.

3. Results

A total of 26 samples were evaluated from the 30 women who underwent AEM between 6/8/2006 and 7/31/2008 at the São Paulo Hospital. Four patients identified as 2, 3, 6, and 8 did not return for reevaluation, and eighteen patients identified as 1, 4, 5, 7, 10, 11, 13, 15, 16, 17, 19, 20, 21, 24, 25, 26, 27, and 28 were excluded from the study because of insufficient mRNA samples for cDNA synthesis. Eight women, identified as 9, 12, 14, 18, 22, 23, 29, and 30, underwent biopsis 6 months post-AEM and had samples with sufficient mRNA for cDNA synthesis.

Table 1 presents the distribution of some demographic data (race, parity) and uterine volume measurements, before and after AEM, in the 8 participants. A statistically significant reduction in uterine volume was observed at 6 months after AEM ($p < 0.0001$, paired t test).

The samples quantified using the Nano Drop kit (Thermo Scientific) showed a 260/280 nm ratio of 1.9. These samples composed two groups of pooled RNAs: Pre-EAM group (control) and Post-EAM group (six months).

Figure 1 shows ECM gene expression after pairing results between control and test groups. The results show that the following genes were significantly overexpressed at 6 months after AEM: MMP (1, 3, 10, 11, and 14), CTGF1, ICAM1, TBHS1, ITGA2, ITGA3, ITGB3, COL7A1, COL12A, SPP1, and TNC. The ADAMTS8 gene was the only underexpressed at 6 months after AEM (**Table 2**).

Figure 2 shows CYT gene expression after pairing results between control and testing groups. The results show that the SPP1, BCL6, CXCL12, IL-8, and CEBPB genes were significantly overexpressed, and CCL21 and CXCL13 were significantly underexpressed (**Table 3**).

Table 1. General data from study participants at 6 months after AEM.

	Testing group at 6 months after AEM	
Age (years) ± DP	34.2 ± 7.5	
Number of cases	8	
Race	White-37.5%	Black-62.5%
Parity	Gestations = 0 7	Gestations ≥ 1 1
UV pre-AEM (cc^3) ± DP	318.1 ± 69.0	
UV post-AEM (cc^3) ± DP	232.7 ± 81.7	
Reduction rate (%) ± DP	27.9 ± 16.3	

*UV: uterine volume; SD: standard deviation; AEM: arterial embolization of myoma.

Table 2. RT² *Profiler* PCR Array™ (ECM and adhesion genes) at 6 months after AEM. Underexpressed (in blue) and overexpressed genes (in red).

Expression of ECM and adhesion genes at 6 months after AEM (8 cases)		
Genes	**p value***	**post-AEM/pre-AEM**
ADAMTS8	**0.046738**	**−9.53**
CLEC3B	0.089017	**−4.98**
LAMA2	0.207394	**−4.24**
COL16A1	0.079158	**3.03**
COL1A1	0.181751	**3.23**
CTGF	**0.029376**	**3.76**
MMP14	**0.008037**	**3.79**
ICAM1	**0.021413**	**3.84**
THBS1	**0.024441**	**4.45**
TIMP1	0.089500	**4.50**
ITGA3	**0.000257**	**6.89**
MMP11	**0.000965**	**9.03**
TNC	**0.025995**	**10.46**
ITGA2	**0.000524**	**11.09**
ITGB3	**0.001069**	**11.40**
COL7A1	**0.000078**	**12.50**
COL12A1	**0.003783**	**13.43**
SPP1	**0.003325**	**19.08**
MMP10	**0.000009**	**31.07**
MMP3	**0.003447**	**46.98**
MMP1	**0.000007**	**134.74**

Source: GNC Prosystem™ Gene Network Central (SA Biosciences); *Student t test.

Table 3. RT² *Profiler* PCR Array™ (CYT genes) at 6 months after AEM. Underexpressed (in blue) and overexpressed genes (in red).

