Research Article

Prostacyclin Synthase: Upregulation during Renal Development and in Glomerular Disease as well as Its Constitutive Expression in Cultured Human Mesangial Cells

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1. Introduction

Prostacyclin synthase (PGIS) is an atypical cytochrome p450 enzyme [1], which generates prostacyclin (PGI₂) from prostaglandin H₂ (PGH₂), provided by cyclooxygenase-1 (COX1) or COX2. Prostaglandin-I synthase is expressed constitutively, consistent with its TATA-less and GC-rich promoter [2]. Modulation of constitutive expression with strong upregulation has been observed in uterine development [3]. However, in vitro studies have failed to identify specific factors that induce consistently PGIS-protein expression. Prostacyclin, which has a half-life of 30 sec in vivo, activates adenylyl cyclase through interaction with its G-protein-coupled receptor, dubbed IP [4]. In contrast to certain synthetic and stable prostacyclin analogues, known to activate the nuclear receptor peroxisome proliferator-activated receptor, β/δ (PPAR-β/δ) [5], there remains controversy over whether this is also true for endogenous prostacyclin [6, 7].

Development of severe glomerular, vascular, and interstitial abnormalities in PGIS-knockout-mice only serves to confirm the critical role that prostacyclin plays in normal renal development [8]. Such defects are not observed in the IP-knockout-mice [9], fitting with the notion of a second PGI₂-receptor, possibly PPAR-β/δ. Numerous glomerular actions of PGI₂ have been reported, including effects on local hemodynamics, renin secretion, cell proliferation, and matrix turnover [4]. The potential beneficial effects of various synthetic ligands on slowing progression of renal disease have been observed in experimental animal models as well as in humans [4].

Despite the fact that prostacyclin has been shown to play several roles in the kidney, the pattern of PGIS expression in
humans during renal development and in glomerular disease is virtually unknown. There are also few studies describing the in vitro expression of prostacyclin synthase. In human endothelial cells, biosynthesis of prostacyclin is controlled primarily through the induction of cyclooxygenase by growth factors or mitogens whereas expression of PGIS remains unchanged [10]. Others have shown that bovine endothelial cells translate tumour necrosis factor (TNF) binding into an increase of COX2 enzyme expression and subsequently into the induction of prostacyclin synthase mRNA [11]. Finally, incubation of human endothelial cells with acidic fibroblast growth factor in the presence of heparin resulted in a marked diminution in PGI2 synthesis caused by a decrease in expression of both prostacyclin synthase and cyclooxygenase protein [12].

Most studies that have reported on prostacyclin synthesis in the scientific literature have focused on expression and regulation of cyclooxygenases, the enzymes that provide PGH2 and thus cyclooxygenases are considered to be the rate-limiting step during prostanoid synthesis. Two isoforms of cyclooxygenase have been identified: a constitutive COX1 that is thought to be involved in housekeeping functions of prostaglandins and an inducible COX2 that is believed to be involved in the synthesis of high amounts of prostaglandins under pathological conditions [13].

The present series of studies were conducted under various different circumstances in order to gain further insight into the regulation of prostacyclin synthase. We analyzed the mRNA and protein expression of prostacyclin synthase in normal "developing" and adult human kidneys, as well as in glomerular disease. We also investigated prostacyclin synthesis and synthase in human mesangial cells in vitro.

2. Material and Methods

The current work was conducted with permission of our institution’s local medical ethics committee. During the course of normal clinical practice, renal tissue was obtained from the unaffected poles of kidneys surgically removed as part of treatment of renal carcinoma (n = 5 patients). Human fetal tissue was collected from medical abortions [14]. Unused paraffin-embedded renal biopsy tissue samples remaining after sectioning were also identified for study. In total 21 patients, 12 patients with IgA-nephropathy, seven patients after sectioning were also identified for study. In total 21 paraffin-embedded renal biopsy tissue samples remaining obtained from Santa Cruz (Santa Cruz, CA: COX1, C20: sc#1752, and COX2: C-20, sc#1745).

2.2. Immunohistochemistry of Human Fetal Tissue. The characterization of the monoclonal antibodies for prostacyclin synthase has been published previously [17]. In brief, approximately 5 µm tissue sections were sliced from snap-frozen samples, thaw-mounted on poly-L-lysine-coated glass slides, air-dried, and fixed in acetone for 10 min at 4 °C. The presence of primary antibodies was confirmed with the alkaline phosphatase antialkaline phosphatase method using rabbit antiserum or mouse anti-rabbit antibodies.

