Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) stimulates PTGS2 expression and PGF$_{2\alpha}$ synthesis through NFKB activation via reactive oxygen species in the corpus luteum of pseudopregnant rats

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Abstract

This study was undertaken to investigate how prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) increases PGF$_{2\alpha}$ synthesis and PTGS2 expression in the corpus luteum of pseudopregnant rats. We further investigated the molecular mechanism by which PGF$_{2\alpha}$ stimulates PTGS2 expression. PGF$_{2\alpha}$ (3 mg/kg) or phosphate buffer as a control was injected s.c. on day 7 of pseudopregnancy. $Ptgs2$ mRNA expression and PGF$_{2\alpha}$ concentrations in the corpus luteum were measured at 2, 6, and 24 h after PGF$_{2\alpha}$ injection. PGF$_{2\alpha}$ significantly increased $Ptgs2$ mRNA expression at 2 h and luteal PGF$_{2\alpha}$ concentrations at 24 h. PGF$_{2\alpha}$ significantly decreased serum progesterone levels at all of the times studied. Simultaneous administration of a selective PTGS2 inhibitor (NS-398, 10 mg/kg) completely abolished the increase in luteal PGF$_{2\alpha}$ concentrations induced by PGF$_{2\alpha}$. PGF$_{2\alpha}$ increased NFKB p65 protein expression in the nucleus of luteal cells 30 min after PGF$_{2\alpha}$ injection, and electrophoretic mobility shift assay revealed that PGF$_{2\alpha}$ increased binding activities of NFKB to the NFKB consensus sequence of the $Ptgs2$ gene promoter. Simultaneous administration of both superoxide dismutase and catalase to scavenge reactive oxygen species (ROS) inhibited the increases of nuclear NFKB p65 protein expression, lipid peroxide levels, and $Ptgs2$ mRNA expression induced by PGF$_{2\alpha}$. In conclusion, PGF$_{2\alpha}$ stimulates $Ptgs2$ mRNA expression and PGF$_{2\alpha}$ synthesis through NFKB activation via ROS in the corpus luteum of pseudopregnant rats.

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Introduction

The control mechanisms for functional luteolysis are complex and differ among animal species (Wiltbank & Ottobre 2003, Sugino & Okuda 2007). Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) is a critical factor for functional luteolysis in various species. PGF$_{2\alpha}$ increases in the corpus luteum during the regression phase (Olofsson & Selstam 1988, Olofsson et al. 1990) and inhibits progesterone production, suggesting that PGF$_{2\alpha}$ has a role in triggering and accelerating functional luteolysis. In fact, in PGF$_{2\alpha}$ receptor-deficient mice, serum progesterone concentrations do not decline, and parturition does not occur (Sugimoto et al. 1997).

Luteolytic PGF$_{2\alpha}$ is of uterine origin in various animal species including rodents and domestic animals, but not in humans and primates. Interestingly, the corpus luteum has also been found to produce PGF$_{2\alpha}$ in those species, indicating that luteal PGF$_{2\alpha}$ has an important role in the regulation of luteal function (Wiltbank & Ottobre 2003). PGF$_{2\alpha}$ is synthesized via PTGS2, which is the rate-limiting enzyme in PG biosynthesis. PTGS2 is induced by a variety of stimulants such as cytokines and peptides. Interestingly, it has been reported that PGF$_{2\alpha}$ induces PTGS2 expression in the corpus luteum in various animal species (Narayansingh et al. 2002, Wiltbank & Ottobre 2003), suggesting the presence of a positive feedback pathway in the corpus luteum undergoing functional luteolysis. However, the molecular mechanism by which PGF$_{2\alpha}$ induces PTGS2 is not fully characterized.

Bonello et al. 2007, Kobayashi et al. 2008). Interestingly, we recently found that ROS activate NFKB, which in turn increases PTGS2 expression and PGF2α production in human endometrial stromal cells (Sugino et al. 2004). PGF2α stimulates ROS production in the rat corpus luteum (Sawada & Carlson 1994, Shimamura et al. 1995, Aten et al. 1998, Motta et al. 2001). NFKB stimulates PTGS2 expression by binding to its response element on the Ptgs2 gene promoter (Nakao et al. 2000, 2002, Tsai et al. 2002, Nadjar et al. 2005). These findings, taken together, suggest that there is a close relationship among PGF2α, ROS, NFKB, and PTGS2 in the corpus luteum. This study was undertaken to investigate how PGF2α increases PTGS2 expression and PGF2α synthesis in the corpus luteum during functional luteolysis.

