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Luteolysis and Thrombus Formation in Ovaries of Immature Superstimulated Golden Hamsters

K. Spanel-Borowski[A] *and Ch. Heiss*

Abteilung für Klinische Morphologie der Universität Ulm, Postfach 4066, D-7900 Ulm, Federal Republic of Germany.

[A] Present address: Institut für Anatomie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-2400 Lübeck 1, Federal Republic of Germany.

Abstract

Thrombus appearance during luteolysis with and without exogenous prostaglandin ($PGF_{2\alpha}$) was studied in immature golden hamsters between days 4 and 7 after stimulation with pregnant mares' serum gonadotrophin (PMSG) followed by human chorionic gonadotrophin. Both ovaries were weighed and cut in series for light-microscopic evaluation. The fibrinolytic activity was determined by the fibrin slide method after treatment with $PGF_{2\alpha}$ on day 4 after PMSG stimulation and compared with controls of days 3 and 4 after PMSG stimulation.

There was a marked decrease in ovarian weights in the experimental and the control group between days 4 and 7 after PMSG. Few necrotic cells were seen in corpora lutea on day 5, but many on day 6. All of them had disappeared on day 7. The number of ovaries with thrombi was 80–100% in both groups on day 4 and declined to approximately zero levels on day 7. The amount of thrombus formation appeared to be higher in the $PGF_{2\alpha}$-treated groups than in controls. Fibrinolytic activity was high in controls on day 3 and low in controls and in $PGF_{2\alpha}$-treated animals on day 4 after PMSG. It is concluded that (i) thrombus formation occurs in superstimulated ovaries during luteolysis; and (ii) thrombus formation is related to a decrease in fibrinolytic activity.

Introduction

While prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is unanimously accepted as a luteolytic substance in many mammals, the luteolytic mechanism of $PGF_{2\alpha}$ is still under investigation. One group of authors claims that $PGF_{2\alpha}$ causes vasoconstriction of ovarian vessels, thus affecting luteal cells indirectly via a reduced blood flow (Gutknecht *et al.* 1970; Pharriss *et al.* 1970; Nett *et al.* 1976). Another group favours the idea that $PGF_{2\alpha}$ induces biochemical alterations directly at the luteal cell level (Behrman *et al.* 1971; Baird 1974; Janson 1975; Hossain *et al.* 1979). The morphological course of luteolysis is attributed to auto- and heterophagocytosis in sheep and guinea pigs (Bjersing *et al.* 1970; McClellan *et al.* 1977; Paavola 1977, 1979). No observations have been available to date which describe the macro- and microscopic sequence of luteolysis in immature superstimulated golden hamsters. It is the aim of this study to investigate the effect of luteolysis on the morphology of corpora lutea in immature superstimulated golden hamsters and to gain further insight into the luteolytic role of $PGF_{2\alpha}$. An unexpected finding in the beginning of the study was the recurring appearance of ovarian thrombi, and this led to a thorough examination of this coagulation phenomenon during normal or $PGF_{2\alpha}$-induced luteolysis. Histological observations were supplemented by determinations of the ovarian fibrinolytic activity (FA) using the fibrin slide technique.

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Material and Methods

Experimental Methods

Up to five female golden hamsters (strains C-lac and Bom) were laboratory-reared in transparent plastic cages and weaned at the age of 21 days. Dried food and water were freely accessible. Lighting and temperature were automatically controlled (14 h of light, 10 h of dark; 21 ± 2°C). Experimental protocol for the induction of superovulation by PMSG (pregnant mares' serum gonadotrophin, Sigma) followed by hCG (human chorionic gonadotrophin, Primogonyl, Schering) and for the $PGF_{2\alpha}$-treatment is given in Fig. 1.

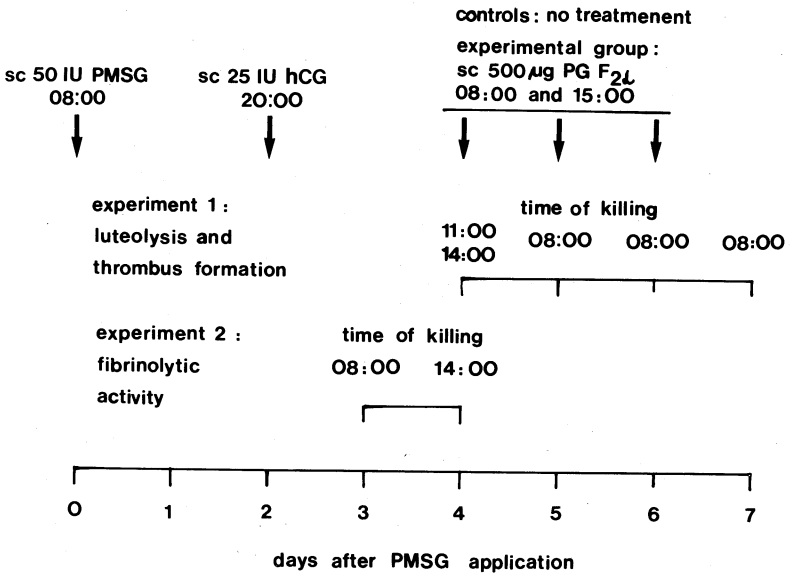


Fig. 1. Scheme for the conduction of experiments 1 and 2 in 28–32-day-old golden hamsters (body weight 50–80 g). The animals were superovulated with PMSG and hCG.

