Changes in growth plate extracellular matrix composition and biomechanics following in vitro static versus dynamic mechanical modulation

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Abstract

The objective of this study was to investigate the effects of mechanical modulation parameters on structural proteins biomecomposition and mechanical properties of the growth plate. Establishing these parameters is a crucial step in the development of fusionless treatment of scoliosis. In this study, ulna explants from 4-weeks-old (pubertal) swines were used. The biomecomposition was characterized using biochemical content evaluation and immunohistochemistry. Mechanical properties were characterized by fitting the data of the stress relaxation curves using a fibril reinforced biphasic model. Mechanical properties were characterized by fitting the data of the stress relaxation curves using a fibril reinforced biphasic model. For the mechanical loading, one static modulation condition and three different dynamic modulation conditions, with similar average stress but different amplitude and frequency values, were performed using a bioreactor. Results showed that static loading triggers a decrease in proteoglycan content and type X collagen in specific zones of the growth plate. These changes can be associated with the observed decrement of permeability in the static group. None of the three conditions evaluated for dynamic modulation affected the growth plate biomecomposition and biomechanical responses. Results of this study provides an improved understanding of growth plate responses to mechanical environment, which will be useful in finding the optimal and non-damaging parameters for fusionless treatments based on the mechanical modulation of bone growth.

Keywords: Growth Plate, Mechanobiology, Compressive Loading, Quantitative Immunohistochemistry, Biochemical Content Evaluation

Introduction

Longitudinal growth of long bones and vertebrae occurs in growth plate cartilage, located at their extremities¹. Based on experimental and clinical evidences, mechanical forces are a key factor of longitudinal bone growth regulation and one of the main reasons for the progression of developmental growth angular deformities such as juvenile and adolescent scoliosis². The concept of mechanical modulation of bone growth is currently used for the development of novel fusionless devices to correct spinal deformities (i.e. adolescent idiopathic scoliosis) by applying compression on the rapidly growing part of vertebrae located in the convex part of the curvature in order to retard growth³.

Experimental in vivo and in vitro studies are trying to find optimal but non damaging loading parameters for mechanically modulating bone growth by comparing the effects of static vs. dynamic loading⁴,⁷ or comparing dynamic loading parameters⁴,⁸ or comparing dynamic loading parameters⁴,⁸ on bone growth rate⁷,⁹, growth plate histomorphology⁸,⁹, extracellular matrix protein synthesis⁸,⁹, biomechanical responses⁸ and chondrocyte viability⁸. In these studies, stress-equivalent static and dynamic mechanical loading protocols were applied. Average stress values of 0.1-0.2 MPa were used for static and dynamic loadings, this last one with sinusoidal waveform oscillating at ±30-100% of the average amplitude and with a frequency range of 0.1-1 Hz. The overall results suggest that dynamic loading is less detrimental for tissue biomechanics and integrity while it has the same growth reducing effect as static loading. In our
Table 1. Experimental groups and mechanical modulation parameters.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Stress (MPa)</th>
<th>Amplitude (%)</th>
<th>Frequency (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Fresh samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Cultured for 48 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Static</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dynamic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dyn1</td>
<td>0.1</td>
<td>30</td>
<td>0.1</td>
</tr>
<tr>
<td>Dyn2</td>
<td>0.1</td>
<td>30</td>
<td>1.0</td>
</tr>
<tr>
<td>Dyn3</td>
<td>0.1</td>
<td>100</td>
<td>0.1</td>
</tr>
</tbody>
</table>

recent in vitro study, we also observed that static loading is more detrimental for tissue biomechanics as it changes the mechanical strain patterns within the growth plate tissue when compared to dynamic loading. Previous studies on growth plate mechanobiology from our group and some studies on articular cartilage, which is a structurally very similar tissue to growth plate, have shown that static and dynamic loading regulates protein synthesis differently. However, to date no study has evaluated the effect of static vs. dynamic modulation and the effects of frequency and amplitude of dynamic loading on protein expression of growth plate cartilage. Since growth plate mechanical properties are also related to protein contents, we hypothesized that changes in the growth plate biomechanical responses are related to changes in protein syntheses. Therefore, the objective of this study was to evaluate the effect of static vs. dynamic compression as well as dynamic compression parameters (frequency and amplitude of loading) on the main structural growth plate proteins (aggrecan, type II collagen and type X collagen). Immunohistochemical signal reaction strengths were evaluated on bright field images of the tissue for the three main structural proteins. Moreover, quantification of the overall content of proteoglycan and collagen was performed for each zone based on the protocol developed by Hoemann et al., 2004. Finally, to link changes in protein expressions and growth plate biomechanical responses, a fibril reinforced poroelastic model was used to analytically extract the matrix modulus, the fibril modulus and the permeability of the tissue.