Results

To investigate whether PGF2α increases PGF2α synthesis by inducing the expression of PTGS2 in the corpus luteum, PGF2α was administered on day 7 of pseudopregnancy, and the expression of Ptgs2 and Ptgs1 mRNA and PGF2α concentrations in the corpus luteum were measured. PGF2α significantly decreased serum progesterone concentrations compared with the control at 2 h (P<0.01), 6 h (P<0.05), and 24 h (P<0.01; Fig. 1A). There was no significant change in the weight of the corpus luteum by PGF2α treatment at any of the times studied (date not shown). PGF2α significantly increased PGF2α concentrations in the corpus luteum at 24 h (P<0.05), but not at 2 and 6 h (Fig. 1B). Ptgs2 mRNA levels were dramatically increased in the corpus luteum at 2 h (P<0.01) after PGF2α administration, but not at 6 and 24 h (Fig. 1C), whereas there was no significant change in Ptgs1 mRNA levels at any of the times studied (Fig. 1D).

To investigate whether the transient increase of PTGS2 expression by PGF2α administration is responsible for the increase in PGF2α concentrations in the corpus luteum at 24 h, a selective PTGS2 inhibitor, NS-398, was administered simultaneously with PGF2α. NS-398 completely abolished the increase in PGF2α concentrations in the corpus luteum induced by PGF2α administration (P<0.05; Fig. 2). Serum progesterone concentrations in the control group, PGF2α group, and PGF2α NS-398 group were 101.7 ± 12.1, 30.3 ± 5.4, and 29.9 ± 6.7 ng/ml (mean ± S.E.M.) respectively.

To examine the transcriptional factor responsible for the increase in Ptgs2 mRNA expression induced by PGF2α, we focused on NFKB. Since NFKB is rapidly activated within 30 min by some stimulation and is translocated into the nucleus, we decided to examine nuclear NFKB protein contents 30 min after PGF2α administration. NFKB p65 protein expression was increased in the nucleus 30 min after PGF2α administration (Fig. 3A). In addition, electrophoretic mobility shift assay (EMSA) revealed that PGF2α increased the binding activity of NFKB to the Ptgs2 promoter region (Fig. 3B).

To examine whether PGF2α activates NFKB through the production of ROS, both superoxide dismutase (SOD) and catalase were administered just before PGF2α administration. The increase in NFKB p65 protein levels in the nucleus 30 min after PGF2α administration was decreased by treatment with SOD and catalase (Fig. 4A). The increase in lipid peroxide (LPO) levels in the corpus luteum 30 min after PGF2α administration was significantly (P<0.05) suppressed by SOD and catalase (Fig. 4B). The increase in Ptgs2 mRNA levels in the corpus luteum 2 h after PGF2α administration was also significantly (P<0.05) suppressed by SOD and catalase (Fig. 4C).

Discussion

This study showed that PGF2α increased PGF2α synthesis in the corpus luteum by stimulating PTGS2 expression. It has been reported that PGF2α induces PTGS2 expression in the corpus luteum in various
species (Tsai & Wiltbank 1997, Narayansingh et al. 2002, Hayashi et al. 2003, Arosh et al. 2004, Zerani et al. 2007), suggesting the presence of a positive feedback pathway in which PGF2α stimulates PGF2α synthesis in the corpus luteum. This may contribute to accelerating and completing functional luteolysis to initiate the development of a new ovulatory follicle of the next reproductive cycle. There was a time lag between the increase in Ptgs2 mRNA expression and luteal PGF2α concentrations in this study. Since the increase in PGF2α concentrations induced by PGF2α administration was completely blocked by a selective PTGS2 inhibitor (NS-398), the transient increase of Ptgs2 mRNA levels 2 h after PGF2α administration seems to be responsible for the increase in PGF2α concentrations at 24 h. Our result is also consistent with the report by Kobayashi et al. (2007), in which the inhibitory effect of the gene silencing of PTGS2 with siRNA on PGF2α production appeared 24 h after the treatment in bovine granulosa cells in vitro. However, we cannot eliminate other potential intracellular mechanisms involved in PGF2α-induced production of intraluteal PGF2α, such as an activation of cytosolic phospholipase A2 mediated by MAP kinases or protein kinase C (Diaz et al. 2002).

