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Abstract

Introduction and Objective. The congenital disease Fibrodysplasia Ossificans Progressiva (FOP) is extremely rare, characterized by irreversible and intractable skeletal malformations, with devastating heterotopic ossifications. FOP is associated with a change in the amino acid of the ACVR1 protein at position R206H due to a mutation in its respective genetic code. Previous research has observed distinct gene expression profiles between FOP peripheral blood mononuclear cells (PBMC) versus control cells. However, invasive access to biological material in these FOP patients is an obstacle due to possible collection trauma that can cause flare-ups, with undesirable consequences, such as the formation of ectopic ossification. There is, however, a need to obtain biological specimens for research or monitoring of experimental medications. The aim of the study was to obtain total RNA from buccal mucosa epithelial squamous cell scrapings (BEC) from FOP patients.

Materials and Method. BEC samples were collected by scraping the oral mucosa on the inner side of each cheek, using tongue depressor spatulas, followed by stabilization in RNAlater and Trizol extraction of total RNA. The expression profile of eight putative target Genes were analyzed from FOP BEC (n=7) and healthy volunteers (n=5), by mRNA expression through qPCR.

Results. The results showed differences in basal mRNA expression of *ACVR1*, *TNF- α* and *COL1* (p < 0.05) genes in FOP, compared to control, characterizing a distinct phenotypic profile of FOP BEC.

Conclusions. The use of BEC may be an innovative non-invasive biological material for further clinical and molecular analyzes in patients with FOP.

Key words

buccal mucosa, epithelial cells, Fibrodysplasia Ossificans Progressiva, FOP mRNA expression, squamous cell scrapings

INTRODUCTION

Fibrodysplasia ossificans progressive (FOP), characterized by the extensive and progressive formation of heterotopic bone (OH), is a rare (1 case per 2x10⁶ people) intractable autosomal dominant genetic disease that manifests in early childhood and confers cumulative disability with each crisis or flare-up. Late diagnosis of FOP or inadequate therapeutic management can prematurely confine the patient to a wheelchair or bed [1, 2].

The disease was genetically mapped in 2006. Mutations were identified in the *ACVR1* locus on chromosomes 2 (2q23–24), the gene that encodes the type I activin A receptor, also known as ALK2, a receptor type I for bone morphogenetic proteins. In the mutated gene, both in hereditary and

sporadic cases, in most cases, an identical heterozygous single nucleotide substitution (c.617G>A – guanine for adenine at position 617) is observed, which causes changes of amino acid arginine to histidine at 206 (R206H) position, in the glycine-serine domain [3, 4]. Additional variations may occur within these sequences. The *ACVR1* gene mutation leads to changes in its protein structural configuration, allowing BMP/Smad signaling pathway unregulated activation triggering osteogenic differentiation processes, at an inappropriate time and location [2, 5, 6].

In vivo and *in vitro* experimental models demonstrated the activation of the mutated *ACVR1* receptor by changes in pH resulting from tissue hypoxia [2, 7]. Acute crises at local inflammations often culminate in heterotopic bone formation, which can compromise tendons, ligaments, aponeuroses, fascia, and other connective tissues of the voluntary musculoskeletal system [8]. The eyes, heart, diaphragm, sphincter muscles, and visceral smooth muscles are characteristically spared. Acute exacerbations can last

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weeks or months, and can be accompanied by low-grade fever [9, 10].

The frequency of flare-ups is unpredictable, ranging from repeated flare-ups to long latency periods. The rate of evolution of FOP is highly variable from patient to patient, with adults already confined to bed or in a wheelchair, or with less restriction in adulthood [4, 10, 11].

The progression of FOP is typically axial to appendicular, cranial to caudal, and proximal to distal. Increased risk of involvement of the hips, wrists and knees follows age, with the mandible typically being one of the last areas critically involved [12].

Without curative treatment, therapeutic trials of multidisciplinary approaches aim to control symptoms while maintaining mobility and functionality. Limited use of high doses of corticosteroids, non-steroidal anti-inflammatory drugs and leukotriene inhibitors constitute the most used therapy during flare-ups, providing pain relief and reasonable control of inflammation, without, however, preventing heterotopic ossification [1, 13, 14].

