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In Vivo Chronic and In Vitro Acute Effects of Di(2-Ethylhexyl) Phthalate on Pseudopregnant Rabbit Corpora Lutea: Possible Involvement of Peroxisome Proliferator-Activated Receptor Gamma[1]

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ABSTRACT

The in vivo chronic and in vitro acute effects of di(2-ethylhexyl) phthalate (DEHP) on the reproductive function of peroxisome proliferator-activated receptor gamma (PPARG) were studied in rabbit corpora lutea (CL) at early stage (Day 4), midstage (Day 9), and late stage (Day 13) of pseudopregnancy. The rabbits were in vivo treated with DEHP for 15 days before induction of pseudopregnancy. Immunohistochemistry provided evidence for the presence of PPARG, prostaglandin endoperoxide synthase 1 (PTGS1), PTGS2, prostaglandin E2-9-ketoreductase (PGE2-9-K), and 3beta-hydroxysteroid dehydrogenase (3beta-HSD) in all the luteal cells during pseudopregnancy. DEHP decreased progesterone plasma levels and CL production in all the luteal stages and PPARG protein and gene expressions in early and mid-CL. DEHP in vivo treatment reduced PTGS2 protein expression at the late stage and that of PGE2-9-K at all the stages, whereas PTGS1 and 3beta-HSD were not affected. In in vitro cultured CL, DEHP alone, the PPARG antagonist T0070907 alone, or DEHP plus T0070907 diminished progesterone production and 3beta-HSD activity and increased PGF2alpha and PTGS2 in early and mid-CL, whereas DEHP plus the PPARG agonist 15d-PGJ2 did not affect these hormones and enzymes. All the in vitro treatments did not affect PGE2 secretion as well as PTGS1 and PGE2-9-K enzymatic activities in all the luteal stages. These results provided evidence that DEHP favors functional luteolysis of pseudopregnant rabbit CL, with a mechanism that seems to involve PPARG expression down-regulation, an increase of PTGS2 activity and prostaglandin F2alpha secretion, 3beta-HSD down-regulation, and decrease in progesterone.

3beta-HSD, corpora lutea, corpus luteum, DEHP, endocrine disruptors, PGF2alpha, PPARG, PTGS2, progesterone/progesterone receptor, prostaglandins, rabbit

INTRODUCTION

Animals housed in confined habitats face a wide range of potentially harmful biotic and abiotic environmental stressors, among them endocrine-disrupting chemicals (EDCs). Dietary intake of traces of these contaminants contributes to varied male and female animal health problems worldwide [1–3]. Di(2-ethylhexyl) phthalate (DEHP) is extensively used as a plasticizer in many mass-produced products, including food packaging, toys, electrical equipment, medical devices, paints, and cosmetics [4, 5]. Phthalates are not chemically bound to plastic materials and so are easily leached into the environment with time and use [5]. DEHP is the most widespread phthalate in the environment and easily contaminates livestock feed, causing profound and long-lasting changes of reproductive functions [6–10]. While the exact mechanisms by which phthalates induce these effects are not clear, they may involve interaction with multiple receptor systems, including peroxisome proliferator-activated receptors (PPARs) [11–14].

PPARs, a family of three (alpha, delta, and gamma) nuclear receptor/transcription factors, are involved in several processes, some of which are critical for normal ovarian function [15]. PPARs have been identified in the ovary of many mammalian species, such as rats [16], mice [17, 18], swine [19], ovine [20], cattle [21, 22], rabbits [23, 24], and humans [25, 26]. It has recently been suggested that PPAR gamma (PPARG) plays a luteotropic role in pseudopregnant rabbits [24]. Various studies showed that EDCs, such as DEHP, bind to PPARs and that the interaction with PPARG interferes with several reproductive functions [14, 27].

The renaissance of the laboratory rabbit as a reproductive model for human health [28] suggests that the corpora lutea (CL) of this species are a good model for investigating both long- and short-term effects of DEHP on mechanisms regulating luteal function because ovulation can be induced by exogenous gonadotropin-releasing hormone (GnRH) administration [29] and the CL can be staged precisely. Therefore, the goal of the present investigation was to examine in vivo and in vitro both the chronic and acute effects of DEHP on rabbit CL during early, mid, and late luteal stages of pseudopregnancy. In particular, the experiments were performed to better understand the effects of DEHP on the protein and gene expressions of PPARG, on the production of progesterone, prostaglandin E2 (PGE2), and PGF2alpha, and on the activity of the enzymes involved in the regulation of CL life span: 3beta-hydroxysteroid dehydrogenase (3beta-HSD), prostaglandin endoperoxide synthase 1 (PTGS1), PTGS2, and PGE2-9-ketoreductase (PGE2-9-K).

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MATERIALS AND METHODS

Reagents and Hormones

Mouse monoclonal anti-PPARG (sc-7273) and mouse monoclonal anti-3beta-HSD (sc-100466) were obtained from Santa Cruz Biotechnology; mouse monoclonal anti-PTGS1 and mouse monoclonal anti-PTGS2 were purchased from Acris Antibodies and BD Transduction Laboratories, respectively; goat polyclonal anti-PGE2-9-K (CBR1) was purchased from Abcam. Biotin-conjugated goat anti-mouse immunoglobulin G (IgG) and biotin-conjugated rabbit anti-goat IgG secondary antibodies were obtained from Santa Cruz Biotechnology. Avidin-biotin complex (ABC Kit), chromogen 3,3′-diamino-benzidine tetrachloride, and mouse and goat IgG were purchased from Vector Laboratories.

Random hexamer primers, deoxyribonuclease I (DNase I Amplification Grade), RNase H-reverse transcriptase (Superscript III Reverse Transciptase), *Escherichia coli* RNase H, DNA ladders as well as reagents for the isolation and purification of total RNA (TRIzol), Taq DNA polymerase (Platinum), RNase-free tubes, RNase-free water, and deoxynucleotide triphosphates were obtained from Invitrogen. Primers for *18S* rRNA and corresponding competimers (QuantumRNA 18S Internal Standards) as well as *PPARG* were also acquired from Invitrogen. Tritiated hormones and [^{3}H] arachidonic acid (AA) were purchased from Amersham Biosciences Ltd., while progesterone, PGF2alpha, and PGE2 antisera, DEHP, dimethylsulfoxide, and nonradioactive hormones came from Sigma. The endogenous PPARG agonist 15d-PGJ2 and the selective antagonist T0070907 were purchased from Tocris Bioscience. Selective PTGS2 inhibitor (NS-398) and nonselective PTGS inhibitor (acetylsalicylic acid) were purchased from Sigma-Aldrich. Incubation wells were obtained from Becton Dickinson Co., while medium 199 and Earle Balanced Salt Solution were from GIBCO. HEPES and bovine serum albumin (BSA) were purchased from Sigma, whereas all the other pure grade chemical and reagents were obtained locally. The following hormonal preparations were administered via intramuscular injection: eCG (Folligon; Intervet) and GnRH analog (Receptal; Hoechst-Roussel Vet).

Animals and Hormonal Regimen

Sexually mature New Zealand white female rabbits (3.5–4 kg body weight and 5 mo of age), raised in premises owned by the University of Perugia were used for all the experiments. The rabbits were housed individually in wire mesh cages under controlled light (14L:10D, lights off at 2100 h) and temperature (18°C–24°C) conditions. Each animal had free access to food and water. Pseudopregnancy was induced with 20 international units eCG followed 3 days later by 0.8 μg GnRH analog (buserelin) [30]. The day of buserelin injection was designated as Day 0. The protocols for the use of the animals for these experiments were approved by the Bioethics Committee of the University of Perugia.

In Vivo Experiments

The rabbits (n = 15/group) were daily administered orally either DEHP in corn oil (treated group, 1 mg/kg/day in 0.2 ml/kg body weight) or corn oil alone (control group) for 15 days before induction of ovulation by buserelin injection. We purposely employed the NOAEL (no observed adverse effect level) dose suggested by Andrade et al. [31] and, more recently, by Kay et al. [32] to avoid any possible pharmacological actions. Feed intake and body weight were recorded every day. From each rabbit, blood samples were collected by venous puncture of the marginal ear vein [33]; the first just before DEHP treatment (Day −15 before ovulation) and then Day −7, Day 0 (buserelin injection), and Days 4, 9, and 13 of pseudopregnancy. The samples, collected in ethylenediaminetetraacetic acid (EDTA) vacutainers, were centrifuged at 3000 × *g* for 15 min, and plasma were stored frozen until assayed for progesterone concentrations. No differences were evidenced in the number of CL between treated and nontreated rabbits.

