Research Article

Effects of Vitamin D Supplementation during the Induction and Progression of Osteoarthritis in a Rat Model

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Received 18 April 2012; Revised 4 September 2012; Accepted 5 September 2012

Academic Editor: Angelo Antonio Izzo

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Epidemiological studies correlate low levels of vitamin D with the osteoarthritis (OA) progression. Cytokines and metalloproteases play a major role in OA promoting the inflammation and degradation of the cartilage and can be induced through the Toll-like receptor (TLR) pathway. The aim of this study was to evaluate the protective effect of vitamin D supplementation on the development of osteoarthritis (OA) through examining the genetic regulation of TLRs, cytokines, and metalloproteases in chondrocytes as well as the width of cartilage in rats with OA. Our results demonstrate that the signaling through TLR-4 is a proinflammatory mechanism in osteoarthritis that drives the upregulation of MMP-3, IL-1β, and TNF-α gene expression, leading to cartilage degradation and inflammation. Vitamin D supplementation had a protective effect during the onset but not during the chronic stage of OA in the rat model.

1. Introduction

It has been shown that TNF-α synthesis decreases in monocytes treated with vitamin D (1,25(OH)2D3) due to the downregulation of TLR-2 and TLR-4 signaling [1]. In this context, epidemiological studies and experimentally induced animal models suggest that the status of vitamin D is associated with the severity of inflammatory diseases such as rheumatoid arthritis [2] and OA [3]. In particular, vitamin D deficiency has been positively associated with knee [4] and hip [5], OA progression [2, 6], cartilage loss [7], and low bone mineral density [3]. However, it has also been shown that vitamin D deficiency is associated with only certain age groups [4, 6, 8] and with certain factors, such as pain, rather than with radiographic changes [9, 10], while other studies have found no association between vitamin D and OA [8, 11, 12], even in patients diagnosed with OA who were using vitamin D supplements [13].

Because epidemiological studies can only evaluate the effect of vitamin D in certain stages of OA, we aimed to define whether vitamin D has a protective effect during the onset or progression of OA using a rat model. The effect of vitamin D was evaluated by measuring condyle wideness and the gene expression of key cytokines, MMPs, and TLRs that are involved in the development of OA.

2. Methods

2.1. Rats and Cartilage Samples. Male Wistar rats weighing 130–150 g were kept in acrylic cages with a stainless steel cover. The rats were housed in a controlled environment with a 12 h light/dark cycle, constant temperature (22°C) and humidity (70%), and free access to food and water. Their diet was standard chow (Formulaib Diet 5008) containing 1% calcium, 0.65% phosphorous, and 3.3 IU/g of vitamin D3.
Cartilage samples were obtained from 10 knees of healthy (control (CTL)) or OA rats per experimental group. The local ethics committee of the UPEAL, CINVESTAV-IPN (NOM-062-ZOO-1999), approved this study. The rats were anesthetized with a ketamine 60 mg/kg and xylazine 4 mg/kg solution, and OA was induced in the knee of the right hind leg by a partial meniscectomy, which is the transecting of the medial collateral ligament (MCL) [14], and high-impact exercise (HIE) for 1, 3, 6, 8, 10, or 20 days. For the progression groups, the rats exercised for 3, 10, or 20 days and then were maintained for 20 additional days without exercise (Figure 1), resulting, study periods of 23, 30, and 40 days, respectively. Explants were obtained from the femoral condyles and tibial plateau cartilage from joints where the partial meniscectomy was performed.

The high-impact exercises began two days after surgery. The rats were forced to slide from side to side for 2 minutes in a box, and then the rats were dropped continuously for another 2 minutes. Finally, the rats were forced to jump as the box was continually shaken vertically for 1 minute. This exercise cycle was repeated 3 times each day.

2.2. Vitamin D Administration. Capsules of vitamin D (1,25-dihydroxyvitamin D₃) from Member’s Marck (Bentonville, AR) were dissolved in corn oil (vehicle). The doses were 100 ng (4 IU), 1 μg (40 IU), 10 μg (400 IU), and 100 μg/kg/day (4000 IU) and were administrated orally with an esophagogastric cannula 14 h after lights on (14HALO [15]). Each dose was administered 3 days before surgery, and daily administration continued until the last day of OA induction. For the evaluation of 4 IU of vitamin D supplementation during OA progression, three experimental subgroups were included: (1) rats without vitamin supplementation during HIE + progression without vitamin supplementation (nV + PnV), (2) rats supplemented with the vitamin during HIE + progression without vitamin supplementation of vitamin (sV + PnV), and (3) rats supplemented with the vitamin during HIE + progression with vitamin supplementation (sV + PsV) (Figure 1).