In advanced clinical trial phases, Palovarotene, REGN 2477 and Rapamycin being prominent [13, 15, 16]; side-effects are still being clarified, as is the effectiveness in preventing progression of the disease [13, 17]. Other studies still in progress suggest therapeutic approaches for the use of ascorbic acid and association with propranolol, targeting certain benefits in alleviating the frequency of flare-ups and inflammatory consequences [18].

Monitoring classic and current treatments still requires biochemical and molecular analyzes to verify the effects of medications on processes of inflammation, fibrosis, and heterotopic ossification [19–21]. The continuity of these studies is strengthened by the suggestion that FOP is an inflammatory disease [22, 23], with participation of the immune system having already been established [24]. Yet, the participation of the autonomic nervous system in regulating inflammation and in the stages of ectopic bone formation, remains to be clarified in FOP [25], as well as the possible role of other adjuvants in controlling neo-angiogenesis required in the pathophysiological pathway of ectopic bone formation.

Previous research has observed distinct gene expression profiles between FOP peripheral blood mononuclear cells (PBMC) versus control cells [20, 21]. However, invasive access to biological material in these FOP patients is an obstacle due to collection trauma that can cause flare-ups and undesirable consequences, such as ectopic ossification. In this regard, the present study aims to present a new biological approach using BEC as specimens for research or monitoring of experimental medications.

MATERIALS AND METHOD

The study was approved by the Research Ethics Committee of the Federal University of Mato Grosso do Sul (Approval No. 4763072). All healthy volunteer participants (n=5) and patients with FOP (n=7) were duly informed by Free and Informed Consent Form (ICF), with participants aged between six years and incomplete 18 years were duly authorized by their caregivers through the ICF.

Mouth squamous cell (BEC) collection. To obtain total RNA, BEC samples were collected by scraping the oral mucosa on

the inner side of each cheek, using tongue depressor spatulas (THEOTO – 14 cm/1.4 cm/0.5 mm; 11 cm/0.8 cm/1.0 cm) of smooth conventional format, sterilized, for paediatric use. Briefly, the epithelial mucus was obtained by scraping with light manual pressure in the longitudinal, horizontal posterior-anterior direction of the inner cheek, both on the right and left side, in two separate collections approximately 5 centimeters in length, The epithelial cell mucus was dispensed into Eppendorf-type micro-centrifuge tubes and dissolved in 500 µl of Ambion RNAlater® nucleic acids stabilizing solution (Ambion™, RNAlater®). The microtube with the sample was gently shaken before storage at -80 °C and kept frozen until RNA extraction processing.

Total RNA extraction and DNase treatment. To obtain total RNA, BEC samples stored at -80 °C, in stocks of 500 µl of RNAlater, subsequently thawed at 4 °C, and then centrifuged at 700xg for 15 min/4 °C in a refrigerated micro centrifuge (Eppendorf 5427 R), for cell pellet concentration. Subsequently, total RNA was extracted using Trizol® method, following protocol described by the manufacturer, with small volume changes. Total RNA was resuspended in 100 µl of RNase treated water (diethyl pyrocarbonate – DEPC H₂O). Aliquots of 2 µl were used for quantification at 260 nm in a spectrophotometer NanoDrop™ One (Thermo Scientific™). Total RNA aliquots were stored at -80 °C for long-term storage, while ~2 µg of total RNA aliquots were further DNase treated (DNase I (TURBO DNA-free kit, Ambion Inc., Foster, California, USA), according to the manufacturer's protocol. The total RNA DNase treated pellet was resuspended in 25 µl of DEPC H₂O and concentrations readjusted subsequently for reverse transcription and quantitative PCR usage.