Expression of cytokine genes at 6 months after AEM (8 cases)		
Genes	**p value***	**post-AEM/pre-AEM**
CCL21	**0.009355**	**−3.92**
CXCL13	**0.003215**	**−3.17**
CCL1	0.422165	**3.07**
CXCL6	0.207664	**3.23**
CCR9	0.344158	**3.37**
CEBPB	**0.009985**	**3.62**
BCL6	**0.013785**	**3.69**
CRP	0.337765	**4.00**
IL-8	**0.019358**	**4.30**
CXCL12	**0.002386**	**4.95**
CCL20	0.081574	**7.50**
SPP1	**0.000081**	**18.60**

Source: GNC Prosystem™ Gene Network Central (SA Biosciences); *Student t test.

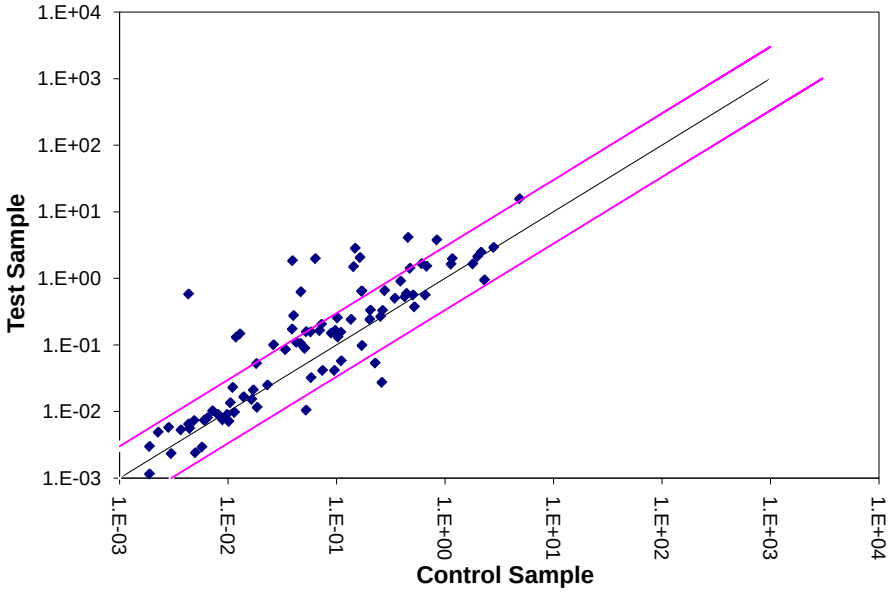

Figure 1. ECM gene expression of the studied genes at 6 months after AEM. The black line indicates the desired average (FC) = 2 − ΔΔCof 1. The red line indicates the desired change of threshold in gene expression.

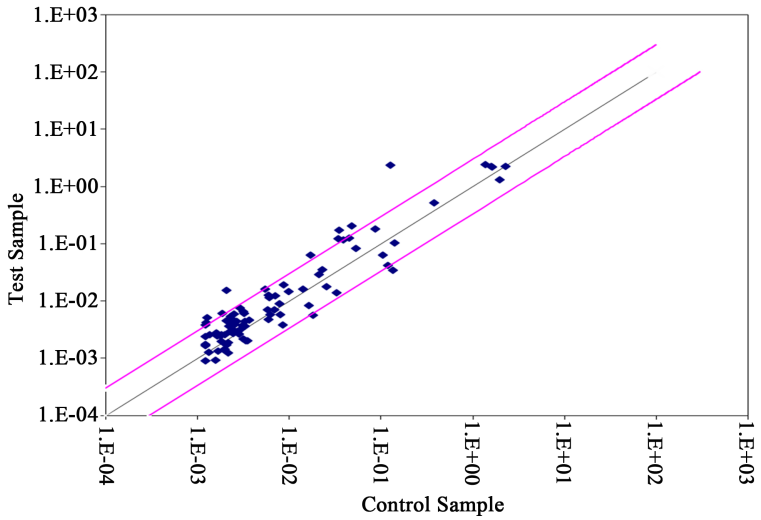

Figure 2. CYT gene expression of the studied genes at 6 months after AEM. The black line indicates the desired average (FC) = 2 − ΔΔCof 1. The red line indicates the desired change of threshold in gene expression. Source: GNCPro™ Gene Network Central (SA Biosciences).

