

Effect of hypoxia on progesterone production by cultured bovine early and mid luteal cells

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Abstract. A major role of the corpus luteum (CL) is to produce progesterone (P4). The CL has immature vasculature shortly after ovulation, suggesting it exists under hypoxic conditions. To elucidate the mechanism involved in regulation of luteal cell function during CL development, we compared the effect of hypoxia on P4 production by cultured bovine early and mid luteal cells. Luteal cells obtained from early and mid CL were incubated under different O₂ concentrations (20% and 3%) with or without hCG (1 U/ml) for 6 h and 24 h. After 6 h of culture in the presence of hCG, P4 production was not affected by hypoxia whereas decrease in its production by mid luteal cells was observed. After 24 h of culture, P4 production was significantly decreased by hypoxia in both stages of luteal cells regardless of the use of hCG. At 6 h of culture, hypoxia increased mRNA expression of hydroxyl- Δ -5-steroid dehydrogenase, 3 β - and steroid Δ -isomerase 1 (*HSD3B1*) in early luteal cells, and decreased mRNA expression of cytochrome P450 cholesterol side chain cleavage (*CYP11A1*) enzyme in mid luteal cells. At 24 h of culture, mRNA expressions of steroidogenic acute regulatory protein (*STAR*), *CYP11A1*, and *HSD3B1* were not affected by hypoxia in both stages of luteal cells. The overall results suggest that early luteal cells maintain P4 production under hypoxic conditions, and hypoxia-induced HSD3B1 may support this P4 production in the bovine early CL.

Key words: Corpus luteum, Early and mid luteal cells, Hypoxia, Progesterone

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The corpus luteum (CL) is an organ that is temporarily formed during the female reproductive cycle. It is formed from the empty follicle left behind after ovulation and produces progesterone (P4), which is required for the establishment and maintenance of pregnancy in female mammals [1, 2]. The CL develops after ovulation, which is accompanied by active angiogenesis, and if conception does not occur it regresses with the decrease of P4 synthesis and apoptosis of luteal cells [3]. Changes in the ovarian blood flow result in the changes in transport of nutrients, hormones, and gases including O₂ to the ovary [3]. In cows, ovarian blood flow has been reported to decrease during luteal regression and maintain at low levels during luteal formation after ovulation [3]. These conditions represent hypoxic conditions in newly formed CL due to bleeding and immature vascularization. Hypoxia-inducible factor-1 α (HIF-1 α), a subunit of hypoxia-specific transcription factor, also known as a marker for hypoxia highly expresses in bovine early CL [4].

Follicular granulosa cells and theca cells start luteinizing and producing P4 before ovulation. The key proteins in P4 biosynthesis include steroidogenic acute regulatory protein (STAR), cytochrome P450 side-chain cleavage enzyme (CYP11A1) and hydroxyl- Δ -5-steroid dehydrogenase, 3 β - and steroid Δ -isomerase 1 (HSD3B1). STAR transports cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane [5–7], CYP11A1 converts cholesterol into pregnenolone and HSD3B1 converts pregnenolone into P4 [8, 9]. Exposing cultured bovine mid luteal cells to hypoxic conditions (3% O₂) has shown to decrease P4 production, expression and activity of CYP11A1 [10]. These findings suggest that hypoxia facilitates down-regulation of P4 synthesis during luteal regression [10, 11]. Hypoxia increases P4 production and expression of *STAR* and *HSD3B1* mRNA in bovine luteinizing granulosa cells [12]. However, the importance of hypoxia in the regulation of luteal cell function during CL development is unclear.

In the present study, to clarify the physiological roles of hypoxia during CL development in cattle, we investigated the effects of hypoxia on P4 production and expression of *STAR*, *CYP11A1* and *HSD3B1* mRNA in cultured bovine early luteal cells and compared the effects with mid luteal cells.

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Materials and Methods

Collection of bovine CLs

Ovaries with CL from cows were collected at five different luteal stages from a local abattoir within 10–20 min after exsanguinations. Luteal stages were classified as the early (Days 2–3), developing (Days 5–6), mid (Days 9–12), late (Days 15–17) and regressed (Days 19–21) stages by macroscopic observation of the ovary and uterus as described previously [13, 14]. For cell culture experiments, the ovaries with early and mid-stage CLs were submerged in ice-cold physiological saline and transported to the laboratory.