Tissue Collection and Processing

On Days 4 (early stage), 9 (midstage), and 13 (late stage) of pseudopregnancy, animals (n = 6 control and n = 6 treated for each luteal stage) were euthanized by cervical dislocation in accordance with the guidelines and principles for the care and use of research animals. Upon euthanasia, the reproductive tracts were promptly removed and thoroughly washed with saline. After a few minutes, the CL were excised from ovaries and, after careful dissection of nonluteal tissue by fine forceps under stereoscopic magnification, immediately processed for in vitro experiments or, after rinsing with RNase-free PBS, frozen at −80°C for later evaluation of gene and protein expression. For the immunohistochemical analysis, the ovaries of two animals for each luteal stage were fixed by immersion in 4% (w/v) formaldehyde in PBS (pH 7.4) for 24 h at room temperature and subsequently processed following routine tissue preparation procedures [34, 35].

Immunohistochemistry

The immunohistochemical procedures were performed as previously reported [36]. Ovaries were embedded in paraffin and sectioned serially at a thickness of 5 μm. Sections were deparaffinized, rehydrated through graded concentrations of alcohol to distilled water, and submitted to antigen retrieval by microwaving in 1 mM EDTA (pH 8.0) for PPARG and in 10 mM citrate-buffered solution (pH 6.0) for PTGS1, PTGS2, and PGE2-9-K, for 10 min. The slides were then cooled to room temperature. After rinsing with TBS (Tris-buffered saline), they were dipped in 3% H_2O_2 in methanol for 1 h to quench the endogenous peroxidase activity and rinsed in TBS. Background labeling was prevented by incubating the sections with normal goat serum (for PPARG, PTGS1, PTGS2, and 3beta-HSD) and with normal rabbit serum (for PGE2-9-K) diluted 1:10 for 1 h at room temperature. Subsequently, the sections were incubated overnight at 4°C in a moist chamber with the following primary antisera diluted in TBS containing 0.2% Triton X-100 and 0.1% BSA: anti-PPARG (1:50), anti-PTGS1 (1:50), anti-PTGS2 (1:50), anti-PGE2-9-K (1:500), and anti-3beta-HSD (1:100).

The next day, the slides were rinsed three times in TBS (5 min each), treated again with normal goat or rabbit serum and incubated with biotin-conjugated goat anti-mouse (for PPARG, PTGS1, PTGS2, and 3beta-HSD) or biotin-conjugated rabbit anti-goat (for PGE2-9-K) secondary antibodies diluted 1:200 for 30 min at room temperature. After TBS washes, the slides were exposed to the ABC Kit for 30 min and rinsed again with TBS. The peroxidase activity sites were visualized using the 3,3′-diaminobenzidine tetrachloride kit as the chromogen; the sections were rinsed with distilled water and, in some cases, were counterstained with Mayer hematoxylin, washed in running tap water, dehydrated by passing through graded ethanol (70%, 95%, and 100% v/v), cleared in xylene, and finally mounted with Eukitt medium for light microscopy. Tissue sections in which the primary antibody was omitted or substituted by mouse or goat IgG were used as negative controls of nonspecific staining. The intensity of immunostaining was assessed and compared microdensitometrically as previously described [37] using an image analysis system (IAAS 2000 image analyzer; Delta Sistemi).

RT-PCR

Total RNA was extracted from CL of three rabbits for each luteal stage of treated and control animals as previously described [38]. Five micrograms of total RNA were reverse transcribed in 20 μl of SuperScript III Reverse Transcriptase cDNA synthesis mix using random hexamer according to the protocol provided by the manufacturer. Genomic DNA contamination was checked by developing the PCR without reverse transcriptase. The PCR reaction (25.0 μl) was performed with 0.2 μl Taq DNA polymerase (5 units/μl), 1.0 μl deoxynucleotide triphosphates (10 mM), 1.5 μl $MgCl_2$ (50 mM), 5.0 μl Taq buffer 10×, 2.0 μl mixed primers for *PPARG* gene (product size 200 bp; forward 5′-TGAAGGATGCAAGGGTTTCT-3′, reverse 5′-CCAA CAGCTTCTCCTTCTCG-3′, accession no. U84893.1), and *18S* housekeeping gene (product size 489 bp, forward 5′-TCAAGAACGAAAGTCGGAGGTT-3′, reverse 5′-GGACATCTAAGGGCATCA-3′) with competimers (1:9 v/v) [24].

Cycling conditions consisted of an initial denaturizing cycle at 94°C for 75 sec, followed by 35 cycles for each target gene at 94°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec, and a final extension step at 72°C for 10 min [24]. Within each experiment, the complete set of samples was processed in parallel in a single PCR using aliquots of the same PCR master mix. The amplified PCR-generated products (20 μl of 25 μl total reaction volume) were analyzed by electrophoresis on 2% agarose gel using ethidium bromide staining. Analysis of amplification products was carried out as reported elsewhere [38]. The amplified products, collected from agarose gel after electrophoresis, were purified with Nucleospin Extract II kit, and their identities were confirmed by DNA sequencing with the Sanger method.

Western Blot Analysis

Total luteal proteins were extracted from 100 mg of CL collected at the midstage of pseudopregnancy as previously described [39]. Briefly, 20 μg proteins were separated by SDS-PAGE, and the blocked membrane was then probed with mouse monoclonal anti-PPARG, mouse monoclonal anti-3beta-HSD, mouse monoclonal anti-PTGS1, mouse monoclonal anti-PTGS2 and goat polyclonal anti-PGE2-9-K overnight at 4°C. All the antibody dilutions were 1:1000. After being washed with TBS, the membrane was incubated with

horseradish peroxidase-labeled goat anti-mouse IgG and rabbit anti-goat IgG secondary antibodies (1:20 000 dilution) at room temperature for 1 h.

In Vitro Experiments

A previously reported method was used for the in vitro study [40, 41]. Day 4, 9, or 13 CL of control (in vivo untreated) and DEHP in vivo treated rabbits (four rabbits per treatment for each luteal stage) were randomly distributed (one CL/well) into incubation wells (Becton Dickinson Co.) in 1 ml of culture medium 199 with Earle Balanced Salt Solution containing 2.2 mg/ml sodium bicarbonate, 2.3 mg/ml HEPES, and 3% BSA (w/v), referred to here as M199. Before treatment, the CL was quartered inside each well using fine forceps. Stock solution of DEHP was prepared at concentrations of 2 mM in dimethylsulfoxide [42]. Each set of incubation wells was divided into six experimental groups, each formed of six wells as follows: 1) CL of in vivo untreated rabbit (control) in medium alone; 2) CL of DEHP in vivo treated rabbit in medium alone; 3) control CL in medium plus PPARG agonist (200 nM 15d-PGJ2); 4) control CL in medium plus DEHP (100 μM); 5) control CL in medium plus DEHP (100 μM) plus 15d-PGJ2 (200 nM); and 6) control CL in medium plus DEHP (100 μM) plus PPARG antagonist (T0070907, 50 nM). The culture plates were incubated at 37°C in air with 5% CO_2. The media of each well were collected after 4 h of incubation and stored immediately at −20°C for later determination of progesterone, PGF2alpha, and PGE2. Preliminary evidence led to our choosing the incubation conditions and the minimum effective dose for 15d-PGJ2 and DEHP used in the present in vitro study (data not shown).

To evaluate whether DEHP treatment in vitro has any acute effect on PPARG expression, CL harvested at Days 4, 9, and 13 of pseudopregnancy from control rabbits (three rabbits for each luteal stage) were randomly distributed (one CL/well) into incubation wells in 1 ml of culture medium M199 alone (control) or medium containing DEHP (100 μM, treated). After 4 h of incubation, the CL were collected, rinsed with RNase-free PBS, and processed for RT-PCR analysis.

Hormone Determination

Progesterone, PGF2alpha, and PGE2 were determined following previously reported radioimmunoassay protocols [43]. Intra- and interassay coefficients of variation and minimum detectable doses were: progesterone (6.1%, 8.9%, 11 pg); PGF2alpha (7.5%, 12.4%, 18 pg); and PGE2 (6.4%, 12.2%, 16 pg).

Enzyme Activity Determination

Activities for PTGS1 and PTGS2 were determined by measuring the disappearance of the radiolabeled substrate [^{3}H]AA using a previously reported method [44, 45]. The selective PTGS2 inhibitor (NS-398, 1 μM) and nonselective PTGS inhibitor (acetylsalicylic acid, 1 mM) were used. For each sample, PTGS1 activity was determined by calculating the rate of loss of [^{3}H]AA incubated with the selective PTGS2 inhibitor. Conversely, the PTGS2 activity of each corresponding sample was determined by calculating the rate of loss of [^{3}H]AA incubated without the selective PTGS2 inhibitor and subtracting from this the value for PTGS1. The values for PTGS1 and PTGS2 were corrected by subtracting the [^{3}H]AA disappearance values resulting from other enzymatic activities and to nonenzymatic reactions.