Methods

Animal model and mechanical modulation

Growth plate samples were extracted from 4-week-old swine distal ulnae under sterile conditions. The samples were harvested from a local abattoir within two hours post mortem. The upper and lower surfaces of explants were trimmed using a vibratome (Vibratome1500 Sectioning System) to obtain two parallel surfaces. The samples were randomly distributed among the six experimental groups (Table 1): baseline (BL), control (Ctrl), static (Stat), and 3 dynamic groups (Dyn1, Dyn2, Dyn3); each group including 10 samples. Samples of all groups, except baseline, were placed in the chambers of a bioreactor (Tissue Growth Technology, Instron, Norwood, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 20% heat deactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) at 37°C in 95% humidity and 5% CO₂ for 48h hours. For the mechanically modulated groups (static and dynamic), the compression was performed using the parameters indicated in Table 1 for 12 hours during the 48 hours of culturing. The samples were modulated using the piston of bioreactor and the amount of force was controlled by a load cell integrated in the bioreactor system. The full experimental set up is described in a previous study. Shortly, samples were placed in the bioreactor chamber immersed in the culture medium. Static loading was applied with an average constant compressive stress of 0.1 MPa, similar to previous in vivo studies, being near biological ranges that modulate the growth rate without stopping it. Dynamic loading was applied using a sinusoidal waveform with an average compressive stress of 0.1 MPa and a frequency of 0.1 or 1.0 Hz and amplitude (i.e. variation around the average stress) of ±30% (±0.03 MPa) or ±100% (±0.1 MPa). Samples were modulated during 6 cycles of 8 hours throughout 48 hours: for each 8 hours period, samples were loaded for 2 hours (at conditions indicated in Table I) and then loading was removed for the remaining 6 hours. During unloading periods, only the preload of 0.001 MPa was maintained. Control explants were cultured in the same chambers as loaded explants for 48 hours, with only the preload applied. These conditions were selected from previous studies in the literature including those from our group. After mechanical modulation and/or culturing period (or right after dissection for the baseline group), the explants were divided into four parts. One part was used for viability assessment, another for strain map characterization, histomorphological analysis and mechanical characterization and one part was fast frozen and preserved for biochemical content evaluation. The last part was fixed in 4% paraformaldehyde (Sigma-Aldrich), decalcified in 10% EDTA, and prepared in paraffin for immunohistochemistry.

Determination of sulfated glycosaminoglycan and hydroxyproline contents

Each frozen part of the explants was further divided into its three zones of reserve, proliferative and hypertrophic using a Microtome Cryostat HM 500 O (GMI, Minnesota, USA) based on the individual histomorphological measurements performed in our previous study. Each zone of each sample was digested overnight at 60°C with papain enzyme (Sigma-Aldrich Oakville, ON, Canada) at pH 6.0. For determination of sulfated glycosaminoglycan content, the Dimethylmethylene Blue Assay (DMMB assay) was performed on papain digested samples based on the protocol suggested by Hoemann et al., 2004. Different dilutions of chondroitin sulfate A sodium salt from shark (C4384, Sigma, Aldrich) were used to produce the standard
Figure 1. Quantification of the immunohistochemical (IHC) reaction signal intensity: a) original image; b) segmented parts containing reactions; c) grayscale indexed image and d) scaled indexed images.