2.3. RNA Isolation, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction (PCR) Amplification. The cartilage samples were enzymatically digested, and the total RNA was isolated from chondrocytes for real-time reverse transcriptase PCR analysis (qPCR) of TLR-1, TLR-2, TLR-4, TLR-6, IL-1β, TNF-α, IL-6, MMP-3, MMP-9, and MMP-13 (Real-Time PCR System, model 7500, Applied Biosystems, Carlsbad, CA, USA) using the TRIzol LS Reagent (Invitrogen, USA). The first strand of cDNA was synthesized from 1.0 μg total RNA using a high capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, Carlsbad, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an endogenous control to allow for the relative quantification of the genes of interest. The qRT-PCR was performed with the fast SyBR-Green master mix (Applied Biosystems, Carlsbad, CA, USA) on both the targets and the endogenous control (the probes used are shown in Table 1). The amplified PCR products were
quantified by measuring the calculated cycle thresholds (CT). The amounts of specific mRNA in the samples were quantified by measuring the calculated cycle thresholds.

The quality of the samples was determined by the difference in the absorbance at 260 and 280 nm. The RNA had an average A260/A280 ratio of 1.87. The RNA quality was also assessed by the electrophoresis of the total RNA followed by staining with ethidium bromide. In all samples, bands of 5 kb and 2 kb, corresponding to the 28S and 18S rRNAs, were observed. The cDNA obtained had an A260/A280 average value of 1.86.

2.4. Histological Analysis. Cartilage samples were obtained from 2 knees of healthy (control; CTL) or 20 days OA rats with or without vitamin D supplementation. Samples from each group were fixed with 4% formol, embedded in paraaffin, cut at a thickness of 4 μm and stained with hematoxylin and eosin (HE).

2.5. Statistical Analysis. The data are shown as the mean ± the standard error (standard error of the mean; SEM). The statistical significance is indicated on the graphs as follows: P < 0.05 (*), P < 0.01 (**), or P < 0.001 (***) with a 95% CI. The expression levels of TLRs, MMPs, and cytokines were measured in five independent experiments (n = 5) for the OA kinetic analysis and in three independent experiments (n = 3) for the vitamin D dose-response and the OA progression analysis. The statistical analyses were performed by the Graph Pad Instat program (Graph Pad Software Inc., San Diego, CA, USA). A two-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was performed to compare means between the experimental groups without vitamin supplementation and the CTL group or a test for linear trend when applicable. A one-way ANOVA analysis using Dunnet’s post hoc test was performed to compare the differences of vitamin D. Student’s t-test was used to compare between the progression groups without vitamin supplementation and the progression group supplemented with vitamin D since the induction. Finally, the correlation analysis was performed using Pearson’s correlation.

3. Results

3.1. Expression of TLRs, Proinflammatory Cytokines, and MMP Genes in Chondrocytes during OA Induction. To evaluate TLR-1, TLR-2, TLR-4, and TLR-6 gene expression during OA development in this rat model, we isolated cartilage chondrocytes from rats subjected to 1, 3, 6, 8, 10, and 20 days of OA induction and assessed TLR mRNA levels using quantitative RT-PCR. Figure 2(a) shows that TLR-1, TLR-2, and TLR-6 were expressed in the chondrocytes throughout the OA induction with no differences in expression levels compared to the control group. Meanwhile, an increase in the level of TLR-4 mRNA expression was observed (linear trend, P = 0.0041; R² = 0.8339) from day 8 (1.6-fold; P < 0.05) to 20 (2.1-fold; P < 0.001) with maximal expression on day 10 (2.8-fold; P < 0.001).

