

Biphasic Change in Correlation between Ovarian Lipid Peroxides and Progestational Activity during Pseudopregnancy Induced in Immature Rats

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ABSTRACT. We measured ovarian lipid peroxide (LP) levels and plasma progestins, progesterone (P_4) and 20α -dihydroprogesterone, throughout pseudopregnancy in gonadotropin-primed immature rats. Plasma P_4 fluctuated, with two peaks on days 5 (PSP5) and 8 of pseudopregnancy, and then declined to the basal level by PSP12. Ovarian LP increased from PSP1 to PSP4, decreased temporarily until PSP8, and then rose gradually until PSP14. From PSP1 through PSP7, ovarian LP was positively correlated with total progestins according to the Spearman ranked correlation coefficient ($r=+0.829$, $p<0.05$). In contrast, a negative correlation between ovarian LP and plasma P_4 was apparent ($r=-0.816$, $p<0.05$) from PSP8 to PSP14. These results show the biphasic correlation of LP with luteal progestational activity depending on the luteal stage.—**KEY WORDS:** lipid peroxide, progestin, pseudopregnancy, rat.

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Superoxide radicals (SOR) and other reactive oxygen species have been assumed to play pathological roles in inflammation, cancer and radiation damage. Recent evidence has demonstrated their physiological roles, for example in the regulation of ovarian corpus luteum (CL) function [2, 9].

SOR and related compounds can be generated by both luteal cells [23, 24] and white blood cells [2] in the CL. They preferentially attack unsaturated lipids in the plasma membrane and induce the formation of lipid peroxide (LP). On the other hand, they are scavenged to hydrogen peroxide by superoxide dismutase. The implication of SOR and related compounds in luteolysis was intensively studied in prostaglandin (PG) $F_{2\alpha}$ -induced luteolysis in rats [20–22]. The relationship between SOR and spontaneous luteal regression as well as luteal growth has been investigated only by Shimamura *et al.* who utilized adult pseudopregnant rats [26]. The findings on the nature of these reactive agents throughout the luteal phase (the formation, maintenance and spontaneous regression of CL) are still limited. In this study, we have utilized gonadotropin-primed immature pseudopregnant rats and measured both plasma progestins and ovarian levels of LP, one of the reaction products generated by SOR, throughout pseudopregnancy.

MATERIALS AND METHODS

Reagents: eCG and hCG were purchased from Shionogi (Osaka, Japan) and Sankyo (Tokyo, Japan), respectively. Sephadex LH-20, which was used for the chromatography of progestins, was purchased from Pharmacia (Uppsala, Sweden). Both $1,2,6,7\text{-}^3\text{H}$ -progesterone (P_4) (0.38 TBq/mmol) and $1,2\text{-}^3\text{H}$ - 20α -dihydroprogesterone (20α -OHP) (2.06 TBq/mmol) were purchased from Dupont-NEN (Boston, MA, U.S.A.).

Animal and tissue preparation: Immature female rats of the Wistar-Imamichi strain were used in this study. They were kept in an air- and light-conditioned room (temperature, $23 \pm 3^\circ\text{C}$; lights on, 0500–1900 hr). Superovulation and pseudopregnancy were induced by the earlier method [21, 22] with a little modification. Twenty six-day-old animals weighing 50–73 g were injected with eCG (50 IU, sc) at 1700 hr followed by hCG (50 IU, ip) 46 hours later. These animals ovulated 34.2 ± 9.0 (mean \pm SEM, $n=5$) ova the next morning. On vaginal smear examination, the rats exhibited proestrus on the 28th, estrus on the 29th day, and diestrus thereafter for 12.9 ± 0.3 ($n=24$) days. The first diestrous day was designated as day 1 of pseudopregnancy (PSP1) in this report. The rats were killed by decapitation on each day from PSP1 to PSP14 except for PSP2. Systemic blood plasma was kept at -20°C until the steroid assay. Ovaries were kept at -80°C until assay of the LP level.

Determination of plasma progestins and ovarian LP: Plasma P_4 and 20α -OHP were extracted with n-hexan and isolated by column chromatography. They were then determined in duplicate by radioimmunoassay [6, 14, 16]. The working range of assays was 12.5–1000 pg for both progestins.