The PGE2-9-K activity was determined by measuring the conversion of [^{3}H]PGE2 into [^{3}H]PGF2alpha using a previously reported modified method [45], whereas that of 3beta-HSD was determined by measuring the conversion of [^{3}H]pregnenolone into [^{3}H]progesterone [24]. Briefly, each pool of CL was homogenized in 1 ml cold fresh homogenizing buffer (20 mM K_2HPO_4, 1 mM EDTA, and 10 mM beta-mercaptoethanol, pH 7.4). The total homogenate was filtered and immediately used for the assay of enzymatic activity. One hundred microliters of homogenate and 50 μl of homogenizing buffer containing 150 000 dpm [^{3}H]pregnenolone and NADPH (3 mg/ml) were added to the incubation tube. The mixture was incubated at 37°C for 10 min. Termination was achieved by addition of 100 μl 0.1 M HCl. Steroids were extracted with diethyl ether and resuspended with 500 ml RIA buffer (74.5 mM Na_2HPO_4, 12.5 mM Na EDTA, and 0.1% gelatine, pH 7.5). Two hundred microliters of RIA buffer containing progesterone specific antiserum were added to duplicated samples, and the mixture was incubated at 4°C for 16 h. The [^{3}H]progesterone-antiserum bound fraction was separated with charcoal-dextran suspension, and the radioactivity was quantified by liquid scintillation counting.

Statistical Analysis

All the data were examined by Levene test and one-way ANOVA followed by Tukey post hoc comparisons. Differences were considered significant at $P < 0.01$.

RESULTS

Immunohistochemistry

In both control and treated rabbits, PPARG immunoreactivity was localized intensely in the perinuclear cytoplasm and with lesser intensity in the nucleus of luteal cells (Fig. 1). In does treated with DEHP, PPARG immunoreactivity decreased ($P < 0.01$) in early CL and mid-CL compared to controls (Fig. 1). At late stage of pseudopregnancy, the majority of the nuclei of luteal cells were immunonegative in both control and treated CL (Fig. 1). The blood vessels were always negative (Fig. 1).

In control animals, PTGS1 immunostaining was intensely evidenced in the perinuclear cytoplasm and weakly evidenced in the nucleus of all luteal cells at the early stage of pseudopregnancy (Fig. 2). The immunodensity decreased ($P < 0.01$; Fig. 2) from early to late stages (Fig. 2); in particular, at the late stage, the cytoplasm luteal cell immunoreactivity diminished whereas that of the nucleus increased (Fig. 2). Immunosignals for PTGS1 were also observed in the nucleus of endothelial cells during all the phases of CL. In the treated animals, PTGS1 immunodensitometric analysis showed that the treatment with DEHP did not produce significant effects compared to controls (Fig. 2).

Immunosignals of PTGS2 were detected in the cytoplasm of all luteal cells during all the CL stages of pseudopregnancy in control animals (Fig. 3); the immunodensity was higher ($P < 0.01$; Fig. 3) at late and mid stages (Fig. 3). Additionally, endothelial cells of arteries, but not of veins (Fig. 3), displayed intense immunoreactivity during all the CL stages considered. In treated does, PTGS2 immunosignals decreased ($P < 0.01$; Fig. 3) only at late stage compared to controls (Fig. 3). The immunopositive reaction of arterial endothelial cells was not modified by DEHP treatment (Fig. 3).

In control animals, PGE2-9-K immunosignals were observed in the cytoplasm and nucleus of luteal cells during all the CL stages of pseudopregnancy (Fig. 4); the immunodensity was higher ($P < 0.01$) (Fig. 4) at the early stage. The immunopositive reaction was also detected in the nuclei of endothelial, smooth musculature, and stromal cells of both veins and arteries. In treated does, PGE2-9-K immunoreactivity decreased ($P < 0.01$) during all the CL stages compared to controls (Fig. 4); the densitometric levels of early treated does were similar to that of middle and late control animals (Fig. 4). The immunostaining of endothelial, smooth musculature, and stromal cells of blood vessels were similar to the controls.

In control animals, 3beta-HSD immunosignals were localized in the cytoplasm of all the luteal cells (data not shown). The immunodensity did not change in all the luteal stages (data not shown). In treated animals, 3beta-HSD immunodensitometric analysis showed that the treatment with DEHP did not produce significant effects compared to controls (data not shown). The blood vessels were always negative (data not shown). No staining was detectable in negative control rabbit ovary, where the primary antibodies were omitted or substituted with mouse or goat IgG (data not shown).

Western Blot Analysis

The Western blot analyses confirmed the specificity of the antisera used for immunohistochemistry in CL samples with a single band for each protein examined at approximately 55 kDa

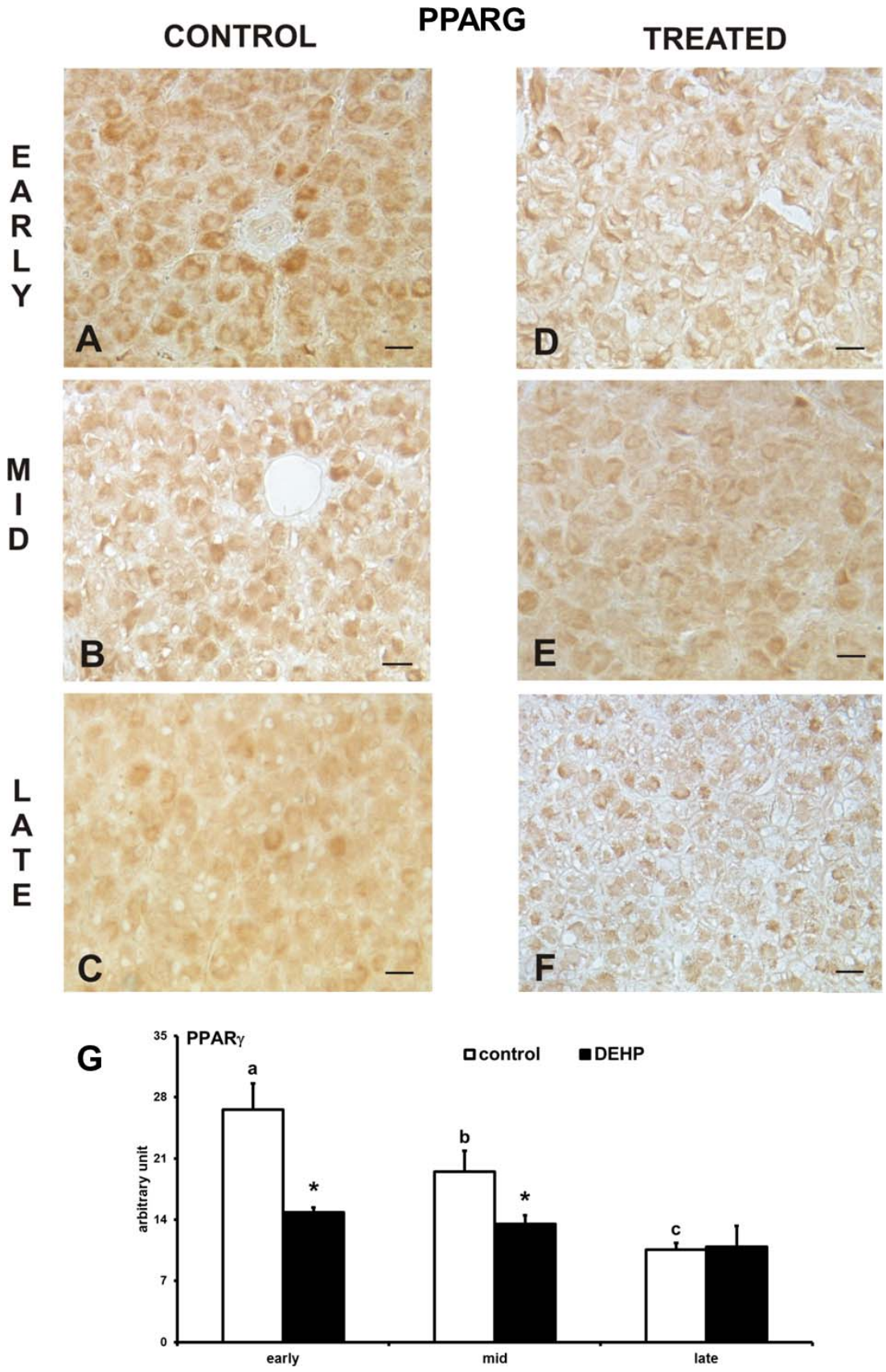

FIG. 1. Immunohistochemical detection of PPARG (PPAR$_\gamma$) in CL collected from untreated (control) and DEHP-treated rabbits at early, mid, and late stages of pseudopregnancy. **A**–**C**) Control animals. **D**–**F**) DEHP-treated does. **G**) Immunodensitometry. Data are expressed in arbitrary units; values are the means ± SD of 30 replicates; different letters above the bars indicate significantly different values ($P < 0.01$) among control CL; and the asterisks indicate significantly different values ($P < 0.01$) versus the control. Original magnifications ×20, bars = 20 μm.