TLR-4 activation results in the expression of molecules associated with damage, such as MMPs and inflammatory cytokines. Therefore, we evaluated the mRNA expression of MMP-3, MMP-9, MMP-13, IL-6, TNF-α, and IL-1β. The expression of MMP-9 and MMP-13 was detected during the progression of OA, but there were no major changes in their expression between the OA and the CTL groups (Figure 2(b)). In contrast, MMP-3 expression was higher in the OA groups on days 8 (1.8-fold; P < 0.01) and 10 (2.1-fold; P < 0.01), with a linear trend suggesting an increase in expression (R² = 0.5883; P = 0.0219), as shown in Figure 2(b). Similar to MMP-9 and MMP-13 expression, the IL-6 expression level remained similar to that of CTL, while the expression level of TNF-α (R² = 0.4173) and IL-1β (R² = 0.375) was elevated throughout OA induction (Figure 2(c)). There was a peak in the IL-1β expression on day 8 (18-fold; P < 0.001), suggesting that this cytokine plays an important role during the onset of OA pathogenesis.

TLR-4 expression correlated with the expression of MMP-3 (R² = 0.7692; P = 0.0095), TNF-α (R² = 0.7127; P = 0.0169), and IL-1β (R² = 0.3954; P = 0.1304), as shown in Supplementary Figure 1, (see Supplementary Material available online at doi:10.1155/2012/156563). These results

| Gene    | Sense          | Antisense               |
|---------|----------------|-------------------------|
| TLR1    | 5'-TACCCCTGAACGACGTGAC-3' | 5'-ATCGACAAAGGCCCTAGAGA-3' |
| TLR2    | 5'-GGGACTCTTGGAAGAGGC-3' | 5'-CGCTAAGAGCAGATCACA-3' |
| TLR4    | 5'-CCAGAGCCGTGGTGATATC-3' | 5'-TCAAGAGCTTTTCCATCAGCA-3' |
| TLR6    | 5'-GCTTGGCCTACCTGCAGCA-3' | 5'-CCACGGTCTTCCCCTC-3' |
| TNFa    | 5'-AAACTCCAGTGACAGGCGCC-3' | 5'-GCACGCTTGTCCCTTGAGA-3' |
| IL1β    | 5'-CAGCTTCTCGACGCAAGC-3' | 5'-GGGTTCATGGTAAAGTCAC-3' |
| IL6     | 5'-TCTCTACCCCAACTTCATGTC-3' | 5'-TTGGAATGCTTGTGCCTTGGAC-3' |
| MMP-3   | 5'-AGACAGGACCTTTGCGG-3' | 5'-CTCTATGACCTCCTGGATAGCC-3' |
| MMP-9   | 5'-TCAGATCGAGGAGAAGC-3' | 5'-CTCTAGCCCAACTTATCCAGC-3' |
| MMP-13  | 5'-CAAGGACCTCTTGAGCCCTTG-3' | 5'-TGAGGGTGCAGACGCAGCA-3' |
| GAPDH   | 5'-TCTACTCCCCAAATGTATCCG-3' | 5'-GGTTGAGAAGATGGGAGTTGC-3' |
suggest that the activation of the TLR-4 pathway plays an important role in OA pathogenesis by inducing MMP-3, TNF-α, and IL-1β expression. However, IL-1β expression may not be entirely dependent on TLR-4 because it also depends on the NOD-like receptors pathway [16].

3.2. Effect of Vitamin D on OA Cartilage (Dose Response). If the activation of TLRs and the synthesis of cytokines and MMPs are associated with OA and if vitamin D modulates TLR-4 activation, then there should be detectable changes in the joint in response to such modulation. Some of the main characteristics of OA are joint hypertrophy and cartilage and bone erosion, which are associated with increased fibrotic tissue at the borders of the joint. For that reason, we next evaluated the effect of supplementation with different vitamin D doses (4, 40, 400, and 4000 IU/kg/d) during the induction of OA for 20 days (OA20). We measured the OA condyles and nonmeniscected condyles (right and left, resp.), and the difference between them was considered an indicative value of OA hypertrophy severity (Figures 3(a) and 3(b)). The widening of the condyles in OA20 rats without vitamin D
was used as the control (Figure 3(a)). We observed that the 4 IU/kg/day dose was the only dose that caused a reduction in the width of the OA condyles \((P < 0.01; 0.34 \pm 0.047 \text{ mm})\) (Figure 3(c)).