Cell isolation

Early and mid-luteal tissues were enzymatically dissociated and the luteal cells were cultured as described previously [15]. After enzymatic dissociation, the luteal cells were suspended in culture medium, DMEM and Ham's F-12 medium (D/F medium; Life Technologies, Grand Island, NY, USA; Catalog No. 12634-010) containing 5% calf serum (CS; Life Technologies; Catalog No. 16170-078) and 20 µg/ml gentamicin (Wako pure Chemical Industries, Osaka, Japan; Catalog No. 078-06061) to attach cells to culture plate. Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells in the cell suspension consisted of about 70% small luteal cells, 20% large luteal cells, 10% endothelial cells or fibrocytes and no erythrocytes [15].

Cell culture experiments

To measure P4 production and mRNA expression, dispersed luteal cells were seeded at 4.0×10^5 viable cells per ml in 24-well cluster dishes (Greiner Bio-One, Frickenhausen, Germany; No. 662160). Luteal cells were cultured in D/F medium containing 5% CS in a humidified atmosphere of 5% CO₂ in air at 37.5°C in a N₂-O₂-CO₂-regulated incubator (ESPEC, Osaka, Japan; Model No. BNP-110). After 12 h of culture, the medium was replaced with fresh D/F medium containing 5 ng/ml sodium selenite and 0.1% BSA with or without human chorionic gonadotropin (hCG; ASKA Pharmaceutical, Tokyo, Japan; 1 U/ml) instead of luteinizing hormone (LH) which is important for luteal P4 production [9] and cells were cultured for 6 h and 24 h. The characteristics of early luteal cells may change during cell culture, so that we set short (6 h) and long (24 h) times of culture. Following incubation, the cell culture supernatant and extracted total cellular RNA was used for the determination of P4 concentrations and mRNA expression of *STAR*, *CYP11A1* and *HSD3B1*, respectively.

RNA isolation and cDNA synthesis

Total RNA was extracted from cultured luteal cells using RNeasy according to the manufacturer's instructions (Qiagen, Hilden, Germany; Catalog No. 74106). Total RNA (1 µg) was reverse transcribed using a PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Otsu, Japan; Catalog No. 6110A).

Real-time polymerase chain reaction (PCR)

Gene expression was measured by real-time PCR using 7500 FAST thermal cycler (Applied Biosystems, Tokyo, Japan; Catalog No. 7500-05) and the KAPA FAST ABI prism qPCR kit (KAPA, Boston,

MA, USA.; Catalog No. KK4604) using 1 ng of reverse-transcribed total RNA. The expression of 18S ribosomal RNA (*18SrRNA*) was used as an internal control. Specific primers with 50–60% GC-contents were synthesized for *STAR*, *CYP11A1*, *HSD3B1* and *18SrRNA*. The PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. KAPA FAST ABI prism qPCR mixture was used at elevated temperatures to achieve reliable and sensitive quantification of the RT-PCR products with high linearity (Person correlation coefficient $r > 0.99$). Analysis of the relative level of expression of each mRNA was done using the 2^{-ΔΔCT} method as described previously [16].

P4 concentration determination

Concentrations of P4 were determined directly from the cell culture supernatant with an enzyme immunoassay (EIA) as described previously [10]. The standard curve ranged from 0.391 to 100 ng/ml, and the effective dose of the assay for 50% inhibition (ED 50) was 4.5 ng/ml. The intra- and interassay coefficients of variation were 9.2% and 9.7%, respectively.

Statistical analysis

The statistical significance of differences in the effects of hypoxia and hCG on P4 production (Fig. 1) and on the amount of *STAR*, *CYP11A1* and *HSD3B1* mRNA (Fig. 2) was assessed by two-way factorial analysis of variance (ANOVA). The two factors analyzed were oxygen concentrations (3%, 20%) × treatment (without and with hCG). Since there was no interaction between the two factors in all experimental data, statistical significance of the effect of hypoxia or hCG on P4 production and on the amount of mRNAs was assessed by paired *t*-test. Statistical significance was set at $P < 0.05$.