In this study, although the increase in luteal PGF2α contents caused by PGF2α injection was inhibited 24 h after NS-398 treatment, serum progesterone levels were not affected by NS-398 treatment. This result may be due to that the effect of PGF2α injection on serum progesterone levels lasted for 24 h.

Regarding a possibility that PGF2α affects corpus luteum in an endocrine manner through some hormonal mediators, in vivo experiments always involve such a possibility. However, it is likely that PGF2α directly affects the corpus luteum because NFKB activation was immediately observed 30 min after PGF2α injection. Furthermore, although a well-established animal model was used in this study, we may be in a dilemma as to whether the dose of PGF2α used in this study is physiological. The dose of PGF2α is based on a number of published reports, but we cannot completely neglect the pharmacological effects of PGF2α.

The basal level of PGF2α in the corpus luteum despite low luteal PTGS2 expression suggests that it is from the other organs than from corpora lutea. It is most likely of uterine origin. This may be supported by the finding that intraluteal PGF2α levels after NS-398 treatment tended to be lower than the control, although it was not significant. In addition, PTGS1 may also contribute to PGF2α synthesis in the uterus in rodents (Stocco et al. 2007). However, further studies are needed regarding how much basal luteal PGF2α is from the uterus or what extent of basal PGF2α is synthesized via PTGS2 in the corpus luteum.

This study showed the molecular mechanism by which PGF2α induces PTGS2 expression in the corpus luteum. The Ptgs2 gene has several consensus sequences for binding of transcriptional factors, including NFKB, C/EBP, CRE, and E-box in the promoter region (Sirois et al. 1993). NFKB stimulates PTGS2 expression in a variety of cells (Nakao et al. 2000, 2002, Tsai et al. 2002, Sugino et al. 2004, Nadjar et al. 2005). In this study, PGF2α increased nuclear NFKB p65 protein expression and binding activities of NFKB to the Ptgs2 promoter region. Our results suggest that PGF2α induced PTGS2 expression through NFKB activation in the corpus luteum. On the other hand, an in vitro study with ovine luteal cells showed that E-box in the Ptgs2 promoter was critical for PGF2α-induced Ptgs2 gene transcription besides C/EBP and CRE (Wu & Wiltbank 2001). In addition, it is reported that C/EBP plays an

**Figure 2** Effects of a selective PTGS2 inhibitor on the increase in PGF2α concentrations in the corpus luteum induced by PGF2α administration. A selective PTGS2 inhibitor, NS-398, was administered simultaneously with PGF2α. PGF2α concentrations were measured in the corpus luteum 24 h after PGF2α administration. Each bar represents the mean ± S.E.M. for the number of animals given in the column. a: P < 0.05 compared with the other groups.

![Figure 2](image-url)

**Figure 3** Effects of PGF2α on NFKB p65 protein expression in the nucleus (A) and on the binding activities of NFKB to the Ptgs2 promoter (B). (A) Corpora lutea were collected 30 min after PGF2α administration on day 7 of pseudopregnancy. The NFKB p65 protein expression in the nucleus was analyzed by western blotting. The immunoblot is a representative of three independent experiments. Histone H1 was used as an internal control. (B) NFKB binding activities were analyzed by EMSA. Unlabeled AP-1 oligonucleotides and nuclear extracts of HeLa cells were used as a nonspecific competitor (NSC) and a positive control (PC) respectively. The NFKB binding was increased by PGF2α (lane 2) and was competed by the addition of the excess cold (lane 3), but not by NSC (lane 4). The autoradiogram is a representative of three independent experiments.
Further studies are needed to know the detailed mechanism regulating PGF2 expression in the corpus luteum. Previous reports that ROS activate NFKB in a variety of cells (Sirois & Richards 1993). However, in our unpublished data, PGF2a treatment did not influence the binding activity of C/EBP to the Ptgs2 promoter region using EMSA with an oligonucleotide sequence corresponding to the C/EBP site (−283 to −264 bp) of the rat Ptgs2 promoter. Further studies are needed to know the detailed transcriptional mechanism regulating PGF2a-induced PTGS2 expression.