2.3. Generation of 35S-Labelled Riboprobes and In Situ Hybridization. Antisense and sense probes for human prostacyclin mRNA were prepared as follows. A polymerase chain reaction (PCR) fragment (420 base pairs) generated the amplification of mesangial mRNA with the primer pair depicted below that was ligated and cloned in a PCR 2.1 plasmid (Invitrogen, USA). The following primers were used to generate a murine-PGIS riboprobe: forward-GGCTGGCTG-GGTTGAGAATC and reverse-GACCTGGCGAAGTTT-GGTAT. Cloned cDNA fragments were sequenced according to the dideoxy method to confirm the identity and orientation of the inserts. In situ hybridization was performed as described previously [16]. After development in Kodak D-19, slides were counterstained with hematoxylin. Photomicrographs were taken with a Zeiss Axioskop microscope using bright field optics.

2.4. Human Mesangial Cells. Normal human kidney tissue was obtained from tumor nephrectomy surgery. Glomeruli were obtained from different donors by passage through serially graded sieves and incubated in growth medium which consisted of RPMI-1640 supplemented with insulin (5 µg/mL), transferrin (5 µg/mL), sodium selenite (5 µg/mL), L-glutamine (1%), penicillin (100 U/mL), and streptomycin (100 µg/mL) containing 10% fetal calf serum and HEPES (20 mM). Cellular outgrowths of mesangial appearance were subcultivated and characterized as follows: (a) morphological criteria with elongated appearance; (b) staining with antivimentin; (c) staining with smooth muscle cell actin; and (d) absence of staining with anti(factor VIII) and antidesmin. Cells were grown to confluence and growth was arrested by low serum conditions (0.5% fetal calf serum) in the absence of supplements for 24–36 h. All experiments were performed using cells between the third and sixth passages.

2.5. Cell Culture. Cell culture media were obtained from PAA (Coelbe, FRG). Recombinant human (rh) interleukin (IL) types 1β IA-1β and rh-TNFx were purchased from Endogene (MA, USA). A commercial enhanced chemiluminescence detection kit with nitrocellulose membranes (Hybond-C) was obtained from Amersham (Braunschweig, FRG). Diclofenac and bovine serum albumin were from Sigma (Deisenhofen, FRG). Primers were synthesized by MWG-Biotech (Ebersberg, FRG); AmpliTaq polymerase was obtained from Perkin-Elmer (Weiterstadt, FRG) and SuperScript reverse transcriptase from GIBCO (Eggenstein, FRG). Insulin, transferrin supplements, and recombinant human interferon (IFN) type-γ rh-IFN-γ were from Boehringer (Mannheim, FRG).
Figure 1: Mesenchymal expression of prostacyclin synthase in the developing human (24 weeks of gestation) and murine (postnatal day 0) kidney. (a) shows a low power view of a developing human kidney. (b) High power reveals absence of PGIS in epithelial structures. (c) Low power shows high subcapsular expression of PGIS-mRNA. (d) confirms the absence of labeling over epithelial structures.

Primary antibodies to characterize cellular cultures were obtained from Dako (CA, USA); secondary antibodies were from Dako and Dianova (Hamburg, FRG).

2.6. Western Blot and mRNA Analysis. Analysis of prostacyclin synthase expression was performed on human mesangial cells. In brief, lysates (80 μg) of cells stimulated for 20 h with rh-TNFα (100 ng/mL), IFN-γ (350 U/mL), IL-1β (1 nM), or control were solubilized in phosphate buffered saline containing 1% Triton X-100. Equal amounts of protein were separated by 10% SDS-PAGE. Immunoblot analysis was performed with different purified polyclonal antibodies against PGIS as described previously [17]. Total RNA was isolated from mesangial cells using the guanidinium thiocyanate method with acidic phenol. Reverse transcription and PCR were performed as described recently for COX1, COX2, β-actin, and thromboxane synthase. Accordingly, for prostacyclin synthase, 1 μg of total RNA was used for target-specific reverse transcription with reverse transcriptase and the primer (5′-ATGCGGTAGCGACGAGGCACG-3′). Polymerase chain reaction amplification was performed using the cDNA with the antisense (5′-ctgcatcaggcgaagccctacctg-3′) and sense primer (5′-TGCTGAGTGAGACTACGGC-TAGGGTTA-3′). Reactions were cycled 30 times (30 sec at 94°C, 30 sec at 56°C, and 30 sec at 72°C following a 5 min denaturing step at 95°C). Amplification products were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The identity of the fragments was evaluated by their molecular mass and dideoxy sequencing. Samples were assayed at various dilutions to ensure proportionality in the yield of PCR products.