Targeted genes and selected oligonucleotide primers. Specific oligonucleotide primers targeting 8 FOP putative genes (Tab. 1) were previously standardized [21] for reverse transcription (RT) and real-time PCR (qPCR). The primers were synthesized by the IDT company (Integrated DNA Technologies), received lyophilized, resuspended in S.F. H₂O, and aliquoted for storage at -20 °C in stocks of 100 pmol/µl. As an endogenous control, the S26 gene [26] was used as a target for qPCR normalization.

Reverse transcription (RT) and Real Time Polymerase chain reaction. Total RNA (DNase treated) was used for first strand complementary DNA (scDNA) synthesis by reverse transcription (RT) reaction. Briefly, the RT consisted of 6 µl (350 ng) of RNA and 10 pmol of each specific reverse primer and oligo dT18, in a final volume of 10 µl, pre-incubated at 70 °C for 10 minutes and stored immediately on ice. Then, 11 µl of reverse transcriptase enzyme mix (40 U) in RT buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4) containing 2 µl of dNTP mix (10 mM of each) were added, incubated at 45 °C for 1 hour, in a final volume of 21 µl of RT reaction. The reaction was completed at 4 °C and used immediately in qPCR (quantitative real-time PCR). All reagents were from Invitrogen™ (SuperScript™ First-Strand Synthesis System for RT- qPCR).

The scDNA samples resulting from RT were used in qPCR, carried out on QuantStudio™ 6 Flex Real-Time PCR System equipment, using the reaction protocol described by the SYBR Green PCR Master Mix Kit [27]. Samples in duplicates were

applied to 384-well plates (ABI PRISM® 384-Well Optical Reaction Plate with Barcode, Invitrogen Life Technologies, Carlsbad, CA, USA), in a final reaction volume of 25 µl. Two µl of RT scDNA were pipetted into each plate well, followed by reaction Mix (5 µl of SYBR Green PCR Master Mix Kit; 0.15 µl {10 pmol/µl} of each sense and antisense primers) added in a 8 µl final volume adjusted with sterile filtered water. PCR plate was sealed with optical adhesive (ABI PRISM® Optical Adhesive Covers, Invitrogen Life Technologies, Carlsbad, CA, USA). The qPCR reactions occurred in the following thermal cycle: [stage 1] a cycle of 50°C/2 min.; [stage 2] one cycle at 95°C/10 min.; [stage 3] 40 cycles of 95°C/15s, followed by a dissociation curve starting at 60°C to analyze the specificity of the target amplicons.

Relative expression quantification of target transcripts was determined by comparative analysis with endogenous S26 normalizer, using comparative CT method, as $2^{-\Delta\Delta CT}$ method for relative levels of gene expression was applied [28]. Data were analyzed in GraphPad Prism 5 programme for statistics, and unpaired t test plus ANOVA were applied. Results were statistically significant for $p \leq 0.05$.

RESULTS

The results obtained from total RNA extraction showed a ten-fold higher yield in RNA extraction when scraping with larger spatulas (14 cm/1.4 cm/0.5 mm), compared to the yield from smaller spatulas (11cm/0.8 cm/2.0 mm) collections, i.e. 26.5 +11.9 µg (n=9) versus 1.9 + 2.2 µg (n=14) of total RNA yields, respectively. Nevertheless, the present methodology, using the Trizol method, showed that the amounts of total RNA were sufficient for all RT-qPCR studies. Relative expression data from all investigated genes from FOP BEC versus healthy BEC controls are shown as scatter plots in Figure 1.

Results of basal mRNA expression showed significant differences in *ACVR1*, *TNF-α* and *COL1* genes ($p < 0.05$) in FOP, when compared to BEC controls. Additional gene expression representation is shown in Figure 2 and Table 1, characterizing distinct gene expression profiles in BEC in FOP. The expression of the *Col3*, *ADRB1*, *ADRB2* and *RUNX 2* genes showed no significant differences, although there are trends of increased expression of *ADRB1*, *ADRB2* and *RUNX 2* transcripts, represented by the arrows in Table 2.