Figure 2. Proteoglycan and collagen contents of the reserve, proliferative and hypertrophic zones for the six experimental groups. Values are expressed as mean ± SD. Significant differences with respect to the control group are marked with * (p<0.01). BL, baseline; Ctrl, control; Stat, static; Dyn1, dynamic at 30% amplitude, 0.1Hz frequency; Dyn2, dynamic at 30% amplitude, 1% frequency; Dyn3, dynamic at 100% amplitude, 0.1Hz frequency.
Table 2. Proteoglycan and collagen contents of growth plate explants for reserve (R), proliferative (P) and Hypertrophic (H) zones. Significant differences with respect to the control group are marked with *(p<0.01), n=10 for each group. Values are expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Collagen (μg/wet weight)</th>
<th>Collagen (μg/wet weight)</th>
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<tbody>
<tr>
<td></td>
<td>H</td>
<td>P</td>
</tr>
<tr>
<td>Baseline</td>
<td>36 ± 11</td>
<td>67 ± 17</td>
</tr>
<tr>
<td>Control</td>
<td>41 ± 21</td>
<td>60 ± 20</td>
</tr>
<tr>
<td>Static</td>
<td>24 ± 9</td>
<td>36 ± 16*</td>
</tr>
<tr>
<td>Dynamic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dyn1</td>
<td>31 ± 9</td>
<td>56 ± 15</td>
</tr>
<tr>
<td>Dyn2</td>
<td>37 ± 8</td>
<td>56 ± 19</td>
</tr>
<tr>
<td>Dyn3</td>
<td>35 ± 12</td>
<td>58 ± 23</td>
</tr>
</tbody>
</table>

Immunohistochemistry

Antibodies were tested on 5 μm paraffin sections of each explant cut at three different depths in the tissue. The protocol used was similar to the one used in the study by Sergerie et al (2011)\(^\text{i}\). Explant sections were treated with chondroitinase ABC and 10mM pH 10.0 TRIS buffer for 20 min at 60°C for antigen retrieval. The sections were then digested in hyaluronidase (2 mg/ml; Sigma-Aldrich) for 30 min at 37°C and endogenous peroxidase blocked using 0.3% hydrogen peroxide in PBS for 30 min at room temperature. In order to prevent non-specific reactions, samples were incubated with 1.5% goat normal serum in PBS for 60 min at room temperature. The sections were incubated overnight at 4°C in presence of primary antibody: polyclonal rabbit anti-mouse anti-aggrecan (1/50; Chemicon, Temecula, CA), monoclonal mouse anti-chicken anti-type II collagen (1/50; DSHB, Iowa, USA) or monoclonal mouse anti-porcine anti-type X collagen (1/50; Sigma-Aldrich). The next day, the sections were washed and incubated with secondary antibody: biotinylated anti-rabbit or anti-mouse IgG secondary antibodies (1/200; Vector Laboratories, Burlingame, CA) for 60 min at room temperature. The reaction was revealed and developed using the avidin-biotin complex method (Ventaxtain ABC kit; Vector Laboratories) using diaminobenzidine substrate (DAB, Vector Laboratories). In order to test the specificity of reactions, negative control samples were prepared to eliminate the primary antibody from the procedure.

Imaging and quantitative analysis

In order to compare the results from different samples together, all the conditions for preparing the samples (including fixation time, dehydration and paraffin embedding), immunohistochemistry procedure (duration of incubation with different enzymes and antibodies and their concentration) and imaging (parameters used for taking images) were controlled and kept the same for all samples. The images were taken using a Leica DMR microscope equipped with a Retiga Qimaging Camera using the same sets of parameters for each dataset. Quantification of the reaction signal intensity was performed on the resulting RGB images using a custom developed image processing toolbox. The reaction sections were segmented using a K-Mean clustering algorithm and all the other parts were neglected. The resulting segmented RGB image was converted to an indexed image by calculating the mean square root of its RGB value. The average reaction signal intensity over a region of interest was calculated by averaging the indexed values of the segmented reactions over that region. This quantification was performed for the three zones of growth plate (hypertrophic, proliferative and reserve) and for each antibody in triplicate. The performance of the algorithm was validated on artificially produced images. An example of the result of analysis using the developed algorithm is presented in Figure 1.