Determination of prostanoids by gas chromatography is as follows. Prostanoids in the supernatant of mesangial cells were measured by gas chromatography coupled to dual mass spectroscopy (GC/MS/MS) as described previously [18].

3. Results

3.1. Intrarenal Localization of Prostacyclin Synthase

Nephrogenesis. In the developing human kidney, intense expression of PGIS was observed in mesenchymal cells of the nephrogenic cortex (Figure 1(a)). Epithelial structures
corresponding to various stages of renal development and tubular structures were not labeled (Figure 1(b)). An identical pattern was observed when analyzing prostacyclin synthase mRNA expression in the mouse (Figure 1(c), low power view). In situ hybridization revealed strong cortical labeling in kidneys on postnatal day 0. Within the exception of vascular structures, medullary areas showed significantly less labeling. High power views confirmed labeling over interstitial cells and sparing of epithelial structures (Figure 1(d), high power view).

**Normal Adult Kidney.** Radioactive *in situ* hybridization revealed specific signals for prostacyclin synthase in the mesangial region of glomeruli (Figure 2(a)) and over arterial endothelial cells (Figure 2(b)). As expected from biological and pharmacological studies, PGIS immunoreactivity was detected in endothelial cells of blood vessels (Figure 2(c)) of normal kidney as well as in the smooth muscle cells of arteries. Staining of PGIS in the glomerulus was comparatively low in normal human tissue. Note PGIS immunoreactivity in cells of the juxtaglomerular apparatus adjacent to cells of the macula densa (Figure 2(d)).

**Pediatric Renal Disease.** Glomerular expression of PGIS-immunoreactive protein varied considerably in tissues from children with renal disease ranging from completely absent to strong and localized primarily to endothelial cells. However, there was no evidence that would link expression with a particular disease. Shown are high power views from a patient with IgA-nephropathy (Figure 3(a)), minimal change disease with focal segmental glomerulosclerosis (Figure 3(b)), and chronic transplant rejection (Figure 3(b)). Strong capillary expression of prostacyclin synthase in renal medulla was observed in the same biopsy (Figure 3(d)).

### 3.2. Prostacyclin Synthesis in Cultured Human Mesangial Cells

**In Vitro Studies.** The profile of prostanoid synthesis in our cultured human mesangial cells (HMCs) is shown in Figure 4. Under basal conditions, mesangial cells produced predominantly \( \text{PGI}_2 \), determined as 6-keto-PGF\( _1\alpha\), the primary product of \( \text{PGI}_2 \) metabolic breakdown. Considerably smaller amounts of \( \text{PGE}_2 \) and \( \text{PGF}_2\alpha \) were observed and thromboxane (TX) type \( \text{B}_2 \), the stable product of Tx type \( \text{A}_2 \), was only barely detectable. Incubation of growth-arrested HMC from different donors with cytokines consisting of IL-\( \beta \) (1 nM), TNF\( \alpha \) (10 ng/mL), and IFN-\( \gamma \) (250 U/mL) resulted in increased expression of prostacyclin (24-fold) and \( \text{PGE}_2 \) (65-fold). There was no apparent change in \( \text{TXB}_2 \) and \( \text{PGF}_2\alpha \) production. Coincubation of stimulated HMC with the nonsteroidal anti-inflammatory drug, diclofenac (1 \( \mu \)M), resulted in a complete inhibition of prostanoid synthesis. In contrast, addition of the glucocorticoid dexamethasone (1 \( \mu \)M) lowered cytokine-stimulated prostaglandin production to the same levels as seen under control conditions. The profile of exogenously added arachidonic acid metabolism is shown in Table 1. In a concentration-dependent manner,
Figure 3: Expression of prostacyclin synthase in various renal diseases. (a) IgA-nephropathy, (b) focal segmental glomerulosclerosis. Glomerular (c) and medullary (d) expression of PGIS in endothelial cells and peritubular capillaries in a transplanted kidney with chronic rejection.