LP levels in ovarian homogenates were measured by the thiobarbituric acid test [27]. Values were expressed as nmol of malondialdehyde (MDA) per ovary.

Statistical analyses: All data on progestins and LP were expressed as the mean \pm SEM ($n=6$). They were analysed by ANOVA and Student's *t*-test. Correlation coefficients and, in some cases, the Spearman ranked correlation coefficients of ovarian LP were evaluated with several parameters of luteal progestational activity (P_4 , 20α -OHP, sum of P_4 and 20α -OHP, or the ratio of 20α -OHP to P_4). Differences between experimental groups were considered

significant if $p < 0.05$.

RESULTS

Changes in plasma progestins: Figure 1 summarizes changes in plasma progestins throughout pseudopregnancy. Plasma P_4 in the present model fluctuated with two peaks (Fig. 1-A). It rose from PSP1 and reached the first peak level on PSP4 ($p < 0.01$). It then fell transiently (from PSP5 to PSP7) and reached the second peak on PSP8. Circulating P_4 declined to the basal level on PSP12. The inactive metabolite of P_4 , 20α -OHP, increased from PSP1 and reached a peak level on PSP4 ($p < 0.05$), in the same way as P_4 (Fig. 1-B). Then the steroid fell gradually to around 200 ng/ml and had slightly increased on PSP12 and PSP14. In general, total progestins had a similar pattern to that of P_4 (Fig. 1-C). The ratio of 20α -OHP to P_4 was relatively high on PSP1 and PSP3 and was depressed to a low level from PSP4 to PSP9 (Fig. 1-D). It began to rise on PSP10 and showed a remarkable increase on PSP12 ($p < 0.05$), which was maintained until PSP14. These data collectively indicate that, in the present pseudopregnant model, functional luteolysis occurred on PSP12. This was due to both the reduced steroidogenesis and the activated conversion of P_4 to 20α -OHP by 20α -hydroxysteroid

dehydrogenase (20α HSD) [5, 28].

Changes in LP and correlation coefficients with the progestational index: The alteration in ovarian LP during pseudopregnancy is shown in Fig. 2. Ovarian LP rose from PSP1 and reached its peak level on PSP4 ($p < 0.05$). It then decreased until PSP8, marking the lowest level throughout pseudopregnancy. Thereafter it increased gradually toward the end of pseudopregnancy and then on PSP14 reached a level comparable to that on PSP4.

Coefficients of correlation between ovarian LP and several progestational indices are summarized in Table 1. In the first half of pseudopregnancy, LP was positively correlated with P_4 , 20α -OHP, or total progestins, generating a significant correlation with total progestins in terms of the Spearman ranked correlation coefficient ($r = +0.829$, $p < 0.05$). In contrast, the LP level was negatively correlated with the progestin in the second half, generating a significant correlation with P_4 ($r = -0.816$, $p < 0.05$). It also exhibited a significantly positive correlation with the ratio of 20α -OHP to P_4 , a rough indicator of 20α HSD activity ($r = +0.857$, $p < 0.02$).