Mechanical test and curve fitting

In order to find the relationship between growth plate protein expressions and biomechanical responses, a semi-confined stress relaxation test was performed using a custom developed mechanical testing machine on one section of each explant\(^\text{6}\). Samples were tested for mechanical characterization in approximately 30 minutes after mechanical modulation. Following the first contact between the semi-cylindrical explants and platen, a pre strain of 5% was applied to the tissue. After relaxation, the explants were loaded with 5% of strain and the stress data was collected using a load cell until relaxation. The relaxation criterion was set to 1E-6 N/s as explained in our previous study\(^\text{5}\). During the whole stress relaxation procedure, samples were bathed in DMEM at room temperature. Platen displacements were controlled by a custom designed software (Lab View, National Institutes of Health, http://www.ismni.org).
Instruments, Inc., USA) and the force data was recorded using a load cell with resolution of 0.026 N and range of 0 to 40 N.

The MACH-1 analysis software (Biomomentum Inc., Laval, QC, Canada) was used to fit the fibril-network reinforced biphasic model to the stress relaxation curves and calculate the fibril modulus ($E_f$), the matrix equilibrium modulus ($E_m$) and axial permeability ($k$) of explants. This model is derived from the classical biphasic model and is reinforced with a fibrilar network that resists tension only. The parameters required to describe the growth plate mechanical properties in this model are $E_f$, $E_m$, $k$, and Poisson’s ratios ($\nu_m$ and $\nu_f$). In our analysis, $\nu_m$ and $\nu_f$ were fixed to zero and 0.3 respectively. $E_m$ was calculated directly from the experimental data and $E_f$ and $k$ were estimated by curve-fitting.

Figure 3. A) Representative aggrecan immunohistochemical slides; B) Average aggrecan reaction signal intensity. Values are expressed as mean ± SD. Significant differences are marked with * (p<0.01). BL, baseline; Ctrl, control; Stat, static; Dyn1, dynamic at 30% amplitude, 0.1 Hz frequency; Dyn2, dynamic at 30% amplitude, 1.0 Hz frequency; Dyn3, dynamic at 100% amplitude, 0.1 Hz frequency; IHC, Immunohistochemistry.
**Results**

**Proteoglycan and collagen content**

Results of biochemical content analysis for proteoglycan and collagens are summarized in Table 2. Figure 2 depicts the average content of collagen and proteoglycan for each zone and their confidence intervals. For all experimental groups, the highest proteoglycan content was found in the proliferative zone and the lowest in the hypertrophic or reserve zone. In order to isolate the culture effect, the control group was compared with the baselines. No significant differences were observed between the proteoglycan and collagen content of the two groups. In order to isolate the effect of static vs. dynamic loading, static and Dyn1 groups were compared with controls. Compared to the controls, the proteoglycan content of the proliferative zone of static group was significantly reduced to 36.0±15.5 μg/wet weight while the proteoglycan content of the other zones and the three zones of Dyn1 group remained unchanged. Also, the collagen content of both static and Dyn1 group did not have a significant difference compared to the control group. In order to evaluate the effects of frequency and amplitude of loading, Dyn2 and Dyn3 groups were compared with Dyn1. No significant differences were observed in the proteoglycan and collagen content of the two groups with respect to Dyn1.

**Immunohistochemistry reaction signal intensity**

Representative images from immunohistochemical (IHC) analyses for the three antibodies (aggrecan, type II collagen and type X collagen) along with their average reaction signal intensities are presented in Figures 3-5, respectively. The reaction signal intensity is an indicator of the IHC reaction strength. As a result, higher reaction signal intensity indicates more reaction of the antibody of interest or, in other words, more expression of that protein. With this measure, aggrecan expression remained unchanged in response to culturing in the three zones of growth plate. Static loading reduced the expression of aggrecan in the proliferative zone and early hypertrophic zones (the first 30% of hypertrophic zone) of explants while dynamic loading did not alter the expression of aggrecan with respect to controls (Figure 3). Type II collagen expression was not affected neither by culturing nor mechanical modulation (Figure 4).

Type X Collagen was exclusively expressed in the hypertrophic zone for all experimental groups (Figure 5A).