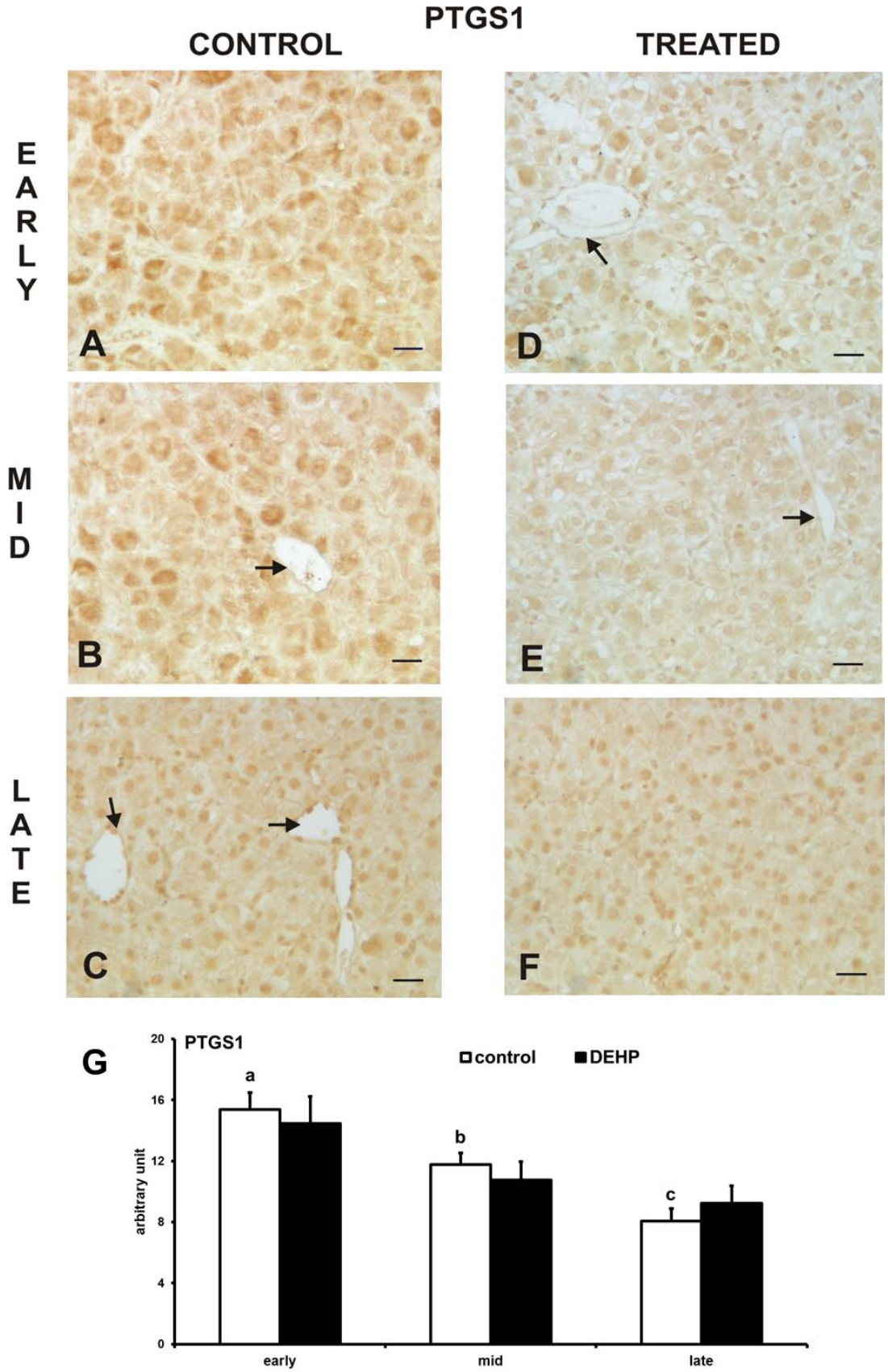

FIG. 2. Immunohistochemical detection of PTGS1in CL collected from untreated (control) and DEHP-treated rabbits at early, mid, and late stages of pseudopregnancy. **A**–**C**) Control animals. **D**–**F**) DEHP-treated rabbits. The arrows indicate the positive reaction of the nucleus of endothelial cells. **G**) Immunodensitometry. The data are expressed in arbitrary units; values are the means ± SD of 30 replicates; different letters above the bars indicate significantly different values ($P < 0.01$) among control CL. Original magnifications ×20, bars = 20 μm.

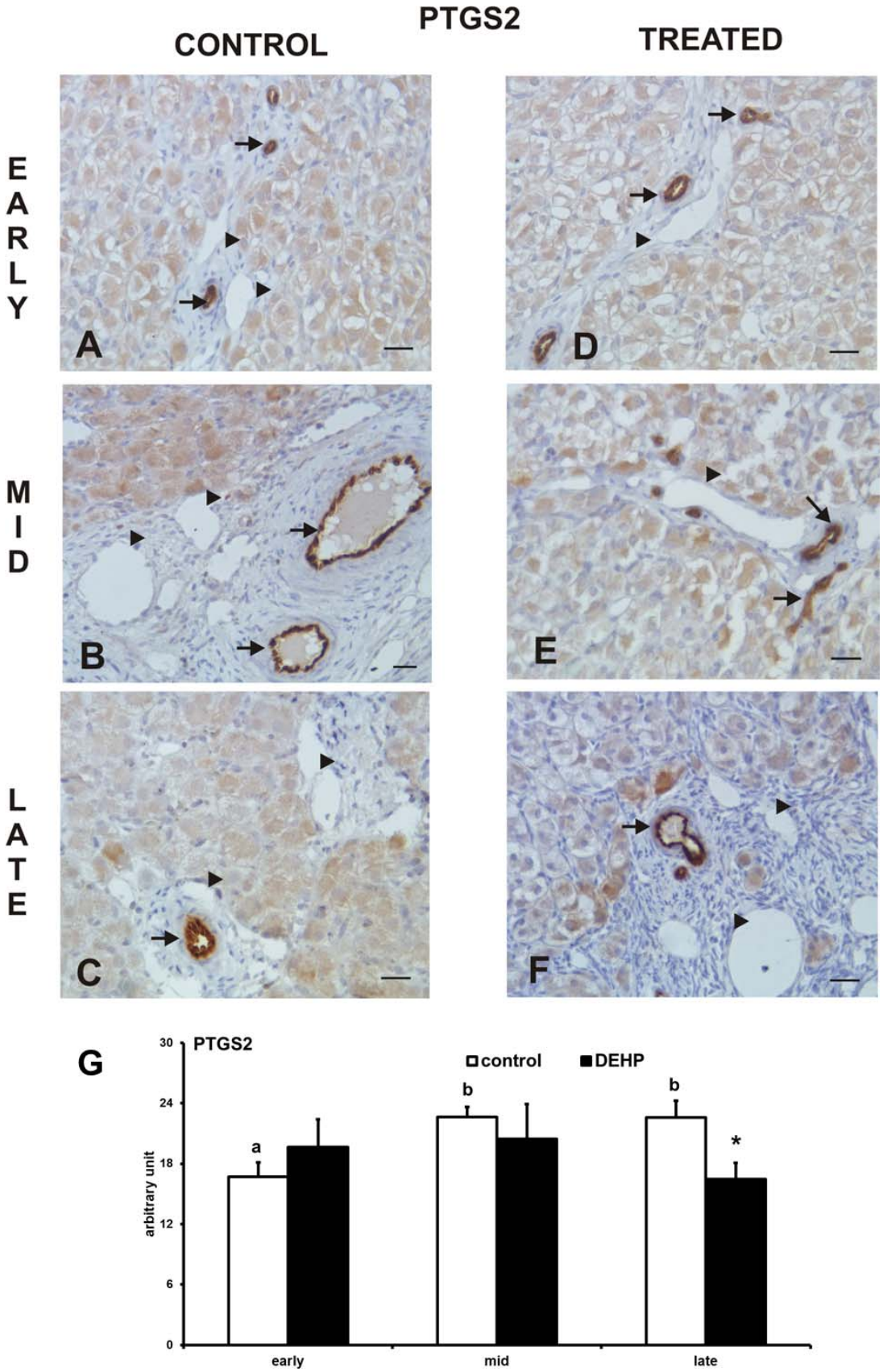

FIG. 3. Immunohistochemical detection of PTGS2 in CL collected from untreated (control) and DEHP-treated rabbits at early, mid, and late stages of pseudopregnancy. **A**–**C**) Control animals. **D**–**F**) DEHP-treated does showing the immunoreactivity of arterial endothelial cells (arrows) and the immunonegative responses of veins (arrowheads). **G**) Immunodensitometry. The data are expressed in arbitrary units; values are the means ± SD of 30 replicates; different letters above the bars indicate significantly different values ($P < 0.01$) among control CL; and the asterisks indicate significantly different values ($P < 0.01$) versus the control. Original magnifications ×20, bars = 20 μm.