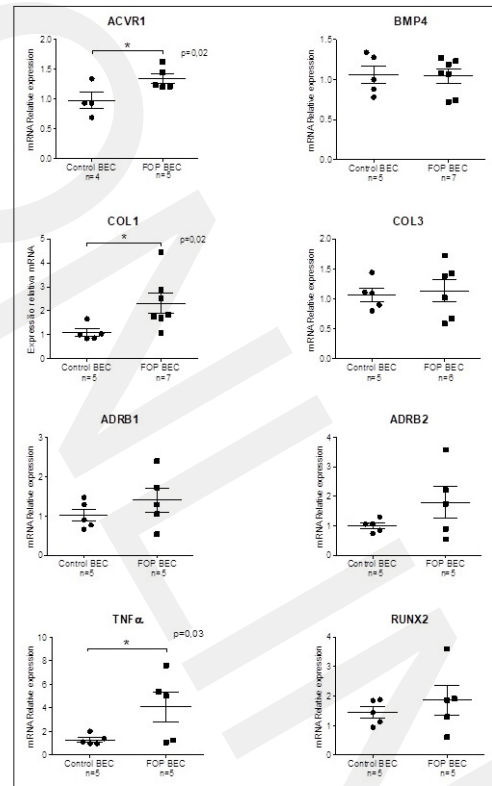


Figure 1. Comparison of expression of target genes in epithelial cells of oral mucosa of patients with Fibrodysplasia Ossificans Progressiva (FOP) versus healthy controls. Significant differences in basal mRNA expressions for *ACVR1*, *TNF-α* and *COL1* genes ($p < 0.05$) of FOP BEC are highlighted

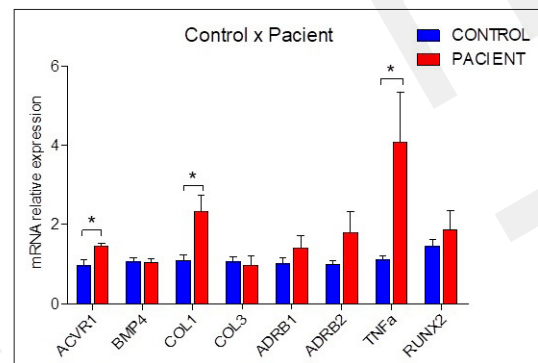


Figure 2. Relative mRNA expression profile for FOP BEC vs. healthy controls. Significant differences in basal mRNA expressions for *ACVR1*, *TNF-α* and *COL1* genes ($p < 0.05$) of FOP BEC are highlighted

Table 1. Targeted genes and selected oligonucleotide primers

Gene	mRNA description	Oligonucleotide primer sequences	Target (bp)
<i>ACVR1</i>	active in A receptor type 1	F<CTGCCTTCGAATAGTGCTGCCAT>R<TAAATCTCGATGGGCAATGGCTGG	100
<i>BMP4</i>	bone morphogenetic protein 4	F<CAGGAGATGGTAGTAGAGGGATGT>R<AGTCTGTGTAGTGTGGGTGA	140
<i>COL1</i>	collagen type I α-1 chain	F<CAAAGGAGACACTGGTGCTAAG>R<CTCCCTCGCTTCTCTCTCTC	89
<i>COL3</i>	collagen type III α-1 chain	F<AGCTGTTGAAGGAGGATGTTCCCA>R<TTTGGCATGTTCTGGCTTCCA;	77
<i>ADRB1</i>	Adrenoceptor β-1	F<TTCTACGTGCCCTGTGCATC>R<GATCTTCTTACCTGCTTCTGG	78
<i>ADRB2</i>	adrenoceptor β-2	F<CTGTGCGTGATCGCAGTGGAT>R<CTTATTCTGGTCAGGCTC	78
<i>RUNX2</i>	RUNX family trans. factor 2	F<CTTGACCATAACCGTCTTAC>R<CGAGGTCCACTACTGTAAC	81
<i>TNF-α</i>	tumour necrosis factor α	F<CCAGGGACCTCTCTAATCA>R<GTTTGTGCTACAACATGGGCTAC	95
<i>S26</i>	S26 ribosomal protein RNA	F<TGTGCTTCCCAAGCTGTATGTGAA>R<CGATTCTGACTACTTTGCTGTG	75