3.3. Effect of Vitamin D Supplementation on OA Induction. To assess whether the protective effect of supplementation with 4 IU of vitamin D involves TLR4-modulation, we first evaluated the expression of TLR4, IL-1\(\beta\), TNF-\(\alpha\), and MMP-3 during different stages of OA induction. We established day 3 of induction as the acute stage, day 10 as the intermediate stage, and day 20 as the chronic stage. This classification was established taking into consideration the previous results [14]. TLR4 expression was upregulated (5.89-fold; \(P = 0.0102 \text{ F test}\)) until the chronic stage (Figure 4(a)), while MMP-3 expression was upregulated from the intermediate stage (4.79-fold; \(P = 0.0246 \text{ F test}\)) to the chronic stage (4.13-fold; \(P = 0.0116 \text{ F test}, \)Figure 4(b)). TNF-\(\alpha\) was also upregulated during the intermediate and chronic stages (10 days: 12.75-fold; \(P = 0.0004\) and 20 days: 5.31-fold; \(P = 0.0041 \text{ F test}\), as shown in Figure 4(c). Meanwhile, IL-1\(\beta\) expression was downregulated in the acute stage (2.21-fold; \(P = 0.0104 \text{ F test}\)), which was followed by an upregulation on day 10 (12.75-fold; \(P = 0.0473 \text{ F test}\)). Finally, IL-1\(\beta\) showed the same levels of expression in the chronic stage (5.31-fold; \(P = 0.2422 \text{ F test}\)) as the nV group (Figure 4(d)). These data suggested that the protective effect \((P = 0.0010 \, t \text{ test})\) of vitamin D supplementation observed during OA induction (Figures 4(e) and 4(f) and Supplementary Figure 2) is not due to the modulation of TLR-4 activation at the transcriptional level. In Figure 4(f), we can observe after 20 days of OA induction a severe damage with intense hypertrophy and hyperplasia of the cartilage, which diminished with vitamin D supplementation.

3.4. Effect of Vitamin D Supplementation on Gene Expression during OA Progression. To mimic the natural progression of OA, we evaluated the effect of vitamin D supplementation in rats with OA progression, as described in Figure 1. The expression of TLR-4 was downregulated at day 30 (28.61-fold; \(P = 0.0125 \text{ F test}\)) in the sV + PsV group compared to the nV + PnV group (Figure 5(a)). MMP-3 expression was similar between the evaluated groups (Figure 5(b)); while, TNF-\(\alpha\) expression was downregulated in the sV + PsV group on day 30 compared to the groups: sV + PnV (39.55-fold; \(P = 0.0042 \text{ F test}\)) and nV + PnV (3.66-fold; \(P = 0.0244 \text{ F test}\)) (Figure 5(c)). In addition, IL-1\(\beta\) was downregulated on day 40 (51.97-fold; \(P = 0.0168 \text{ F test}\)) in the sV + PsV group compared to the sV + PnV group (Figure 5(d)) and upregulated on day 23 (1.09-fold; \(P = 0.0491 \text{ F test}\)) compared to the nV + PnV group (Figure 5(d)).

In contrast to the protective effect of vitamin D observed during the OA induction, we did not find any evidence for a protective effect of vitamin D supplementation during OA progression. As is shown in Figure 5(e) and Supplementary Figure 2, the rats of all groups had the same degree of damage from OA at the end of the experiment. In this case, the damage was correlated with the changes observed in the gene expression of TLR-4, MMP-3, and TNF-\(\alpha\) (Figure 5).
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4. Discussion

The present study shows the involvement of the TLR-4 pathway in the expression of MMP-3, IL-1β, and TNF-α during the development of OA. It also shows that vitamin D supplementation has a protective effect on condyle wideness during the induction of OA despite an increase in the expression of the above genes. In contrast, during OA progression, vitamin D supplementation induced the downregulation of TLR-4, IL-1β, and TNF-α on day 30, but this had no beneficial effect on the disease outcome.

Although human articular chondrocytes express mRNAs for all TLRs (TLRs 1-9) [17] only TLR-2 and TLR-4 expression increased in OA chondrocytes. OA chondrocytes stimulated in vitro with ligands for TLR-2 or IL-1β induced the release of PGE₂, MMPs (-1, -3, and -13), and nitric oxide as a consequence of this stimulation [18–20]. Similarly, the stimulation of TLR-4 expression in normal chondrocytes [17, 21], induces the secretion of TNF-α, IL-1β, MMP-13, and inducible nitric oxide synthase [22]. In this study, we found that TLR-4 was upregulated during OA induction in vivo and that its expression correlates with TNF-α, IL-1β, and MMP-3 gene expression. These data suggest that TLR-4 signaling is important in the pathophysiological response that causes the inflammation and degradation of the cartilage in OA.