Figure 4: Pattern of COX1- and COX2-dependent prostanoid formation of human mesangial cells. HMCs were incubated for 20 h with cytokines in the absence or presence of diclofenac (1 μM), dexamethasone (1 μM), or vehicle. Prostanoid formation was measured in the supernatant. Data represent means (pg/10⁶ cells) ± SEM (n = 5). One-way analysis of variance Bonferroni’s multiple comparison test was performed for each product. ***P < 0.001 versus control; ###P < 0.001 and ##P < 0.05 versus cytokines; ns: nonsignificant.

Conversion to prostacyclin and PGE₂ occurs. Once again, the levels of TxB₂ and PGF₂α, prostanoids known to exert vasoconstrictive actions, were hardly detectable, even when the HMCs were challenged with arachidonic acid (20 μM).

To gain further insight into the regulation of prostacyclin synthase activity following cytokine stimulation, the cyclooxygenase step was bypassed by the addition of exogenous PGH₂, the immediate substrate for PGIS. As can be seen from the data in Table 2, compared to control, there was no significant difference in the conversion of PGH₂ to 6-keto-PGF₁α in any of the cytokine or glucocorticoid treated samples.

These data point towards a constitutive activity of prostacyclin synthase. To investigate this aspect at the protein level, Western blot analysis was performed using cell lysates of cytokine treated HMC or controls. The authenticity of the observed single band of approximately 52 kD was evidenced using the purified bovine enzyme as a positive control (Figure 5(a)).

Similar results, confirming the constitutive nature of the enzyme, were obtained addressing the corresponding mRNA expression (Figure 5(b)). Messenger RNA expression for both PGIS and COX1 was unaffected by cytokine stimulation, whereas expression of COX2 mRNA was markedly induced.
Mediators of Inflammation

Table 1: Prostanoid formation of cytokine-stimulated HMC from different concentrations of exogenously added arachidonic acid.

<table>
<thead>
<tr>
<th></th>
<th>6-Keto-PGF₁α</th>
<th>PGE₂</th>
<th>TxB₂</th>
<th>PGF₂α</th>
</tr>
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<tbody>
<tr>
<td>AA (0 μM)</td>
<td>242 ± 42**</td>
<td>78 ± 8**</td>
<td></td>
<td>40 ± 13**</td>
</tr>
<tr>
<td>AA (2 μM)</td>
<td>6590 ± 300***</td>
<td>6120 ± 370***</td>
<td>110 ± 7**</td>
<td>560 ± 6.6**</td>
</tr>
<tr>
<td>AA (5 μM)</td>
<td>11350 ± 250***</td>
<td>10390 ± 610***</td>
<td>83 ± 7**</td>
<td>116 ± 7**</td>
</tr>
<tr>
<td>AA (20 μM)</td>
<td>14800 ± 100***</td>
<td>13500 ± 200***(a)</td>
<td>100 ± 24**</td>
<td>226 ± 40**(b)§(c)§(d)</td>
</tr>
</tbody>
</table>

HMC were stimulated with cytokines for 20 h and later they were incubated with the depicted concentrations of exogenous arachidonic acid (AA) or only buffer (0 μM) for 15 min at 20°C. Prostaglandins generated were extracted from the supernatant as described and analyzed by GC/MS/MS method. Values are depicted as means ± SEM (pg/10⁶ cells) of three experiments. One-way analysis of variance Bonferroni’s multiple comparison test. **P < 0.001 for all combinations. (a)** P < 0.01 for 5 μM versus 20 μM; (b)** P < 0.01 for 0 μM versus 20 μM; (c)** P < 0.01 for 2 μM versus 20 μM, and (d) P < 0.05 for 5 μM versus 20 μM; ns: not significant.

Table 2: Conversion of the precursor endoperoxide PGH₂ to 6-Keto-PGF₁α in the presence of cytokines or dexamethasone.