(Bp) base pair; (F) forward sense (5'-3'); (R) reverse anti-sense (5'-3')

Table 2. Relative expression profile of target genes in FOP BEC vs. healthy controls

Gene	Expression	P value	2 ^{-ΔΔCT} (mean ± MSE)	Control	(n) (FOP/Control)
ACVR1	↑	0,0227	1,34 ± 0,09*	0,97 ± 0,08	(5/4)
BMP4	-	0,4627	1,04 ± 0,09	1,06 ± 0,11	(7/5)
COL1	↑	0,0191	2,32 ± 0,42**	1,09 ± 0,15	(7/5)
COL3	-	0,3869	1,13 ± 0,18	1,07 ± 0,11	(6/5)
ADRB1	-	0,1538	1,40 ± 0,31	1,02 ± 0,15	(5/5)
ADRB2	↑	0,0919	1,79 ± 0,54	1,00 ± 0,10	(5/5)
RUNX2	-	0,2317	1,85 ± 0,50	1,45 ± 0,19	(5/5)
TNF-α	↑	0,0306	4,08 ± 1,27*	1,30 ± 0,20	(5/5)

(-) same expression; (↑) increased expression; (* = p ≤ 0,05; ** = p ≤ 0,01) statistical test significances are highlighted; (red arrow) tendency; (n) sample number.

DISCUSSION

The present study analyzed the viability of BEC as a non-invasive specimen from which yields above 2 µg of total RNA can be obtained, using oral mucosa scrapings with tongue depressor spatulas for paediatric use. Total RNA obtained by the common Trizol method was sufficient for all real-time quantitative PCR studies after reverse transcription. Immediate storage of scraped squamous cells in an RNA stabilizer was mandatory to preserve total cellular RNA prior to extraction. An extensive discussion of the probable functions of the genes selected here, as well as their role in the pathophysiology of FOP, has already been presented in a previous study by [21]. However, the use of BEC for future genomic studies of a broader spectrum should be important for continued understanding of the molecular mechanisms involved in the pathophysiology of FOP.

In this study, the obtained relative expression data of the 8 genes investigated in the epithelial cells of the oral mucosa, show distinct basal expression differences of *ACVR1*, *TNF-α* and *COL1* in FOP BEC, compared to the healthy control *in vivo*. This is consistent with previous data observed by the authors *in vitro* in PBMC cells and total peripheral blood leukocytes, despite the expression of the *Col3*, *ADRB1*, *ADRB2* and *RUNX 2* genes having no significant differences, although there were trends of increased expression for *ADRB1*, *ADRB2* and *RUNX 2* transcripts. This suggests that these FOP BEC results also corroborate previous observations elsewhere (Tab. 3) of some distinct phenotypic mRNA expression profile of FOP target genes (Tab. 3) [20, 21].

It is well known that the tissue distribution of mRNA expressing different genes is notably tissue specific. A previous study by Nascimento et al. (2021), using 24 – 48-hour cultivation of PBMC (peripheral blood mononuclear cells) *in vitro*, after ficoll-hypaque density gradient separation, these cells entered a basal state in proper culture medium, thus verifying the typical expression pattern for mRNAs relating to 23 different genes, putatively involved in the FOP mechanism, that suggests a distinguishable phenotypic profile. Interestingly, in the present study *in vivo*, some coherences was found in the BEC gene expression profile when compared to PBMC (Tab. 3). Nevertheless, *ACVR1* gene expression was observed as upregulated in BEC *in vivo*, which differed from the description by Nascimento et al., (2021) of *ACVR1* gene downregulated in PBMC *in vitro*.