Our finding that SOD and catalase inhibited the increase in expression of nuclear NFKB p65 protein and Ptgs2 mRNA in the corpus luteum after PGF2a administration indicates that PGF2a-induced PTGS2 expression was mediated by NFKB activation via ROS. NFKB activation via ROS is further supported by our finding of concurrent rapid increases of NFKB p65 protein in the nucleus and ROS production following PGF2a administration. Our hypothesis is also supported by previous reports that ROS activate NFKB in a variety of cells (Altavilla et al. 2001, Li & Engelhardt 2006, Bonello et al. 2007, Kobayashi et al. 2008) and increase PTGS2 expression in bovine luteal cells and human endometrial stromal cells (Nakamura & Sakamoto 2001, Sugino et al. 2004).

Interestingly, SOD and catalase only partly decreased LPO concentrations in the corpus luteum in this study, indicating that they only partly scavenged ROS. This partial inhibition of ROS by SOD and catalase suggests the possible source of ROS in the corpus luteum. If SOD and catalase do not enter luteal cells because of their large molecular weight, it is possible that ROS scavenged by SOD and catalase are derived from the cells other than luteal cells, e.g. phagocytic leukocytes such as macrophages or neutrophils, and vascular endothelial cells. In fact, there are a number of macrophages in the corpus luteum of pseudopregnant rats (Sugino et al. 1996), and PGF2a stimulates superoxide radical production by nonsteroidogenic cells in the corpus luteum of the rats (Aten et al. 1998). It is also reported that ROS such as hydrogen peroxide produced by activated neutrophils can enter the luteal cell and affect luteal function (Pepperell et al. 1992). Furthermore, the importance of vascular endothelial cells and ROS produced by them by PGF2a stimulation in functional luteolysis has been reported in the bovine corpus luteum (Shirasuna et al. 2008, Acosta et al. 2009, Lee et al. 2010). Therefore, PGF2a may have stimulated ROS production by vascular endothelial cells in the corpus luteum in this study.

On the other hand, the partial inhibition of ROS also suggests another source of ROS. ROS that were not scavenged by SOD and catalase may be derived from luteal cells. In fact, superoxide radicals are generated within the rat luteal cells 7−15 min after PGF2a administration in vivo (Sawada & Carlson 1994). Since SOD and catalase do not enter luteal cells, ROS generated in the luteal cells by PGF2a stimulation may have not scavenged in this study.

This study suggested the presence of a positive feedback pathway in which PGF2a stimulates PGF2a synthesis in the corpus luteum. Although this system may not be a main mechanism for the increase in PGF2a in the regressing corpus luteum, it may, at least, in part, contribute to accelerating and completing functional luteolysis. Furthermore, our results suggested the intracellular signaling pathway through which PGF2a stimulates PTGS2 expression in the corpus luteum: PGF2a-induced PTGS2 expression is mediated by NFKB activation via ROS.

**Materials and Methods**

**Animals**

Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan), weighing 220−270 g, were housed at 24°C under controlled conditions (lights on from 0500 to 1900 h) with free access to standard rat chow and water. Vaginal smears were obtained daily, and only the rats showing at least two consecutive 4-day estrous cycles were used. Pseudopregnancy was induced by mechanical stimulation of the uterine cervix for 1 min with...
a glass rod at 1800 h on proestrus and at 0900 h on estrus. The last day on which the rat exhibited an estrous smear was designated as day 1 of pseudopregnancy. Vaginal smears were checked every day. The experiments were reviewed and approved by the Committee for the Ethics on Animal Experiment in Yamaguchi University Graduate School of Medicine.