<table>
<thead>
<tr>
<th></th>
<th>6-Keto-PGF₁α (ng/10⁶ cells)</th>
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<tbody>
<tr>
<td>Control</td>
<td>197 ± 14</td>
</tr>
<tr>
<td>Cytokines</td>
<td>139 ± 10*</td>
</tr>
<tr>
<td>Cytokines + dexamethasone (1 mM)</td>
<td>144 ± 7*</td>
</tr>
</tbody>
</table>

HMC were stimulated for 20 h with cytokines, cytokines plus dexamethasone, or vehicle. Following stimulation, the medium was aspirated and the cells were stimulated for 5 min at 20°C with 20 μM PGH₂ in phosphate buffered saline. The spontaneous decay of PGH₂ in aqueous solution from a control experiment was considered. The figure consists of the mean of three experiments ± SEM. One-way analysis of variance Bonferroni’s multiple comparison test: *P < 0.05 versus control.

Interestingly, RT-PCR analysis failed to detect mRNA for TXS and suggested a negligible role for TXA₃ as mesangial cell derived prostanoid. The extensive increase of inducible nitric oxide synthase mRNA under identical conditions was reported in HMC [19] and served as a control for cytokine activity.

4. Discussion

In the present study, it appears to be a strong expression of PGIS mRNA and immunoreactive protein in mesenchymal cells of developing human and mouse kidney, respectively. The greatest expression appeared to be subcortical, in the most immature part of the kidneys, namely, the nephrogenic cortex, in mesenchymal cells adjacent to ureteric buds. Previously, the regulation of PGIS expression during development has been shown during uterine development, with high levels of expression occurring in the most immature cells [3]. The strong expression of PGIS suggests that promoters of PGIS gene expression that have yet to be identified must be involved. Further studies, possibly including the investigation of miRNAs as potential regulators, are needed to identify the mechanisms leading to the upregulation of PGIS expression. Considering the multiple defects in renal development in PGIS-ko mice, including increased interstitial fibrosis [3], leads to speculate that prostacyclin plays an important role in the prevention of fibrosis and that this process may be modulated via PPAR-β/δ (given the normal renal phenotype in IP-ko-mice [9]).

Our study describes for the first time the intrarenal localization of prostacyclin synthase mRNA and immunoreactive protein in the healthy adult human kidney. Expression of PGIS mRNA and immunoreactive protein in vascular structures appears to confirm pharmacologic findings and is consistent with a role for prostacyclin as a vasodilator and inhibitor of platelet activation [20]. Expression of PGIS in the juxtaglomerular apparatus is in accordance with prostacyclin as mediator of renin release [21]. Expression of PGIS mRNA and immunoreactive protein was less prominent and discernible over mesangial fields. The exact cellular localization (mesangial or endothelial cells) could not be identified. Consistent with previous reports [22], there was no observable tubular expression of PGIS. This agrees with the findings of earlier studies which did not show a role of endogenous prostacyclin on tubular transport [20].

We investigated whether PGIS, like other developmentally regulated genes [23], is upregulated in glomerular disease. We observed variable degrees of PGIS expression in human kidney disease, though the expression was frequently higher than that seen in samples from healthy tissue controls. The interpretation of these observations was limited by the number of biopsies available for study and the multiple underlying diseases, precluding any correlation of PGIS expression with a particular glomerulopathy. The majority of cases showed upregulation of PGIS in glomerular and peritubular endothelial cells, which suggests a common inducer (Figures 3(a) and 3(b)). In some biopsies, however, inter-(Figure 3(c)) and intraglomerular (Figure 3(d)) expression was quite variable, possibly altered by local hemodynamics. Serial sections stained for COX1 and COX2 (data not shown) did not reveal coexpression with PGIS, arguing for differential modes of regulation (of COX1, COX2, and PGIS, resp.). As endothelial cells, for instance, are known to synthesize prostacyclin, absence of COX1 or COX2 containing with PGIS may simply reflect high specificity and reduced sensitivity of our immunostaining procedure.