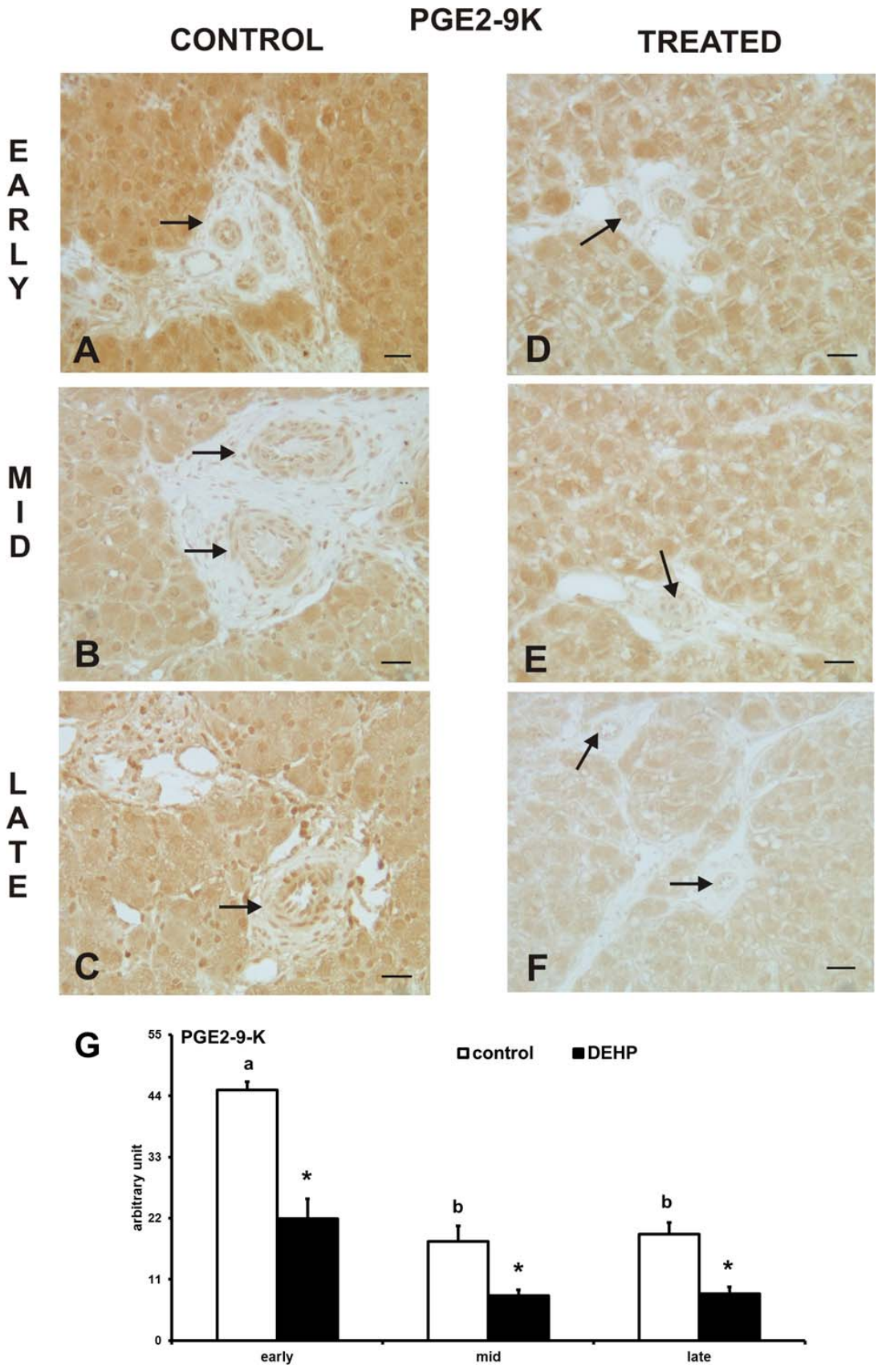

FIG. 4. Immunohistochemical detection of PGE2-9-K in CL collected from untreated (control) and DEHP-treated rabbits at early, mid, and late stages of pseudopregnancy. **A**–**C**) Control animals. **D**–**F**) DEHP-treated does. The arrows indicate the positive reaction of the nuclei of endothelial, smooth muscle, and stromal cells of blood vessels. **G**) Immunodensitometry. The data are expressed in arbitrary units; values are the means ± SD of 30 replicates; different letters above the bars indicate significantly different values ($P < 0.01$) among control CL; and the asterisks indicate significantly different values ($P < 0.01$) versus the control. Original magnifications ×20, bars = 20 μm.

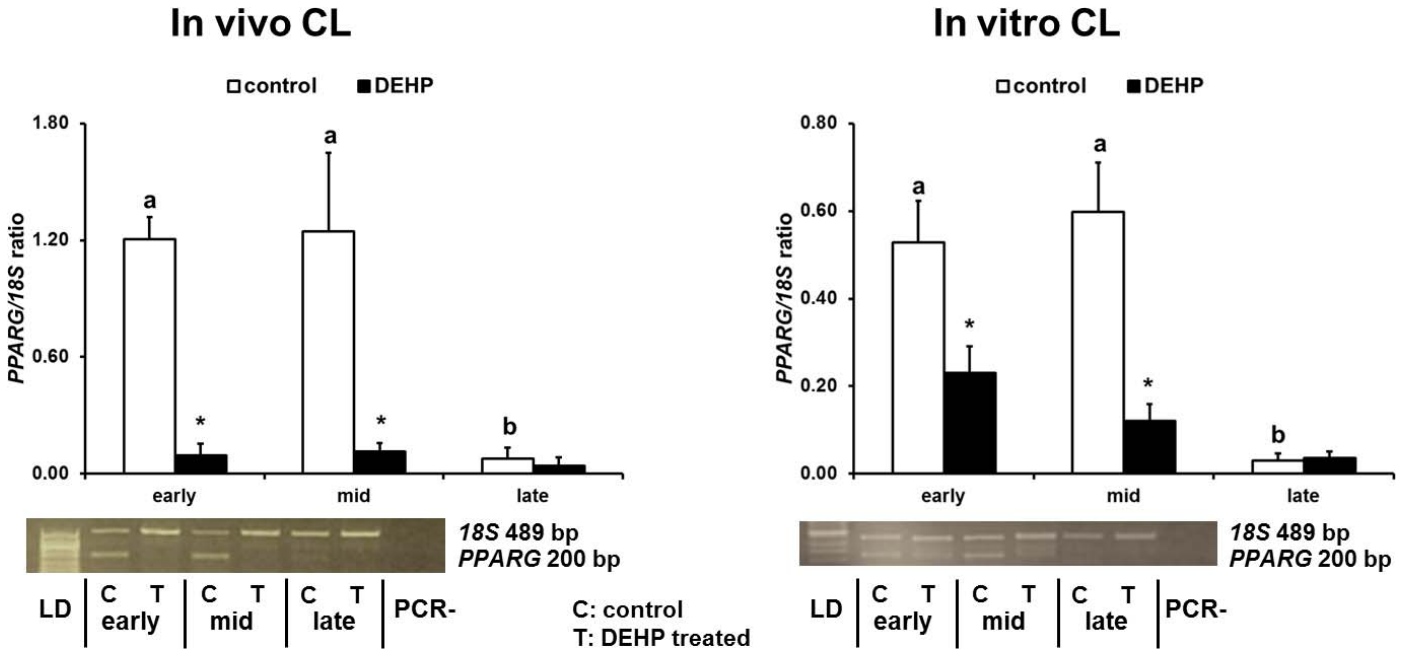

FIG. 5. Expression profile of *PPARG* mRNA in CL of in vivo DEHP-treated rabbit (left panels) and in in vitro DEHP-treated CL (right panels) collected at early, mid, and late stages of pseudopregnancy. The lower panels show a representative photograph of a 2% agarose ethidium bromide-stained gel used to analyze the PCR products. The sizes of the amplified products are shown on the left of the gel. LD is the kilobase DNA marker. The other lanes identify the corresponding pseudopregnancy stages of control (C) and DEHP-treated (T) animals. Lane PCR– represents a negative control of nonreverse-transcribed RNA submitted to PCR amplification.The upper panels show the data derived from densitometric analyses of the gels. For each luteal stage, the values (means ± SD) combine the results from three different rabbits and are reported in arbitrary units of *PPARG* mRNA relative to that of *18S* used as the internal standard. Different letters above the bars indicate significantly different values ($P < 0.01$) among control CL, and the asterisks indicate significantly different values ($P < 0.01$) versus the control.

for PPARG, 70 kDa for PTGS1, 70 kDa for PTGS2, 35 kDa for PGE2-9-K, and 42 kDa for 3beta-HSD (data not shown).