4. Discussion

We found that the AEM treatment uniformly affected the uterine volume because a similar reduction rate was observed among the study participants (average of 27.9%). In addition, the analysis of ECM and CYT gene expression of some genes directly involved in endometrial processes that can affect the reproduction function of this tissue showed over and underexpression at 6 months after AEM treatment.

Messina *et al.* [3] reported a uterine volume reduction of 29% at 3 months after AEM and 41% at one year, after AEM using polyvinyl alcohol, through TVPUS images from 26 women. Sena-Martins *et al.* [9] reported 43.7% uterine volume reduction in 32 women followed up by TVPUS. The assessment of homogeneity in parameters such as uterine volume, spatial presentation of nodules, body mass index, race, smoking, parity, hor-

monal system, and others between patients with uterine leiomyoma is difficult. These difficulties were minimized in our results because a uniform effect from AEM treatment was reflected by a similar volumetric decline between all participants (27.9% of rate reduction average). Moreover, we involved study participants who were nulliparous and with previous gestations to avoid the presence of characteristics particular to patients with primary infertility, which could have skewed the study results.

Zhao *et al.* [7] observed overexpression in some genes in the metalloproteinases matrix family (MMP10, MMP3, and MMP9) when controlled ovarian stimulation followed by luteal phase support was applied. The MMPs (1, 3, 10, 11, and 14) play a role in favoring endometrial reorganization and cell proliferation and the overexpression of these genes observed in our results seems to support the assumption that they are involved in ECM remodeling.

We observed overexpression of the ITGA2, ITGA3, and ITGB3 genes. This result presupposes that the AEM treatment did not negatively affect these genes, which can be responsible for implantation and decidualisation. Tajiri *et al.* [10] reported overexpression of the alpha and beta integrins (ITGA2, ITGA5, and ITGB1) in stromal cells surrounding the embryonic implantation on days 7 and 8.

The overexpression of the THBS and TNC genes observed in this study suggests recovery in endometrial adhesions. Jin *et al.* [11] observed that the underexpression of thrombospondin type 1 (THBS-1) could favor the loss of pregnancy; Tan *et al.* [12] showed that, in the development of endometriosis, the modulation of estradiol in tenascin-C (TNC) can be one of the factors favoring cellular proliferation and invasion.

Collagen (COL7A1 and COL12A1) seems to have a function forming a fibrous anchoring between the outer epithelium and the adjacent stroma [5]. The overexpression of COL7A1 and COL12A1 observed in our results favors the endometrial receptivity and anchoring.

The observed overexpression of the protein encoded by the CTGF gene (connective tissue growth factor) indicates a stabilization of the function of this gene that aids endometrial adhesions. Maybin *et al.* [13] reported that CTGF is overexpressed in the post-menstruation repair period.

Porter *et al.* [14] evaluated the expressions of all 19 forms of metalloproteinases with modified thrombospondin type 1 (ADAMTS), in neoplastic and non-neoplastic breast tumors, and showed that 11 of the ADAMTS genes are deregulators of breast cancers and ADAMTS8, a protease precursor gene, is a poor predictor for this disease. The overexpression of ADAMTS8 observed in our study allows us to affirm that the AEM treatment does not favor endometrial matrix derangements.

Our overexpression results of ECM genes that are responsible for adhesion, invasion, reorganization, and anchoring among other features and underexpression of other genes that are responsible for difficulties in implantation and architectural derangements indicate that AEM does not negatively affect these functions.