The average type X collagen reaction experimental group is presented in Figure 5B. The reaction signal intensity in the controls remained unchanged with respect to the baselines. However, the reaction signal intensity was reduced in all the mechanically modulated groups. Compared to the Dyn 1 group, static and Dyn 3 groups had lower reaction signal intensities.

**Mechanical properties**

No significant differences were observed between collagen fibril moduli of the different groups. All moduli were in the range of 15.5 to 19 MPa (Figure 6A). The matrix moduli of all the cultured groups were decreased with respect to the baseline group (Figure 6B). In the baseline group, the average matrix modulus was 1.39 MPa while it was decreased to 0.45 MPa in the culture control group. The permeability was increased in the culture control group with respect to the baseline group. Moreover, the permeability of static group was significantly increased compared to the culture control group (Figure 6C).

**Discussion**

In vitro static and dynamic compression protocols were performed on growth plate explants to evaluate the relationship between their changes in structural proteins content and mechanical properties. On one hand, significant reductions in proteoglycan and type X collagen content were recorded under static modulation of swine ulnae explants, in parallel with significant changes in matrix modulus and permeability. On the other hand, no changes were observed in growth plate biocomposition and mechanical properties following dynamic modulation.

**Static modulation affects the extracellular matrix protein content and mechanical properties of growth plate.** Compared to the culture control group, the proteoglycan content of the static group was reduced by 40% in the proliferative zone. Also, based on the immunohistochemistry staining, the expression of aggrecan, one of the main proteoglycan in the growth plate extracellular matrix, was also reduced by 21% in the proliferative zone and by 17% in the hypertrophic zone mostly located at early hypertrophic area. In the dynamically modulated samples, aggrecan expression and proteoglycan content were not changed with respect to controls. These results are consistent with a previous study from our lab, where a loss of aggrecan was noted in response to static modulation while aggrecan expression was increased in dynamically modulated samples. However, that previous study lacked a reliable comparative system and the immunohistochemistry images were compared by visual inspection, which might explain the differences between results of the two studies for dynamic modulation. Similar effects have been observed in other cartilaginous tissues, such as intervertebral disc and articular cartilage. In both tissues and for the compressive modulation within a similar range as in our study, aggrecan

http://www.ismni.org
and proteoglycan syntheses were reduced in response to static loading and remained unchanged or were increased in response to dynamic loading\textsuperscript{20,21}. For compressive modulation higher than these values, dynamic loading also had detrimental effects on proteoglycan synthesis\textsuperscript{22}. The loss of proteoglycans occurs through three different mechanisms: increased catabolism, increased porosity of extracellular matrix caused by damages in collagen network or increased deformation-induced convective fluid flow\textsuperscript{23}. In our study, changes in aggrecan expression is not a result of increased catabolism caused be elevated aggrecanase activity as our group previously showed that metalloproteinases ADAMTS-4 and -5 levels do not change in response to static loading\textsuperscript{9}.

Type X collagen is exclusively found in the hypertrophic zone of growth plate and is a marker of chondrocyte maturation and endochondral ossification\textsuperscript{24}. The synthesis of

\begin{figure}[h]
  \centering
  \includegraphics[width=\textwidth]{figure4.png}
  \caption{A) Representative type II collagen (Coll II) immunohistochemical slides; B) Average type II collagen reaction signal intensity. Values are expressed as mean ± SD. BL, baseline; Ctrl, control; Stat, static; Dyn1, dynamic at 30% amplitude, 0.1 Hz frequency; Dyn2, dynamic at 30% amplitude, 1.0 Hz frequency; Dyn3, dynamic at 100% amplitude, 0.1 Hz frequency; IHC, Immunohistochemistry.}
\end{figure}
type X collagen was reduced in the hypertrophic zone of static explants compared to controls and base dynamics (Dyn1). This correlates with results from a previous similar study in our lab. Moreover, increasing the amplitude of loading from 30% to 100% reduced type X collagen expression in Dyn 3 group.