PPARG RT-PCR

In control rabbits, *PPARG* transcript was expressed in the CL at different stages of pseudopregnancy with the lowest abundance ($P < 0.01$) at the late stage of pseudopregnancy (Fig. 5, upper panels). Daily administration of DEHP for 15 days before ovulation reduced ($P < 0.01$) *PPARG* expression in CL collected at early and mid stages of pseudopregnancy (Fig. 5, left panels). In CL harvested from control rabbits and cultured in vitro for 4 h, addition of DEHP to the medium reduced ($P < 0.01$) PPARG expression in early and mid-CL (Fig. 5, right panels).

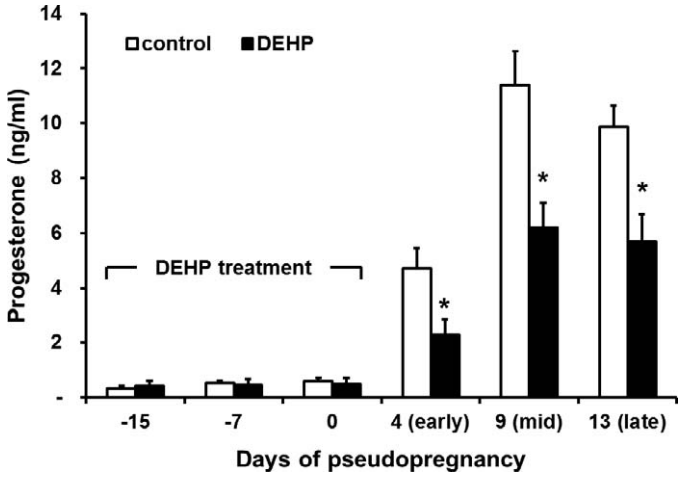

FIG. 6. Progesterone plasma levels in pseudopregnant rabbits in vivo chronically treated with DEHP for 15 days before induced ovulation. Values are the means ± SD of 15 replicates. The asterisks above the bars indicate significantly different values ($P < 0.01$) versus the control.

In Vivo Hormone Plasma Levels

Daily administration of DEHP did not affect feed intake and body weight of the treated rabbits (data not shown). Treatment with DEHP did not interfere with ovulation as assessed by progesterone profiles (Fig. 6), whereas it induced a decrease ($P < 0.01$) of plasma progesterone concentrations in rabbits during early, mid, and late stages (Fig. 6).

In Vitro Hormone Productions

Progesterone release from the CL of control rabbits was the lowest ($P < 0.01$) at the early stage and the highest ($P < 0.01$) at midstage (Fig. 7). The amounts of progesterone released by CL obtained from rabbits treated with DEHP was lower ($P < 0.01$) than those of control (in vivo untreated) CL in all the luteal stages (Fig. 7). Addition of 15d-PGJ2 into the medium increased ($P < 0.01$) while that of DEHP, T0070907, or DEHP plus T0070907 reduced ($P < 0.01$) progesterone in vitro secretion by control CL at the early and mid stages of pseudopregnancy (Fig. 7). Addition of DEHP plus 15d-PGJ2 did not modify progesterone in vitro secretion (Fig. 7).

Control basal PGF2alpha secretion was higher in late CL ($P < 0.01$) than in early and mid-CL, and secretion of mid-CL was higher ($P < 0.01$) than that of early stage (Fig. 7). In vivo treated rabbit CL released greater amounts of PGF2alpha ($P < 0.01$) in vitro compared to control CL in all the luteal stages (Fig. 7). The addition of 15d-PGJ2 reduced ($P < 0.01$) and DEHP, T0070907, or DEHP plus T0070907 increased ($P <$

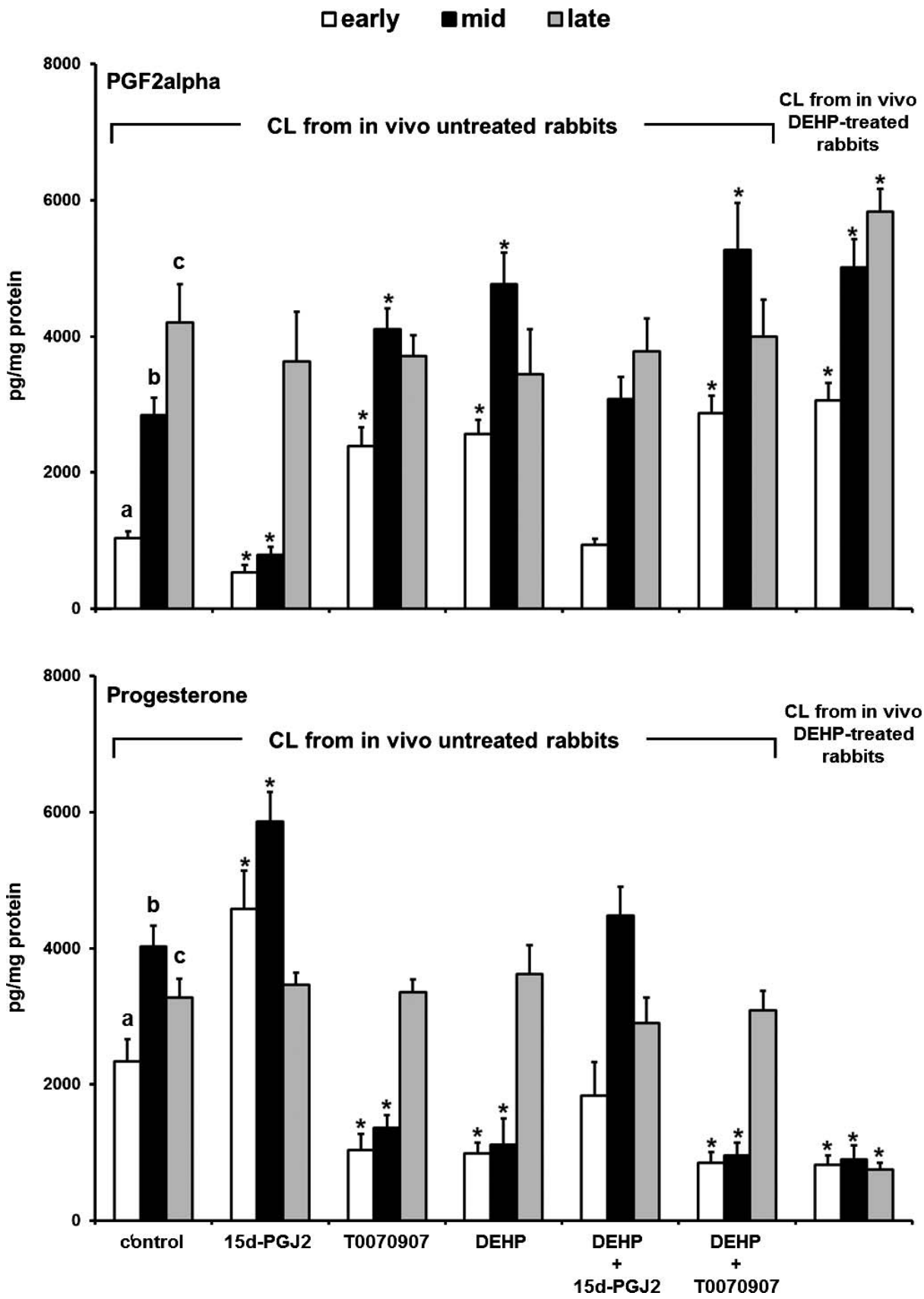

FIG. 7. In vitro releases of progesterone and PGF2alpha by CL collected from in vivo untreated (control) and DEHP in vivo treated rabbits at early, mid, and late stages of pseudopregnancy. The in vitro acute effects of 15d-PGJ2 (PPARG agonist), DEHP, DEHP plus 15d-PGJ2, and DEHP plus T0070907 (PPARG antagonist) were obtained on CL from in vivo untreated rabbits. Values are the means ± SD of six replicates. Different letters above the bars indicate significantly different values ($P < 0.01$) among control CL, and the asterisks indicate significantly different values ($P < 0.01$) versus the control.