The TLR activation pathway induces the expression of the vitamin D receptor (VDR) and CYP27B1 (1α-hydroxylase), the enzyme that converts 25-hydroxycholecalciferol into 1,25(OH)₂D₃, thereby promoting innate immunity through VDR activation [23, 24]. It has been reported that p65, a subunit of NF-κB, interacts directly with the VDR and that this interaction modulates the VDR and TLR responses [25–27]. The outcome of this TLR-VDR transcriptional partnership is the regulation of the target genes of both receptors. For this reason, we decided to evaluate if the suggested protective effect of vitamin D supplementation on OA was due to a modulation of TLRs, cytokines, and MMPs at
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**Figure 5:** Vitamin D has no effect on OA severity. The evaluation of OA was performed during the OA progression phase. (a)–(d). The gene expression of TLR-4 (a), MMP-3 (b), TNF-α (c), and IL-1β (d) from rats in the sV + PsV group (●) compared with the sV + PnV (×) and the nV + PnV (○) groups. The gene expression levels were normalized to GAPDH gene expression and are presented as the n-fold difference in expression. (e) A comparison of the hypertrophy (wideness) of condyles from the rats of the sV + PsV (●), sV + PnV (×), and nV + PnV (○) groups is shown. The results shown are pooled data from three independent experiments with 16 to 18 total rats (a–d) or with 12 to 16 total rats (e). The error bars indicate the SEM. A t-test with an F test was performed; the notations indicate ∗P < 0.05 and ∗∗P < 0.01. The following groups were included: sV + PsV (vitamin supplementation during induction and progression with supplementation of vitamin); sV + PnV (vitamin supplementation during induction and progression without vitamin supplementation); nV + PnV (without vitamin supplementation during induction and progression without vitamin supplementation). For the supplement, 4 IU of vitamin D was administrated daily.

The transcriptional level [1] or if vitamin D was in fact associated with more severe OA because VDR expression has also been associated with MMP-3 expression in OA cartilage [28] and the upregulation of inflammatory cytokines [29]. In this study, we observed that vitamin D supplementation differentially modulated the gene expression of TLR-4, TNF-α, IL-1β, and MMP-3 during OA induction and progression. Vitamin D supplementation induced TLR-4 expression during OA induction but downregulated it during OA progression. These antagonistic responses might be due to the continued mechanical overload that promotes the activation of the inflammatory pathway during OA induction.

This differential response has been observed after inflammatory cytokines modulation. Vitamin D downregulates IL-1β and TNF-α expression in peripheral blood mononuclear cells via TLR-4 downregulation [22]. In contrast, in human monocyte-derived macrophages activated with
lipopolysaccharide, vitamin D stimulates the production of IL-1β [28] and downregulates TNF-α expression [1, 22, 30]. In our results, we observed both positive and negative effects on cytokines. IL-1β increased on day 10 of OA induction (sV) and was downregulated on day 40 of OA progression (sV+PsV). Similar effects on TNF-α expression were observed.

Enhancing and suppressive roles for vitamin D have also been described for MMPs expression. Vitamin D has been associated with MMP-3 expression in human OA cartilage. In vitro studies show that vitamin D induces the production of MMP-1, MMP-3, and PGE2 in chondrocytes stimulated with IL-1β, whereas it reduces MMP-1, MMP-3, and PGE2 induction by IL-1β in rheumatoid synovial fibroblasts [28, 31], which are two scenarios that could be present and contribute to the final state of the cartilage during OA development. Our in vivo results agree with the effect described for MMP-3 in chondrocytes in vitro with regard to the groups of OA induction and at day 40 of OA progression. However, despite that at day 40, MMP-3 expression was upregulated in OA rats, there were no major changes in the other groups. These data suggest that the effect of vitamin D depends not only on the cell type but also on the activation state of the cells and the inflammatory environment.