DISCUSSION

This study showed that ovarian LP levels rose transiently

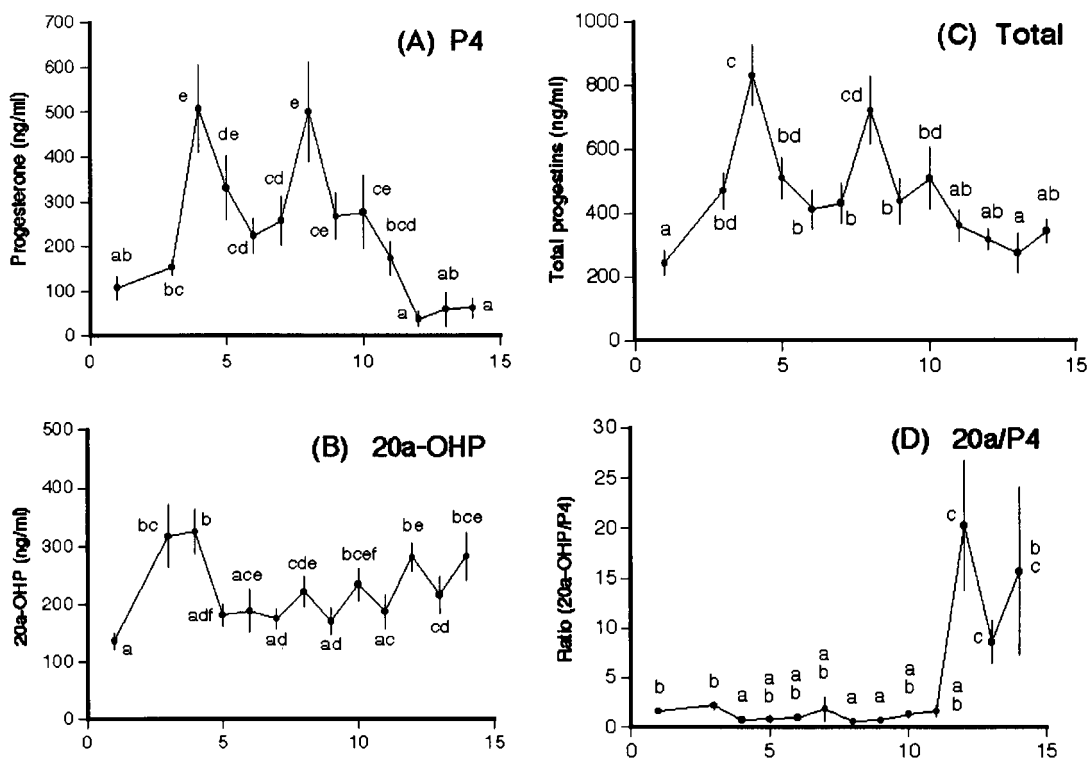


Fig. 1. Plasma progestin levels during pseudopregnancy induced in immature rats. (A) P_4 , (B) 20α -OHP, (C) Total ($P_4 + 20\alpha$ -OHP), (D) Ratio (20α -OHP/ P_4). The abscissa shows the day of pseudopregnancy. Plasma P_4 and 20α -OHP were extracted, separated, and measured by radioimmunoassay with specific antisera. Data are expressed as the mean \pm SEM ($n=6$). Values with different alphabetical letters within each figure mean significant differences from each other ($p < 0.05$).

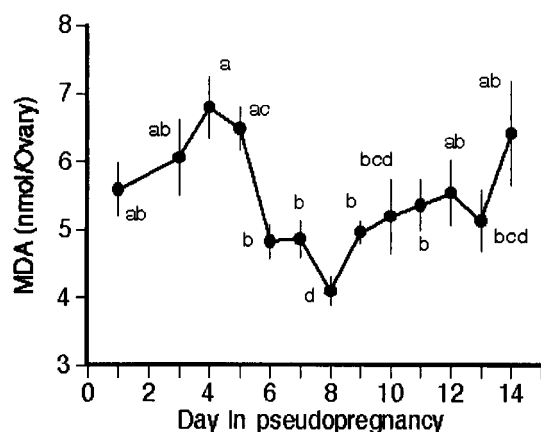


Fig. 2. Changes in ovarian lipid peroxide level throughout pseudopregnancy. Lipid peroxide levels were expressed as malonaldehyde content measured by the thiobarbituric acid test. Data are expressed as the mean \pm SEM (n=6). Values with different alphabetical letters mean significant differences from each other ($p < 0.05$).

during the early stage of pseudopregnancy and then gradually toward the end of pseudopregnancy in gonadotropin-primed immature rats. We found a biphasic change in the correlation between LP and luteal progesterone activity depending on the luteal stage.

In the early stage of pseudopregnancy, ovarian LP was at rather high levels and had reached a peak on PSP4. It changed similarly to that of plasma P_4 , 20α -OHP or total progestins, generating a significantly positive correlation with total progestins. This result suggests that a common regulatory mechanism exist for LP production and luteal steroidogenesis. A possible candidate is hCG/LH, which is a luteotropin in rats. Direct evidence is available that LH stimulates luteal SOR generation in a dose-dependent manner *in vitro* [24].