Our observations with cultured human mesangial cells appear to confirm that prostacyclin [24] is the predominant mesangial prostanoid both under basal conditions and following cytokine stimulation. The increase in PGI₂ synthesis was mirrored by PGE₂ synthesis [24]. Similarly to PGI₂, PGE₂ is a potent vasodilator, dependent on specific receptors located on target cells.
Despite using highly selective and sensitive GC/MS/MS, we could only detect trace amounts of the vasoconstricting prostanoids, TXA₂ and PGF₂α, from HMS. In contrast to cytokine induced PGE₂ and PGI₂ formation, we did not observe changes in TXA₂ and PGF₂α synthesis. There is evidence that these prostanoids are predominately formed products of human glomerular epithelial cells (unpublished observations). Thus, we suggest that the previously reported [25] formation of TXA₂ or PGF₂α from human mesangial cells is possibly a consequence of contamination of the previous authors’ work with visceral epithelial cells. The predominance of the vasodilatory prostanoids PGE₂ and PGI₂ suggests that mesangial prostanoid formation exerts a tonic vasodilatory effect on the glomerular capillary network.

In a different set of experiments, we sought to better understand the mechanisms involved in cytokine induced PGI₂ synthesis. Exposure of HMC to cytokines resulted in a 24-fold increase of PGI₂ synthesis, but there was no evidence of PGIS mRNA and protein having been induced. In contrast, there was a marked induction of COX2 mRNA and protein. These results are in agreement with previous studies [26] and suggest that the limiting step in HMC-derived PGI₂ synthesis is the regulation of COX2 expression. To substantiate the concept that COX2 levels regulate prostanoid synthesis in HMCs, we investigated the effect of dexamethasone on cytokine induced PGI₂ prostaglandin formation. Dexamethasone is known to inhibit stimulated expression of both COX2 protein and mRNA through its action on transcriptional and posttranslational mechanisms. In cytokine-stimulated HMC, dexamethasone repressed COX2 mRNA and protein expression in parallel with a decrease in prostanoid formation to levels seen inside control. As dexamethasone failed to affect expression of the PGIS gene, our data appears to additionally support the concept that COX2 is the key step in controlling prostanoid formation in HMCs. Exposure of cytokine-stimulated HMCs to the nonsteroidal anti-inflammatory drug diclofenac, which inhibits both COX1 and COX2 activities, reduced prostanoid synthesis to levels below those seen in controls. These findings appear to suggest that COX1 contributes to basal HMC-derived prostanoid formation. Accordingly, we also observed constitutive expression of COX1 mRNA.

Our data appear to be in contrast with the observations made in bovine aortic endothelial cells, which has demonstrated the upregulation of prostacyclin mRNA following TNFα stimulation [11]. However, our findings are in line with those from experiments conducted in human venous umbilical endothelial cells [10]. Therefore, we propose that cell-specific regulation of prostacyclin synthase may occur under certain conditions or that the apparent difference in finding is a consequence of species differences. This notion is a supported pattern of organization of the human prostacyclin synthase gene, which was recently determined and uncovered consensus sequences for Sp-1, Ap-2, and, interestingly, NF-κB in the promoter region [26]. NF-κB is known as a transcriptional activator involved in the transmission of proinflammatory responses and could be a target for PGIS transcriptional regulation. The signalling pathway triggering PGIS induction in human systems therefore seems to be more complex and may involve a complex cytokine network (in a fashion similar to that recently proposed for nitric oxide synthase) or the presence of yet unknown mediators.

Nevertheless, our data appear to demonstrate clearly that regulation of PGF1 synthesis is regulated predominantly by COX2 activity, which serves to provide PGH to the constitutively expressed PGIS.

When considering our current findings in conjunction with what is already known about prostacyclin synthase, it appears that our data shows it to be under the influence of a developmentally regulated gene which is often reexpressed in glomerular disease. These in vivo findings appear to be in contrast with the constitutive expression of PGIS in primary human mesangial cells. Nevertheless, our data point to hitherto unknown regulators of prostacyclin synthase expression.
Abbreviations

PGIS: Prostacyclin synthase
TXS: Thromboxane synthase
COX: Cyclooxygenase
HMC: Human mesangial cell
GC/MS: Gas chromatography/mass spectrometry
PPAR-β/δ: Peroxisome proliferator-activated receptor β/δ
IgA-GN: IgA-nephropathy
FSGS: Focal segmental glomerulosclerosis
PGI₂: Prostaglandin I₂
PGH₂: Prostaglandin H₂
TNT₂: Tumour necrosis factor type alpha
PCR: Polymerase chain reaction
IL: Interleukin
Rh: Recombinant human.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