0.01) PGF2alpha in vitro secretion of control CL at the early and mid stages of pseudopregnancy (Fig. 7), whereas these treatments did not affect late CL. Addition of DEHP plus 15d-PGJ2 did not modify PGF2alpha in vitro secretion (Fig. 7).

Control basal PGE2 secretion was higher ($P < 0.01$) in early CL (844 ± 164 pg/mg protein) than in mid-CL (356 ± 116 pg/mg protein) and late CL (295 ± 98 pg/mg protein). In vivo treated rabbit CL released in vitro amounts of PGE2 similar to those of control CL in all the luteal stages (data not shown). All the in vitro treatments did not modify PGE2 secretion of control CL in any of the luteal stages (data not shown).

Luteal Enzyme Activities

Control basal PTGS1 activity was higher ($P < 0.01$) in late CL (15 823 ± 2314 dpm/mg protein) CL than in early CL (6345 ± 776 dpm/mg protein) and mid-CL (7241 ± 9564 dpm/mg protein). In vivo treated rabbit CL showed PTGS1 activity similar to those of control CL in all the luteal stages (data not shown). All in vitro treatments did not modify PTGS1 activity of control CL in any of the luteal stages (data not shown).

Control basal PTGS2 activities were higher in late CL ($P < 0.01$) than in early and mid-CL (Fig. 8), and PTGS2 activity of mid-CL was higher ($P < 0.01$) than that of early ones (Fig. 8). DEHP in vivo treatment increased ($P < 0.01$) PTGS2 activity at early and mid stages of pseudopregnancy (Fig. 8). In vitro treatment with 15d-PGJ2 reduced ($P < 0.01$) and with DEHP, T0070907, or DEHP plus T0070907 increased ($P < 0.01$) PTGS2 activity at early and mid stages of pseudopregnancy (Fig. 8). In vitro addition of DEHP plus 15d-PGJ2 did not modify PTGS2 activity (Fig. 8).

Control basal PGE2-9-K activity were higher ($P < 0.01$) in late CL (40 095 ± 3787 dpm/mg protein) than in early CL (3407 ± 758 dpm/mg protein) and mid-CL (13 651 ± 1947 dpm/mg protein). In vivo treated rabbit CL showed PGE2-9-K activity similar to those of control CL in all the luteal stages (data not shown). All the in vitro treatments did not modify PGE2-9-K activity of control CL in any luteal stages (data not shown).

Control basal 3beta-HSD activity was higher in mid-CL ($P < 0.01$) than those of early and late CL, and activity in early CL was higher ($P < 0.01$) than that of late ones (Fig. 8). DEHP in vivo treatment reduced ($P < 0.01$) 3beta-HSD activity at early and mid stages of pseudopregnancy (Fig. 8). In vitro treatment with 15d-PGJ2 increased ($P < 0.01$) and with DEHP, T0070907, or DEHP plus T0070907 reduced ($P < 0.01$) 3beta-HSD activity at early and mid stages of pseudopregnancy (Fig. 8). In vitro addition of DEHP plus 15d-PGJ2 did not modify 3beta-HSD activity (Fig. 8).

DISCUSSION

PPARG has been found in both granulosa and theca cells and also in CL of rodents, ruminants [16, 20, 46], and pseudopregnant rabbits [24], facts that suggest that it may have a role in the regulation of reproduction. In cattle CL, the *PPARG* gene and protein expressions increased after ovulation, but decreased in case of fertilization or embryo implantation failure [47, 48]. In rabbit CL, PPARG exerted a luteotrophic role via 3beta-HSD up-regulation and PTGS2 down-regulation, with the consequent increase of progesterone production and concurrent decrease of PGF2alpha synthesis [24].

Using immunohistochemistry, positive staining for PPARG was identified in the cytoplasm and nucleus of all the luteal cells during the three stages of pseudopregnancy, with an evident immunonegative response in the majority of nuclei in late CL as already reported [24]. In good agreement with these results, semiquantitative/relative RT-PCR showed the lowest levels of *PPARG* mRNA in late CL. The in vivo DEHP treatments determined a notable drop in PPARG immunosignals in early and mid-CL, whereas they had no effect on late CL when PPARG expression was already down-regulated as well as in control rabbits. The *PPARG* gene expression also was decreased by in vivo and in vitro DEHP treatments in early and mid-CL.

Previous studies showed that the activation of PPARG induces an up-regulation of ovarian progesterone production [27]. Indeed, PPARG activation increased progesterone secretion in rat granulosa cells [16], in porcine theca and bovine luteal cells [48], and in pseudopregnant rabbit CL [24]. Here we found that prolonged DEHP administration before ovulation clearly affected peripheral plasma progesterone concentrations also during the subsequent pseudopregnancy. In fact, progesterone levels were much lower than those of control rabbits during early, mid, and late luteal phases. In agreement with these data obtained in vivo, also the basal in vitro release of progesterone by CL harvested from DEHP-treated rabbits was lower than that of controls for each luteal stage. It remains to be established whether the antiluteotropic effect of DEHP, observed here when administered chronically before ovulation, is due to a direct action on theca and/or granulosa cells that, following luteinization and CL development, will synthesize less progesterone or whether it is related to an indirect action mediated by a reduced synthesis of estrogens, a luteotropic hormone in rabbits, by follicles. On the other hand, because the DEHP administration was orally performed with a consequent systemic distribution, we cannot exclude that the down-regulation of CL progesterone synthesis could be indirectly due also to the DEHP effects on other tissues and/or organs.

Recently, Kay et al. [32], reviewing the reproductive and developmental effects of phthalates in mammal females, proposed for DEHP a NOAEL of 1.0 mg/kg/day, on the basis of studies done on marmosets, mice, and rats, whereas much higher doses were required to cause observable reproductive disorders. More broadly, these authors reported that the animal literature shows that adverse reproductive effects occur at high doses while concentrations representative of human exposure do not produce reproductive or developmental effects, thereby questioning the relevance of current animal models. In the present study, however, we found that DEHP when administered in vivo at the same NOAEL dose influenced the reproductive function of rabbits. Thus, while confirming the high variability of DEHP effects among mammal species, our findings suggest that the rabbit is a suitable animal model for the study of DEHP actions on the reproductive function, being much more sensitive than other species insofar examined.

The in vitro experiment showed that addition to the culture medium of DEHP, PPARG endogenous agonist (15d-PGJ2), or its selective antagonist (T0070907) affected progesterone production in early and mid-CL obtained from untreated rabbits, but they had no effects on late CL. The lack of in vitro acute effects of DEHP and PPARG agonist and antagonist on late CL may be a result of the lower levels of PPARG protein and gene expression found in these luteal cells as demonstrated by densitometry and PCR in control animals. In particular, this PPARG decrease can be ascribed to the loss of this receptor in the luteal cell nucleus during the late stage as revealed by immunohistochemistry.

Our in vitro data suggest that DEHP-induced progesterone decrease could be mediated by PPARG because the in vitro