In addition, it should be noted that luteal LP in adult pseudopregnant rats had a somewhat different pattern, with the lowest level on day 3 of pseudopregnancy [26]. We suppose that the difference between that study and our present one may be due to the method used to induce pseudopregnancy.

The animals in our study were injected with rather high doses (50IU) of both eCG and hCG, thus exhibiting about three-fold increases in ovulation rates and plasma steroid

levels compared to those in immature pseudopregnant rats used elsewhere [12, 13, 15, 16]. eCG would be the determinant for the numbers of ovulated ova and CLs obtained. On the other hand, hCG with a relatively prolonged half-life (8–12 hr) might have not only caused follicular luteinization but also enhanced both luteal progestins synthesis and LP production. The mechanism by which LH provokes SOR production is unclear, but one possible explanation is its reducing effect on an antioxidant ascorbic acid in the ovary [18]. Reduced levels of this antioxidant may help to enhance tissue generation of SOR and LP.

During the late pseudopregnancy, ovarian LP levels increased gradually as plasma P_4 decreased. We found that ovarian LP was negatively associated with P_4 , 20α -OHP or total progestins. Also of interest is a significantly positive correlation between LP and the ratio of 20α -OHP to P_4 . An increase in tissue LP was also observed during the natural luteolysis in adult rats [26] and $PGF_{2\alpha}$ -induced luteolysis in immature rats [22]. $PGF_{2\alpha}$ -induced and spontaneous luteolysis involves increased phospholipase A_2 (PLA_2) activity [20, 29]. We identified 85 kDa cytosolic PLA_2 (c PLA_2) as the luteal primary isozyme and determined its increasing activity during functional luteolysis in pseudopregnant rats [10, 13]. The generation of hydrogen peroxide and LP caused by $PGF_{2\alpha}$ results in the stimulation of PG synthesis through the activation of PLA_2 [19, 25] and cyclooxygenase [3, 8]. The autonomous production of $PGF_{2\alpha}$ and SOR and related agents driven by a positive-feedback loop therefore probably contribute to LP generation in the regressing CL. This mechanism would not operate in the early luteal phase, because c PLA_2 and $PGF_{2\alpha}$ receptor appear to be only modestly expressed in the newly formed CL [7, 11, 13].

Whether SOR and related agents affect luteal P_4 secretion is difficult to determine. LP is known to have inhibitory effects on cellular function, since LP, synthesized preferentially at the plasma membrane, decreases membrane fluidity by disrupting the structural integrity and changing ion permeability [4]. There is also evidence that hydrogen peroxide inhibits luteal steroidogenesis *in vitro* [1, 17]. Sawada and Carlson have shown that SOR is rather luteotropic at low doses and exerts a luteolytic effect over a substantial level [24], but we found high levels of ovarian LP on PSP4 (the functional phase) as well as after PSP12 (the regressed phase). It is unlikely that the SOR effect on

Table 1. Correlation of lipid peroxide with parameters of progestational activity

parameters	PSP1-7 correlation coefficient (SRCC)	PSP8-14 correlation coefficient (SRCC)
MDA- P_4	+0.572	- 0.816*
- 20α -OHP	+0.588	- 0.632
-Total	+0.663 (+0.829*)	- 0.726
-Ratio	-0.367	+ 0.675 (+0.857**)

SRCC: Spearman rank correlation coefficient

Numbers were 6 and 7 for PSP1-7 and PSP8-14, respectively. *, $p < 0.05$; **, $p < 0.02$

luteal function is dose-dependent. The reason why luteal steroidogenesis was increased in the early luteal phase in spite of the substantial amount of LP is not yet clear. The unknown mechanism which protects CL from the inhibitory effects of LP may be suggested. On the other hand, there is a positive correlation between ovarian LP and the indicator of 20 α HSD activity. Whether SOR and related compounds affect this enzyme also requires further study.

In conclusion, this study demonstrates that ovarian LP levels are positively correlated with CL activity early in pseudopregnancy and also supports the finding that LP accumulates late in pseudopregnancy in gonadotropin-primed immature rats. Furthermore, it suggests that the presence of LP does not always inhibit luteal steroidogenesis.

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