Type II collagen, which is found all over the growth plate extracellular matrix, was not affected by the mechanical modulation parameters used in this study. Therefore, we can deduce that collagen network breakdown was not the cause proteoglycan loss neither and that the proteoglycan loss occurred only in response to deformation induced fluid flow. However, our results are not consistent with our previous study on growth plate or studies on articular cartilage, where type II collagen fibril network was reported to be disrupted by mechanical loading in a dose dependent way. This difference in response might be a result of difference

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**Figure 5.** A) Representative type X collagen (Coll X) immunohistochemical slides; B) Average type X collagen reaction signal intensity. Values are expressed as mean ± SD. Significant differences are marked with * (p<0.01). BL, baseline; Ctrl, control; Stat, static; Dyn1, dynamic at 30% amplitude, 0.1 Hz frequency; Dyn2, dynamic at 30% amplitude, 1.0 Hz frequency; Dyn3, dynamic at 100% amplitude, 0.1 Hz frequency; IHC, Immunohistochemistry.
Changes in matrix modulus and permeability of static explants could be associated with changes in their proteoglycan content. The permeability of static explants was increased compared to culture control and dynamic groups. This increment of permeability could be directly correlated with the observed decreased proteoglycan content and aggrecan expression in the proliferative zone and type X collagen reduction in the hypertrophic zone. Based on many studies on growth plate and articular cartilage, proteoglycans are one of the main determinants of the mechanical properties of extracellular matrix in compression\(^\text{26}\). Hence, the decrease in proteoglycan content of the proliferative zone and lower expression of aggrecan might partly explain the increase in the higher mechanical strain observed in this zone in our previous study\(^\text{5}\). Changes in the mechanical properties (permeability) of the tissue in response to mechanical modulation have also been reported in articular cartilage\(^\text{12}\). However, in articular cartilage, the increased permeability has shown to be a consequence of the collagen network breakdown in dynamically loaded samples, which was not observed in our study.

Dynamic loading parameters do not impact the growth plate composition and mechanical properties. Changing the modulation frequency from 0.1 Hz to 1.0 Hz, or changing the amplitude of dynamic loading from 30% to 100% did not affect the growth plate protein expressions, contents or biomechanical responses. These results correlate with previous studies on the effects of frequency or amplitude of dynamic modulation on growth plate responses, where changing the dynamic loading parameters did not affect growth plate in vivo\(^\text{8}\), in vitro\(^\text{8}\) histomorphology, in vivo growth rates\(^\text{8}\), and in vitro compressive strain patterns\(^\text{8}\). Similarly, studies on articular cartilage have shown that synthesis of type II collagen is not affected by frequency\(^\text{27}\).

Culturing changes the mechanical properties without affecting protein composition of growth plate. In order to differentiate the effects of culturing with the effects of mechanical modulation, the culture group was compared with the baselines. Culturing did not affect the protein expression and protein content of growth plate compared to the baselines. However, the permeability of the explants was increased and their matrix modulus was decreased in response to culturing while the fibril modulus remained unchanged. These changes could result from a lack of microcirculation, which prevents nutrition and gas exchange, and/or increased degradation activity in the cultured groups.

Overall, this study provides insights on the effect of static and dynamic parameters on growth plate protein synthesis and biomechanical responses. Results suggest that static mechanical modulation induces more changes in the extracellular matrix protein expression of growth plate compared to dynamic modulation. Indeed, static compression modified the extracellular matrix composition, namely a decrease in aggrecan and type X collagen, which was translated in an increased permeability of the tissue. Conversely, dynamic compression preserved the growth plate extracellular matrix and biomechanical properties. This knowledge is greatly relevant for the improvement and/or development of new fusionless approaches, which are based on the local modulation of bone growth to correct several progressive musculoskeletal deformities. Based on this study, fusionless approaches based on dynamic (vs static) compression would be more promising since less-detrimental to the growth plate tissue.

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Authors contributions

Rosa Kaviani: Design of experiment, experimental procedures, analyses and interpretation of results, article writing and editing, responsible for the integrity of the work. Irene Londoño: Developing protocols for immunohistochemical assays, experimental procedures, analyses, interpretation of results. Stefan Parent: Design of experiment, review of the article. Florina Moldovan: Design of experiment, interpretation of results, review of the article, responsible for the integrity of the work. Isabelle Villemure: Design of experiment, analyses and interpretation of results, review of the article, responsible for the integrity of the work.

References