**Experimental procedures**

**Experiment 1**

A luteolytic dose of PGF$_{2a}$ (3 mg/kg body weight) was injected s.c. on day 7 of pseudopregnancy. The dose of PGF$_{2a}$ used in this study was based on our previous study (Shimamura et al. 1995) and other published reports (Sawada & Carlson 1991, Aten et al. 1998, Motta et al. 2001, Narayansingh et al. 2002). PGF$_{2a}$ was the kind gift of Ono Pharmaceutical Co. Ltd (Osaka, Japan). Control rats received phosphate buffer (vehicle). Each group of rats underwent laparotomy under light ether anesthesia 2, 6, and 24 h later. After blood sampling from the portal vein, the ovaries were perfused with saline via the portal vein during draining of the inferior vena cava to remove the blood and to avoid the contamination of the blood as reported previously (Sugino et al. 1993b). The ovaries were removed, and the corpus luteum was dissected, cleaned of adhering tissue in a watch glass, and weighed. Corpora lutea were stored at −80 °C. Expressions of Ptgs2 and Ptgs1 mRNA and intraluteal PGF$_{2a}$ concentrations were measured as described below. The serum samples were stored at −20 °C for progesterone assay.

**Experiment 2**

A selective PTGS2 inhibitor, NS-398 (10 mg/kg body weight, Cayman Chemical Co., Ann Arbor, MI, USA), was administered simultaneously with PGF$_{2a}$ on day 7 of pseudopregnancy. The dose of NS-398 used in this study was based on previous studies (Futaki et al. 1993, Mikuni et al. 1998). The control rats received phosphate buffer. Each group of rats was autopsied 24 h later as described in Experiment 1, and PGF$_{2a}$ concentrations in the corpus luteum were measured. NS-398 had no effects on Ptgs2 mRNA expression in our preliminary experiments. It is also verified by a manufacturer that NS-398 administration in vivo causes no significant effects on mRNA and protein expression of PTGS2 in the rats (Uno et al. 2004).

**Experiment 3**

To examine the protein expression of NFKB p65 in the nucleus and the binding activities of NFKB to the promoter of Ptgs2 after PGF$_{2a}$ administration, corpora lutea were collected 30 min after PGF$_{2a}$ administration on day 7 of pseudopregnancy. The control rats received phosphate buffer. The NFKB p65 protein expression and NFKB binding activities in the nucleus were analyzed by western blotting assay and EMSA respectively.

**Experiment 4**

Both SOD (50 000 U/kg body weight, Sigma–Aldrich Co.), a specific scavenger of superoxide radicals, and catalase (100 000 U/kg body weight, Sigma–Aldrich Co.), a scavenger of hydrogen peroxide, were administered in the tail just before PGF$_{2a}$ administration on day 7 of pseudopregnancy. The doses of SOD and catalase used in this study were based on our previous study (Sugino et al. 1993a). The control rats received phosphate buffer. The NFKB p65 protein expression in the nucleus and concentrations of LPOs (byproducts of ROS) in the corpus luteum were measured 30 min after PGF$_{2a}$ administration. Ptgs2 mRNA levels in the corpus luteum were measured 2 h after PGF$_{2a}$ administration.

**Real-time RT-PCR**

Total RNA was isolated from corpora lutea using TRIzol reagent (Invitrogen, Inc.) according to the manufacturer’s protocol. For PCR amplification, 3 μg total RNA were reverse transcribed at 42 °C in 20 μl reaction mixture (single strength PCR buffer, 2.5 mmol/l dNTPs, 5 μmol/l random hexamer, 1.5 mmol/l MgCl$_2$, and 200 U Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA)). Real-time PCR was performed as reported previously (Asada et al. 2008) using LightCycler (Roche Diagnostics) with the oligonucleotide primers for Ptgs2 (5’-GATTGACAGCAGCAACTC-3’ and 5’-CGGGATGAACTCTCTCCTA-3’; 174 bp amplified products), for Ptgs1 (5’-GTACTATCCGTAGACTGAGC-3’ and 5’-TGAGTACTCTCCTGGAAGG-3’; 132 bp amplified products), and for Sdhg11 (5’-CTGAAGGTCAAAAAGG-GAATGTG-3’ and 5’-GGACAGAGTCTTGTAGATCTC-3’; 194 bp amplified products) on the basis of the rat Ptgs2, Ptgs1, and Sdhg11 cDNA sequences (Chan et al. 1987, Kelsen et al. 2006). Sdhg11 was used as reference gene for normalization. The reaction mixture contained 10 μl SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), 0.2 μmol/l each of primer sets of Ptgs2, Ptgs1, or Sdhg11 and 2 μl cDNA (a product of RT reaction) in a total volume of 20 μl. The thermocycling program was 40 cycles of 96 °C for 5 s and 60 °C for 20 s with an initial cycle of 96 °C for 10 s.