The regulatory modulation leads to the hypertrophy of the articular cartilage, which is characterized by physical changes, such as fraying, fibrillations with a rough surface, and the formation of osteophytes that become more prominent as the disease progresses [28]. We evaluated hypertrophy in our model by comparing the width of the right and left condyles and observed that the condyle widthness (in millimeters) was not different except when comparing the 30-day group treated with vitamin D during OA progression (sV + PsV) to the 30-day group supplemented only during the induction (sV + PnV). The comparison of these groups also revealed that vitamin D leads to the downregulation of TLR-4, MMP-3, and TNF-α. These findings are in accordance with a study indicating that vitamin D deficiency is greatly associated with the early stages of OA but absent in the advanced stages [4]. This could be explained by a recent report indicating that vitamin D precedes the TLR activation, with no effect observed when vitamin D is administered at the same time or after TLR stimulation [32]. These data and our data suggest that vitamin D supplementation must be administrated before cartilage damage occurs.

Remarkably, we observed hypertrophy protection with low doses of vitamin D (4 IU) that was not observed in the same extent with higher doses (40–4000 IU). This lack of dose response might be due to the dual effect that vitamin D has on the different cells of the joint [28, 31]. In fact, it has been reported that administration of high doses of vitamin D induces side effects like hypercalcemia and hyperphosphatemia [15]. However, there is not an appropriate pharmacokinetic study that establishes the effects of vitamin D on the cartilage. Additionally, there are other factors that have to be taken into consideration, including the vitamin D-dependent induction of the expression of VEGF, growth factors [33], and MMPs [31, 33, 34]. These findings suggest that, in addition to its regulation of inflammation and damage, vitamin D could also contribute to factors highly associated with OA progression [28, 35].

In summary, our findings suggest that vitamin D may play a key role in the pathophysiology of OA. Additionally, the effects of vitamin D on OA may reflect the state of cell activation. Further studies are needed to examine the effects of vitamin D on the various molecules associated with the key degenerative or reparative processes within osteoarthritic cartilage. In addition, it is important to establish whether vitamin D supplementation in healthy subjects with serologically adequate levels of vitamin D yields a better response than supplementation in subjects with lower serum levels. However, it has been reported that healthy women with higher levels of vitamin D in serum had lower concentrations of TNF-α [36]. Moreover, a pharmacokinetic would help to understand the kinetics and effects of different doses of vitamin D.

## Abbreviations

OA: Osteoarthritis
HIE: High-impact exercise
TLRs: Toll-like receptors
CTL: Control
IL-1β: Interleukin-1β
IL-6: Interleukin-6
TNFα: Tumor necrosis factor alpha
MMP: Matrix metalloproteinase
DAMPs: Damage-associated molecular patterns
IU: International units
1,25(OH)2D3: Vitamin D
14 HALO: 14 hours after light on
qPCR: Quantitative real-time polymerase chain reaction
HE: Hematoxylin and eosin
sV: Rats supplemented with the vitamin
nV: Rats without vitamin supplementation
nV + PnV: Rats without vitamin supplementation during HIE + progression without vitamin supplementation
sV + PsV: Rats supplemented with the vitamin during HIE + progression without vitamin supplementation
sV + PnV: Rats supplemented with the vitamin during HIE + progression without vitamin supplementation
ANOVA: Analysis of variance
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
Ct: Cycle threshold
SEM: Standard error of the mean
NOD: Nucleotide oligomerization domain-like receptors.

## Authors’ Contribution

E. C. Castillo conducted all the experiments, analyzed the data, conceived and designed the study, confirmed the data analysis, and wrote the initial draft of the manuscript. J. B. Kouri and V. Ortiz-Navarrete conceived of and designed
study, confirmed the data analysis and reviewed the paper critically for important intellectual content. M. A. Hernandez-Cueto conducted the histopathological analysis. M. A. Vega-Lopez and C. Lavelle reviewed the paper critically for important intellectual content. All the authors read and approved the final paper.

Conflict of Interests

All authors declare that they have no competing interests.

Acknowledgments

The authors thank the support of the CONACyT and CINVESTAV-IPN, Zacatenco. They thank Daniel Hernandez Cueto for his technical assistance. This work was supported by CONACyT Grant 59787 (to J. B. Kouri). E. C. Castillo Cueto for his technical assistance. This work was supported by a CONACyT scholarship 192043 and a CINVESTAV scholarship.

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