Results

Effect of hypoxia on P4 production

hCG stimulated P4 production in early luteal cells after 6 h of culture (Fig. 1A; $P < 0.05$) and in mid luteal cells after 6 and 24 h of culture (Fig. 1A, B; $P < 0.05$). After 6 h of culture, hypoxia did not affect P4 production in cultured bovine early luteal cells (Fig. 1A) whereas hCG-stimulated P4 production was decreased in mid luteal cells (Fig. 1A; $P < 0.05$). After 24 h of culture, hypoxia significantly decreased P4 production in both early and mid luteal cells with or without hCG stimulation (Fig. 1B; $P < 0.05$).

Effects of hypoxia on *STAR*, *CYP11A1* and *HSD3B1* mRNA expressions

Hypoxia increased the expression of *HSD3B1* mRNA in early luteal cells (Fig. 2A; $P < 0.005$) whereas decreased *CYP11A1* mRNA expression in mid luteal cells (Fig. 2B; $P < 0.05$) after 6 h of culture and did not affect the mRNA expressions of *STAR*, *CYP11A1* and *HSD3B1* after 24 h of culture (Fig. 2C, D).

Discussion

The present study found that hypoxia did not decrease P4 production by early luteal cells treated with and without hCG stimulation and increased *HSD3B1* mRNA expression in hCG-treated early luteal

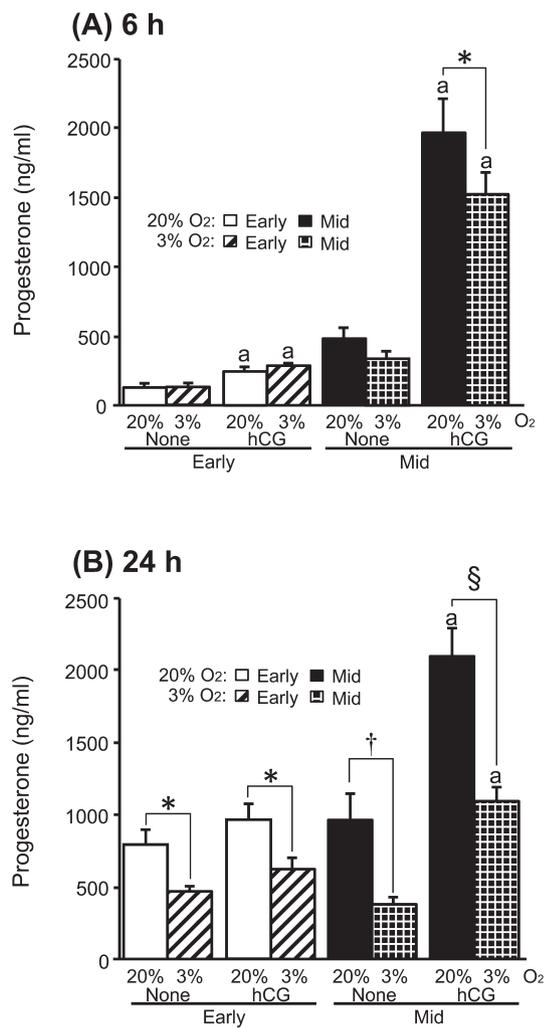


Fig. 1. Effects of hypoxia on P4 production by bovine early and mid luteal cells. The cells were cultured in D/F medium without or with hCG (1 U/ml) under 20% O₂ or 3% O₂ for 6 h (A) and 24 h (B). All values represent mean \pm SEM of separate experiments in early and mid luteal cells after 6 h of culture (early: n = 8, mid: n = 7) and after 24 h of culture (early: n = 4, mid: n = 6). Symbols indicate significant differences (* P < 0.05, † P < 0.01, § P < 0.001) between different oxygen conditions as determined by paired *t*-test. Letters (a) indicate significant effects of hCG (P < 0.05) within each oxygen conditions as determined by paired *t*-test.

cells after 6 h of culture, however hypoxia decreased hCG-stimulated P4 production in mid luteal cells after 6 h of culture as previously reported [10]. These results suggest that bovine early luteal cells can produce P4 under hypoxic conditions due to hypoxia-mediated stimulation of the HSD3B1 mRNA expression. On the other hand, hypoxia decreased hCG-stimulated P4 production in mid luteal cells after 6 h of culture. In these cells, the expression of *CYP11A1* mRNA was also significantly decreased. Our results suggest that bovine early luteal cells are able to maintain P4 production under hypoxic conditions, and this P4 production may be maintained by

hypoxia-induced HSD3B1.