**PGF$_{2a}$ assay**

PGs were extracted as reported previously (Sugino et al. 2000). Corpora lutea were homogenized on ice with PBS (pH 3.5). The homogenate was applied to a C18-LRC solid phase extraction cartridge (Bond-Elute, Varian Co., Harbor City, CA, USA), and the cartridge was rinsed with distilled water and 10% (v/v) acetonitrile. PGs were then eluted with methanol and evaporated under nitrogen. The dried extract was dissolved with ethanol and the kit assay solution, and the kit assay solution was determined by a PGF$_{2a}$ enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI, USA). The results were expressed as pg of PGF$_{2a}$ per cellular mg protein. The protein concentrations in the extracted sample were determined by the Lowry method (Lowry et al. 1951). The sensitivity of the assay was 4.6 pg/ml. The intra- and interassay coefficients of variation (CV) were 7.0 and 7.8% respectively.
Western blot analysis

The nuclear fraction was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. Forty micromgrams of the nuclear extract were separated by 10% (w/v) SDS-PAGE and transferred to the PVDF membranes (ATTO, Tokyo, Japan). The membranes were blocked with 5% (w/v) skim milk in Tween 20–PBS (PBS–T) and incubated with the primary antibodies including anti-rabbit NFκB p65 antibodies (1:500; Upstate, Lake Placid, NY, USA), and anti-histone H1 antibodies (1:1000; MBL, Nagoya, Japan) as nuclear internal control. The membranes were then incubated with HRP-conjugated secondary antibodies. The reacted band was developed on a film with an ECL kit (Amersham Pharmacia Biotech).

EMSA

EMSA was performed using Gel Shift Assay Systems (Promega Co.) as reported previously (Sugino et al. 2002). The oligonucleotide sequence corresponding to the NFκB binding site (−548 to −528 bp) of the rat Pgs2 promoter (5'-GGCCAGGGGATCTTCCATGT-3'; Goren et al. 2004) was annealed with the complementary sequence. Double strand NFκB oligonucleotides (10 pmol) were radio labeled with [γ-32P]ATP (3000 Ci/mmol, 10 mCi/ml) using T4 polynucleotide kinase. The labeled oligonucleotides were separated from unincorporated nucleotides by chromatography through a G-25 spin column (GE Healthcare, Amersham, UK). The radiolabeled probe was incubated with 10 μg nuclear extracts in gel shift binding buffer. To verify the specificity of the binding reaction, an excess of unlabeled oligonucleotides (×100; cold) were added to the reaction before adding the radiolabeled probe. The DNA–protein complexes were separated on native 6% (w/v) polyacrylamide gels in 0.3% (w/v) Tris–borate–EDTA buffer.

LPO assay

Concentrations of LPOs in the corpus luteum were measured by the thiobarbituric acid method as reported previously (Ohkawa et al. 1979). The results were expressed as nmol of malondialdehyde per gram wet tissue weight.

Progesterone assay

Progesterone concentrations in the serum were determined by a specific RIA described as reported previously (Kato et al. 1982). The sensitivity of the assay was 100 pg/ml, and the intra- and interassay CV were 7.0 and 14.4% respectively.

Statistical analyses

Statistical significance was determined by one-way ANOVA. After ANOVA, the Tukey–Kramer test was applied to analyze differences among the groups. As for the comparisons between the two groups, unpaired t-test was performed. All statistical analyses were performed using SPSS for Windows version 11 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at P<0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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