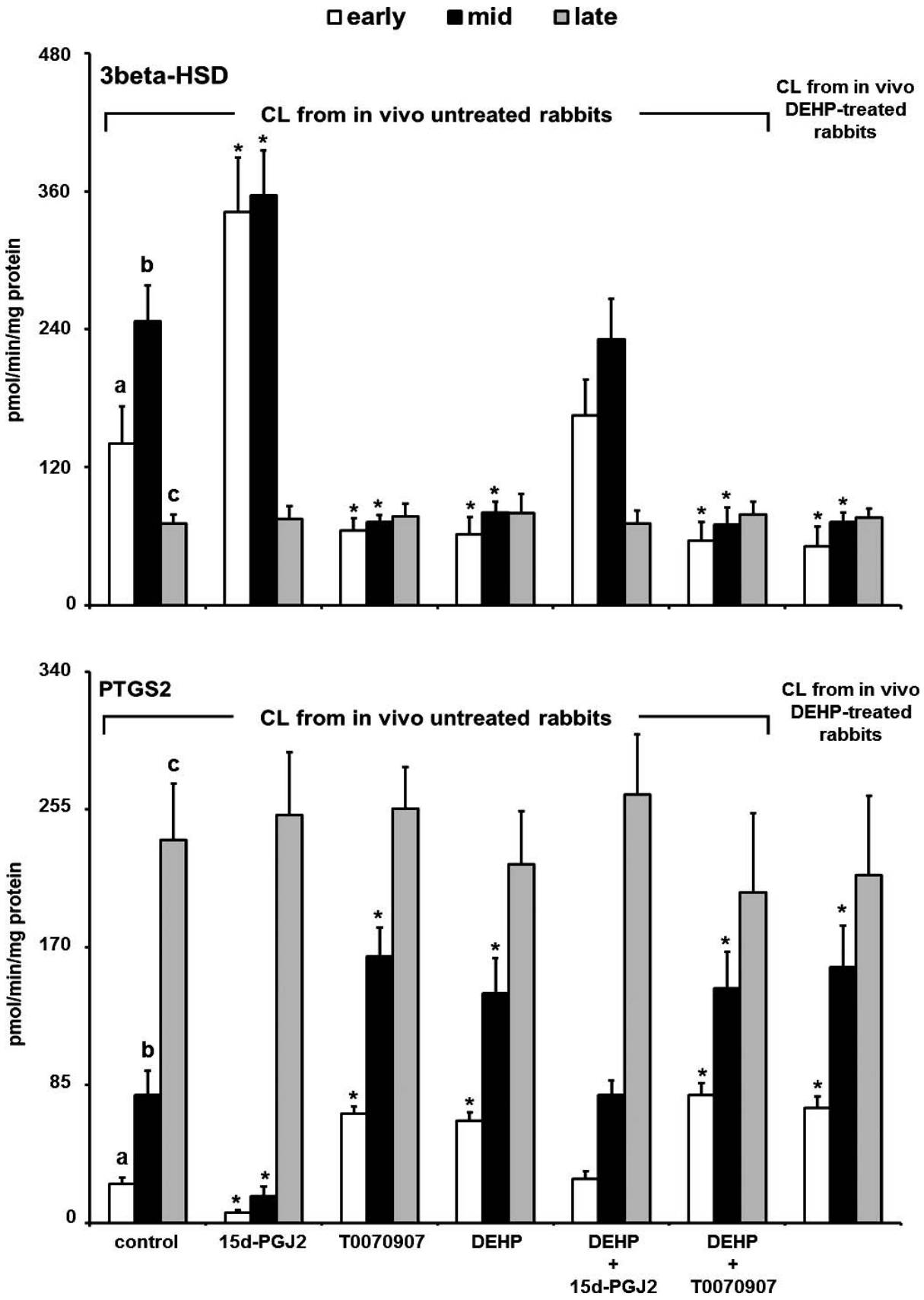

FIG. 8. PTGS2 and 3beta-HSD activities by CL collected from in vivo untreated (control) and DEHP in vivo treated rabbits at early, mid, and late stages of pseudopregnancy. The in vitro acute effects of 15d-PGJ2 (PPARG agonist), DEHP, DEHP plus 15d-PGJ2, and DEHP plus T0070907 (PPARG antagonist) were obtained on CL from in vivo untreated rabbits. Values are the means ± SD of six replicates. Different letters above the bars indicate significantly different values ($P < 0.01$) among control CL, and the asterisks indicate significantly different values ($P < 0.01$) versus the control.

coincubation with DEHP plus 15d-PGJ2 showed that this PPARG agonist counteracted the effects of the EDC. In contrast, the present in vivo results do not support this idea because DEHP affected progesterone production also in late CL when PPARG protein and gene expression were at their lowest level. The discrepancy between in vivo and in vitro data may be explained, as mentioned above, by the possible multiple effects of DEHP systemic in vivo administration.

Furthermore, we cannot exclude that the unchanged progesterone in vitro secretion was a result of two concomitant opposite phenomena and not necessary because DEHP uses the PPARG pathway. An alternative hypothesis could be that DEHP exerts its effect through other cellular pathways, for example, that of aryl hydrocarbon receptor. This is an orphan receptor expressed in ovary follicles and CL [49] that affects steroid synthesis and is activated by phthalates [50]. Thus, in our experiments DEHP would decrease progesterone by means of aryl hydrocarbon receptor activation at the same time as 15d-PGJ2 stimulates this secretion after PPARG activation, a situation that could also result in no significant differences in progesterone levels between the two experimental groups.

The molecular mechanisms of PPARG in ovarian functions are not well understood; one suggestion is that PPARG may directly influence *PTGS2* gene expression [15]. In fact, the *PTGS2* promoter region contains a PPARG response element [51]. On the other hand, there are conflicting studies that report that PPARG stimulates [51] and inhibits [52, 53] *PTGS2* gene expression. More recently, we have found that the PPARG endogenous agonist down-regulated and its antagonist up-regulated PTGS2 luteal activity at both early and mid pseudopregnancy stages [24].

The present results show that DEHP affects PTGS2 protein expression, decreasing the luteal cell immunostaining at late stage, whereas this enzyme does not seem to be influenced by DEHP treatment in early and mid-CL. It is interesting to note that, in early and mid-CL, DEHP increased PTGS2 activity with the concomitant decrease of *PPARG* gene expression, whereas it did not affect immunoexpression of this enzyme. In contrast, in late CL, DEHP decreased PPARG immunoexpression, whereas it did not influence PTGS2 activity and its mRNA level. These conflicting data are difficult to explain; perhaps DEHP at late stage uses other cellular pathways, but at the moment, this is unknown. Our data, also demonstrated that DEHP-induced PTGS2 up-regulation caused an increase of PGF2alpha in vitro secretion by early and mid-CL, whereas both PTGS1 activity and PGE2 release were not modified. The PPARG agonist counteracted also the in vitro DEHP effects on PTGS2 and PGF2alpha, so confirming the possible PPARG mediation on DEHP activity.

In rabbit CL, PGE2-9-K has a modulator role that correlates its enzymatic activity with negative PGE2 and positive PGF2alpha synthesis, respectively [45]. Our data demonstrate that DEHP decreases the PGE2-9-K immunostaining of luteal cells in all the CL types compared to controls, whereas it did not affect the activity of this enzyme in the same CL, as reported in a previous study, showing that PGE2-9-K activity was not modified by both 15d-PGJ2 and T0070907 in vitro treatments in all the luteal stages [24].

While some studies indicated that the activity of 3beta-HSD was inhibited by troglitazone, a member of the class of thiazolidinediones, which are PPARG activators, in porcine granulosa cells [54], other authors stated that thiazolidinediones treatment did not affect the *3beta-HSD* mRNA levels in porcine granulosa cells and protein levels in ovine granulosa cells [55, 56]. The present results confirmed that PPARG affects 3beta-HSD activity because 15d-PGJ_2 increased and T0070907 decreased the activity of this enzyme in early and mid-CL, as we have previous reported [23, 24]. Interestingly, both the agonist and the antagonist of PPARG had no effects on 3beta-HSD activity during the late luteal stage when the immunopresence of this nuclear receptor reached its lowest levels.

Our data indicated that DEHP did not modify the immunopresence of 3beta-HSD in luteal cells, whereas the activity of this enzyme diminished during early and mid stages. The failure of DEHP to affect 3beta-HSD protein expression and the concomitant effect on the activity of this enzyme suggest that DEHP does not directly affect 3beta-HSD activity directly, but rather through PPARG inhibition; indeed, coincubation with 15d-PGJ2 counteracted the DEHP-induced 3beta-HSD down-regulation, as shown by the in vitro data on progesterone and PGF2alpha secretions and PTGS2 activity. Moreover, as noted above for progesterone, other alternative DEHP-activated cellular mechanisms cannot be excluded.

An increasing body of converging experimental evidences indicates that phthalates, including DEHP, cause a pleiotropic range of effects, depending on tissue and species, and alter the reproductive functions acting at different levels as a consequence of their binding and activation of PPARG and downstream effects on gene expression (for reviews, see [57, 58]). Interestingly, however, our present results show that the in vitro effects induced by DEHP on rabbit luteal function are opposite to those observed after treatment with the PPARG agonist. In addition, DEHP was found to mimic the actions induced by an antagonist of the receptor [24], suggesting that the DEHP effects on PPARG-mediated events are controlled by several cellular and molecular mechanisms, depending on tissues and/or species.

In conclusion, our research demonstrates that DEHP has notable effects on the activities of pseudopregnant rabbit CL, favoring functional luteolysis, with a mechanism that seems to involve the down-regulation of PPARG, the increase of PTGS2 activity and of PGF2alpha secretion, the down-regulation of 3beta-HSD, and, finally, the decrease of progesterone. However, though the present data shed new light on possible DEHP effects on physiological mechanisms regulating luteal activity of mammals, further investigations are needed to better understand the fine-tuning of this EDC in modifying CL life span.

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