The decrease of P4 production by hypoxia in early and mid luteal cells was detected after 24 h culture. On the contrary, hypoxia did not affect P4 production in the early luteal cells whereas decreased in mid luteal cells. This indicates that early luteal cells may differentiate into mid luteal cells during 24 h of culture. Further studies are required to examine the dynamics of differentiation and the function of luteal cells.

Our results show hypoxia-mediated decrease in hCG-stimulated P4 production in mid luteal cells at 6 h of culture. Also, in these mid luteal cells, significant decrease in the expression of *CYP11A1* mRNA was observed. In line with our results, our previous study also reported decreased P4 production by bovine mid luteal cells under hypoxia after 8 h of culture regardless of the LH stimulation [10]. Hypoxia led to decreased expression of *CYP11A1* mRNA in mid luteal cells after 24 h of culture and had no effect on *STAR* and *HSD3B1* mRNA expressions at any culture time point [10]. Taken together, these findings indicate that a low-oxygen condition tends to decrease P4 production by suppressing *CYP11A1* mRNA expression in mid luteal cells. We suggest that this suppression as well as P4 production in bovine mid luteal cells are two luteolytic phenomena induced by hypoxia. In the present study, hypoxia decreased P4 production regardless of the use of hCG in both early and mid luteal cells after 24 h of culture. Also after 24 h of culture, *STAR* mRNA expression tended to decrease in the hCG-treated mid luteal cells under hypoxic condition, whereas the expression of *CYP11A1* and *HSD3B1* mRNA was not affected regardless of the use of hCG. The reason for the difference between previous and present results in mRNA expressions of steroidogenic proteins is unclear. One of the reasons for these differences may be a difference in the media used in luteal cell culture system. In our previous study [10], we used a luteal cell culture system that included 5% CS, whereas in the present study media with 5% CS was used only for 12 h after seeding to allow the attachment of cells to culture plates. The media was then changed to a semi-serum-free cell culture system with 0.1% BSA for all the experiments. Since CS contains a number of bioactive substances including steroids, peptides and protein hormones as well as cytokines and growth factors [17], those substances may affect the expression of steroidogenic genes under hypoxic conditions.

Hypoxic conditions (10% O₂ and 5% O₂) were shown to significantly increase P4 production in bovine granulosa cells treated with insulin in combination with forskolin (luteinizing granulosa cells) than non-treated cells (non-luteinizing granulosa cells) [12]. In these cells, the mRNA and protein expressions of *STAR* and the protein expression of *HSD3B1* increased under 10% O₂ rather than under 5% O₂ after 24 h of culture [12]. The present study shows that hypoxia did not affect P4 production in early luteal cells after 6 h of culture but significantly increased the expression of *HSD3B1* mRNA. These results suggest that the hypoxia-induced *HSD3B1* contributes to maintain P4 production under hypoxia. In luteinizing granulosa cells, *HSD3B1* mRNA expression did not increase under 5% O₂ [12], whereas the present study shows increase under 3% O₂. Furthermore, P4 production by luteinizing granulosa cells was shown to increase under 10% O₂ and 5% O₂ [12], whereas in the present study shows decrease in P4 production by early luteal cells under 3% O₂ after 24 h of culture. The difference in P4 production