Among the CYT analyzed genes, we observed overexpression of CXCL12 and under-expression of CXCL13 and CCL21. The microvascular endothelium showed overexpression of the modified CXC chemokine (CXCL13) in the endometrium with an inflammatory process. These findings indicate that the local microenvironment aberrations possibly caused by bacterial infection have a role in the extravasation of circulating B-cells in chronic endometritis [15]. Chand *et al.* [6] noted that the modified C-C chemokine (CCL21) was overexpressed in some women with endometriosis compared with controls ($p < 0.05$). Our observation of the predominant overexpression of genes responsible for chemotaxis and inflammatory process and destructuring development suggests that the endometrium is in the process of healing and stabilization at 6 months after AEM. Moreover, we infer that the possible inflammatory damage caused by AEM undergoes a comfortable regression after 6 months and may not interfere with the endometrial reproductive function.

Dunlap *et al.* [16] observed that the progesterone receptor and the fetus stimulate the glandular epithelium and stroma in the uterus, where the secreted phosphoprotein 1 (SPP1) seems to influence the histotrophic and hematotrophic support in the development of the fetus. The overexpression of CEBPB gene (CCAAT/enhancer binding protein (C/EBP), beta) is a biomarker of endometrial receptivity with a role of conserving this tissue's function during implantation in primates [17]. The overexpression of the SPP1 and CEBPB genes observed in this study suggests that the endometrial receptivity was not compromised in the samples in the testing group compared to those in the control group.

Maruo *et al.* [18] reported overexpression of B-cell CLL/lymphoma (Bcl-2 and Bcl-6) in myoma cells by the progesterone receptor P4. The receptors modulated by + (PRM) not only inhibit the proliferation but also stimulate apoptosis in cultured myoma cells, and suppress collagen synthesis in specific cells. The overexpression of the Bcl-6 gene observed in our results suggests protection against the growth of myomatose.

Maybin *et al.* [19] observed an increase of IL-8 during the menstrual cycle that is consistent with its role in endometrial repair. The overexpression of IL-8 in our results indicates its participation in endometrial repair.

Thus, we infer that the endometrium is in the process of regeneration with overexpression of genes responsible for inflammatory responses (CYT genes); this function could be preserved by the overexpression of genes responsible for receptivity, alloimmunity, and endometrial repair. In this study, we observed over and underexpression of some genes that are functional in the endometrium; however, we did not quantitate their corresponding proteins before and after AEM.

The analysis of gene expressions on RNA samples pooled from a group of participants as opposed to a single individual's RNA samples was a limitation in our study. The small amounts of endometrial tissues obtained and RNA extracted were insufficient for individual analyses.

The molecular analysis of the endometrium allows the assessment of morphology and functionality; however, these on site factors have only been studied in the last two decades, and molecular studies correlating results with clinical post-AEM outcomes in the endometrium are still scarce.

Implantation, invasion, and placentation are complex molecular processes involving several molecules and enzymatic interactions that are still unclear or unknown. New studies elucidating the roles of these molecules and their intricate associations could have a significant contribution.

One limitation in our study was the small number of available cases. Therefore, in order to assess the real impact of AEM on endometrial repair after inflammatory processes, future prospective and large randomized studies with control groups as well as the evaluation of possible changes in protein production post AEM are necessary [20].

5. Conclusion

Participants were evaluated 6 months after AEM for treatment of leiomyoma and during the second phase of their menstrual cycle. The results showed overexpression of ECM genes responsible for adhesion and membrane components, underexpression of protease genes responsible for dysfunction, and destructuration in endometrium morphology. Furthermore, the results demonstrated overexpression of chemokine genes responsible for endometrial repair, underexpression of genes involved in inflammatory processes, and statistically significant uterine volume reduction. Hence, the AEM treatment did not negatively affect the endometrial function at 6 months after embolization. This study broadens the knowledge about using a procedure that is relevant to the treatment of leiomyomas and contributes to the establishment of future guidelines for the decision making process for physicians and patients.

Conflict of Interest and Funding

There is no conflict of interest to disclose.

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