Table 3. Comparative expression profile of FOP target genes in 2 cell sources: (PBMC) peripheral blood mononuclear cells and (BEC) buccal squamous epithelial cell

Gene	Cells	PBMC	BEC
ACVR1		↓	↑
BMP4		-	-
COL1		↑	↑
COL3		↓	-
ADRB1		↑	-
ADRB2		↑	↑
RUNX2		↑	-
TNF-α		↑	↑
Sources		Nascimento 2021	Present study

A plausible explanation for this *in vivo* versus *in vitro* discrepancy expressions may be due to the fact that PBMC cells, when resting in the culture medium, do not undergo any paracrine influence, especially from circulating hormones, such as blood catecholamines, or even the *ACVR1* activators, ligands, such as BMPs, or even activin, as natural agonists, which can occur in BEC cellular specimens susceptible to both paracrine and autocrine circulatory actions. However, more future studies may help to clarify this hypothesis, as well as unpublished ongoing studies by the authors of the current study, based also on the expression data of these genes in peripheral whole blood (PBC).

Invasive access to biological material in patients with FOP is a major obstacle to *in vivo* studies aimed at a better understanding of the molecular biology involved in the pathophysiology of the disease. This is due to possible traumas during collection that can cause flare-ups and undesirable consequences, such as the formation of heterotopic ossification [10, 29]. Cytological specimens from the oral cavity have been suggested as an excellent source of easily accessible biological material for target studies of genetics, genotoxicity, epigenetics, proteomics, metabolomics, and microbiomes. This is due to the rapid, non-invasive and low-cost collection compared to tissues such as the blood [30]. Alternative sources, such as cytological brushes, mouthwashes, and collection cards for extracting genomic DNA from oral cells, have already been tested for genetic studies and epidemiological investigations, comparing their yields, viabilities, qualities and cost. Average yields vary roughly between 2 – 4 µg of genomic DNA [31].

Fluorescence quantification techniques have shown differences in DNA yield with 1.1 and 5.2 µg for cytobrushes and mouthwash, respectively. Protocols have been validated and applied in different studies [32]. Although this method is interesting for genetic studies or for diagnosis by PCR, generally such materials are somehow heterogeneous in terms of cell types in the biological material, which would be more complex for comparative and quantitative analyzes of specific mRNA expression such as target genes of the FOP. This, among others, was the main reason for the authors choosing to work with squamous cells from the internal oral wall.

Nevertheless, quality total RNA is currently one of the most important materials for functional genomics analyses, including transcripts of different RNA species, in their different functional aspects; studies that evaluate the viability of the method for extracting total RNA from

cellular specimens from the mouth are rare. Besides, digestive enzymes, such as amylases and even RNAses from saliva, can be an obstacle to collections with low numbers of cells in different swabs [33–35]. Although there are no references, to the best knowledge of the authors, within studies on FOP pathophysiology regarding collection of BEC, for continuous gene expression analysis, show that this approach is still lacking in FOP studies. Patients with FOP cannot undergo invasive procedures. However, because of this unavailability due to the undesirable consequences for the patient, *in vitro* or *in vivo* FOP studies are hampered by the unfeasibility of obtaining cells or target tissues for continuous, gene modulation studies, or clinical trials monitored with genetic markers, which require specimens for constant analysis.

The most common model used is lymphoblastoid cell lines (LCL) from PBMC by transformation with the Epstein-Barr virus, as trauma to deep connective tissues can be avoided when collecting peripheral blood samples from FOP patients. LCL have been an important and valuable tool for *in vitro* studies of gene expression in FOP [36, 37], although it is still an invasive approach subject to difficulties in certain situations of human error with risks in collection. In this same effort, less risky methods have been addressed, such as isolation of multipotent progenitor cells from the dental pulp of deciduous teeth discarded from children with FOP [38]. This, however, is an approach with limitations if one wants to monitor patients in continuous research. Induced pluripotent stem cells (IPTC) derived from normal dermal fibroblasts and those from patients with FOP [39], have also been considered. However, for these cells there is a need for biopsies from patients, in addition to maintaining these cultures, and it appears that cell responses can change over time. There are some data that show the optimization of biopsy methods and the improvement of CTPI cells [40], but there is always a risk for patients when undergoing biopsies.

In conclusion, the use of BEC as samples for FOP research can be an innovative non-invasive biological material for future clinical and molecular projects, such as risk-free monitoring of gene expression and transcriptome of patients *in vivo*.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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