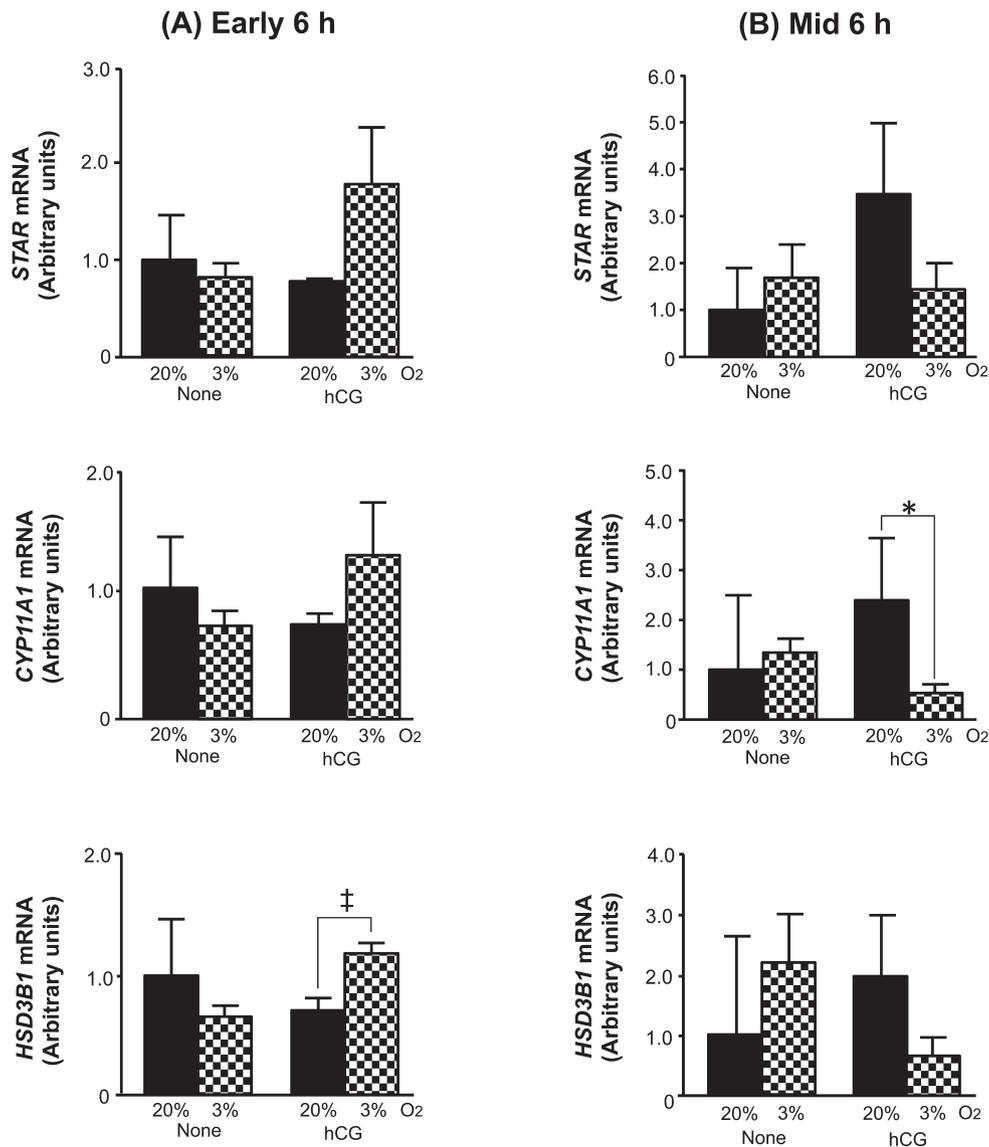


Fig. 2. Effects of hypoxia on *STAR*, *CYP11A1* and *HSD3B1* mRNA expression in bovine early and mid luteal cells. The cells were cultured in D/F medium without or with hCG (1 U/ml) under 20% O₂ or 3% O₂ for 6 h (A, B) and 24 h (C, D). The amounts of *STAR*, *CYP11A1* and *HSD3B1* are expressed relative to the amounts of *18S rRNA* after 6 h of culture (early: n = 4, mid: n = 4) and after 24 h of culture (early: n = 5, mid: n = 4). Symbols indicate significant differences (* P < 0.05, ‡ P < 0.005) between different oxygen conditions, as determined by paired *t*-test.

and *HSD3B1* mRNA expression between luteinizing granulosa cells and early luteal cells may be due to the different characteristics of these cells. In our previous study, we used luteinizing granulosa cells derived from the granulosa cells of healthy follicles with diameters ≤ 6 mm [12]. In the present study, we used the luteal cells isolated from the early stage CL (2–3 days after ovulation). Thus, the early luteal cells used in the present study reflects the function of bovine luteal cells during CL development whereas luteinizing granulosa cells in our previous study reflected the cell function during periovulatory period. After 24 h of culture, hypoxia decreased P4 production regardless of the use of hCG whereas it did not affect the expres-

sions of *STAR*, *CYP11A1* and *HSD3B1* mRNA. Further studies of protein expression and enzyme activities are needed to reveal the relationships between hypoxia and these steroidogenic regulators in bovine early CL.

The overall findings suggest that the response of bovine luteal cells to hypoxia changes from the early to mid luteal stage and that under hypoxic conditions, bovine early luteal cells can maintain P4 production, which may be supported by hypoxia-induced *HSD3B1*.

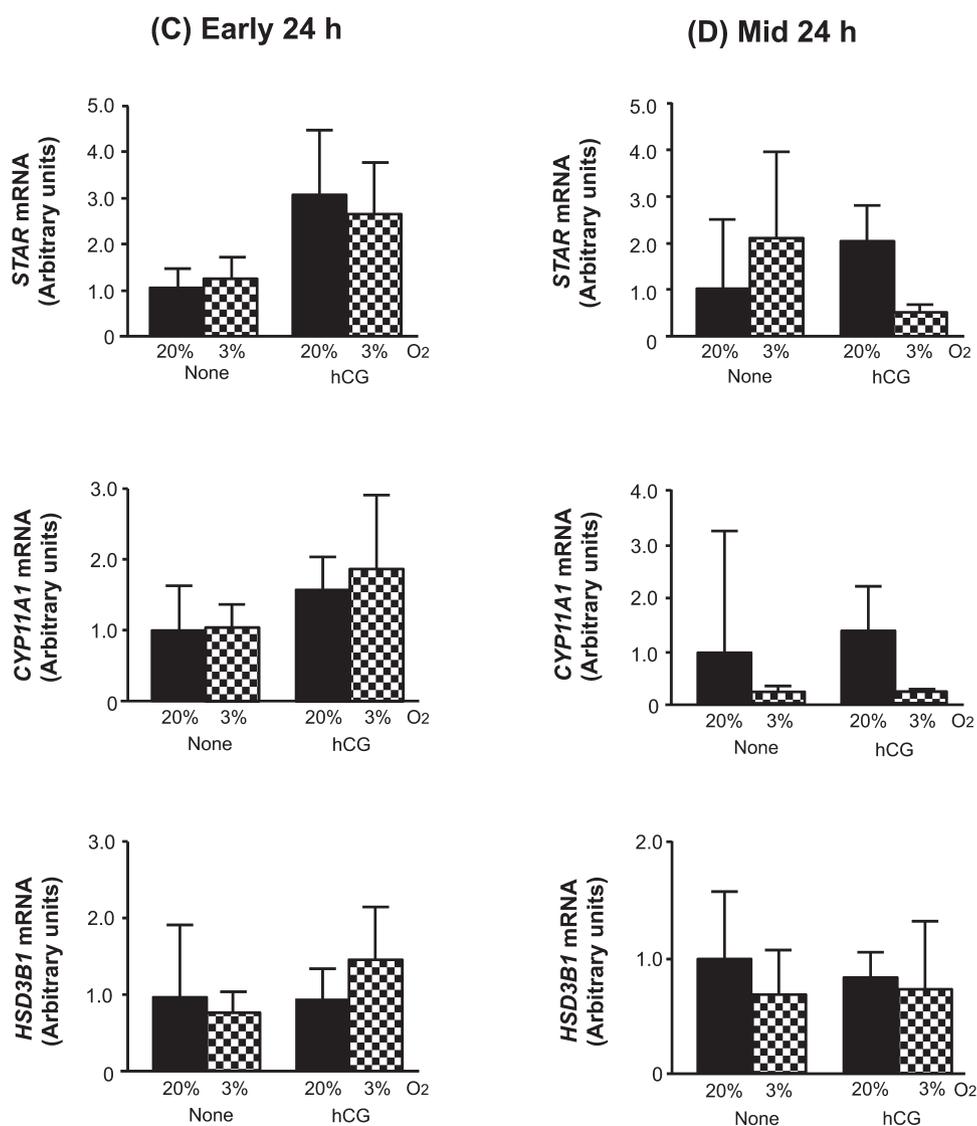


Fig. 2. continued.

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