Experiment 1 was conducted between days 4 and 7 after PMSG stimulation to study luteolysis and thrombus formation. From five to eight animals were available per each time of killing in the control and in the experimental group (see Tables 1 and 2). Body weights and weights of both ovaries – which were dissected free of the Fallopian tube under a stereomicroscope – were recorded for calculation of the relative ovarian weights (mg ovary/g body weight). A few ovaries were discarded because they were cystic. The ovaries were fixed in Bouin's solution and dehydrated in alcohol. They were embedded in paraffin wax, completely cut in series (each section 7 µm thick) and stained with hematoxylin and eosin. Using a light microscope, alterations of the corpora lutea as well as the appearance of thrombi were examined.

The extent of thrombus formation was classified as +, ++ and +++ according to whether up to 15 sections, 16–45 sections, and more than 45 sections were thrombus-positive, respectively. In this semiquantitative method it was not considered whether one large thrombus or numerous smaller ones were found per one section. In accordance with Florey (1970), a thrombus some hours old was regarded as young as long as a fibrin clot with white blood cells was seen. A thrombus more than 24 h old and showing the ingrowth of fibroblasts into the fibrin clot was defined as an organized thrombus. The amount of thrombus formation and the age of the thrombi were noted for both ovaries separately. If the right and the left ovary showed different amounts of thrombus formation, it was decided in favour of the higher amount.

Determination of Fibrinolytic Activity

A preliminary experiment had shown a low FA between days 4 and 7 after PMSG stimulation. This negative outcome did not explain the thrombus formation and the FA study was started on

day 3 after PMSG stimulation, the period of the developing corpus luteum. As $PGF_{2\alpha}$ is known to affect only the developed corpus luteum, no $PGF_{2\alpha}$-treatment was given to five animals on day 3 after PMSG stimulation (expt 2). $PGF_{2\alpha}$ was, however, applied to five animals on day 4 after PMSG, while another five animals served as controls. Both ovaries of each animal were immediately removed, quickly deep-frozen in liquid nitrogen, and used to determine FA using the fibrin slide method developed by Todd (1959) and modified by Pandolfi (1972) and Pandolfi *et al.* (1972). The following materials were required: (1) clean, chrome-alum-gelatine-coated slides to secure attachment of the fibrin film; (2) 2% (w/w) human fibrinogen solution rich in plasminogen (Cat. No. OBKR 10, Behring Werke); and (3) thrombin (Topostasin, Cat. No. T 133, Hoffmann La Roche) dissolved in 0·7 M sodium phosphate buffer, pH 7·4, to a concentration of 25 i.u./ml.

The procedure was as follows: cryostat sections were prepared in such a way that section 1 was mounted on slide A; section 2 on slide B and section 3 on slide C (one section set), section 4 again on slide A and so forth up to section 9. Then a new series of slides was started in a similar fashion. Fibrinogen solution (0·1 ml) was then dropped on each slide on which the cryostat sections had been previously mounted and spread over an area of 4 by 2·5 cm with the edge of another slide. The slide with the fibrinogen solution was slightly tilted to allow liquid to accumulate in one corner. 0·1 ml thrombin solution was added to this liquid mass, then uniformly distributed by quick rotating movements of the slide. The fibrin film was allowed to stabilize at an even level for 10 min and incubated in a moist chamber at 37°C. Slides marked A were incubated 1 h, those marked B for 2 h, and those marked C for 3 h. After incubation, the sections were fixed with 3·5% (v/v) formaldehyde, pH 7·0, for 1 h, stained with Meyer's hematoxylin for 1 h, washed for 30 min in tap water and counterstained with eosin for 15 min. Fibrinolytic areas were observed as unstained areas in the stained fibrin film. The specifity of the fibrinolytic reaction was ascertained in a preliminary test using heat-inactivated fibrin films. Slides were coated with fibrin and incubated in a moist chamber at 80°C for 1 h before cryostat sections were mounted.

The FA was classified into four grades as indicated in the legend of Fig. 11: the fibrinolytic grades determined in every section set (slide A in comparison with slides B and C) were evaluated for both ovaries separately. The percentage of the different fibrinolytic grades was calculated and defined as a fibrinolytic index.

Statistical Analysis

Data of ovarian weights, found in experiment 1, were subjected to linear regression and correlation analysis. In addition, the amount of thrombus size was assessed by the χ^2 test. The values of FA, as seen in experiment 2, were assessed by the U-test of Mann and Whitney.

Table 1. Decrease in absolute ovarian weights in superstimulated immature golden hamsters with days after PMSG stimulation and on treatment with or without $PGF_{2\alpha}$

There were five animals in each treatment group

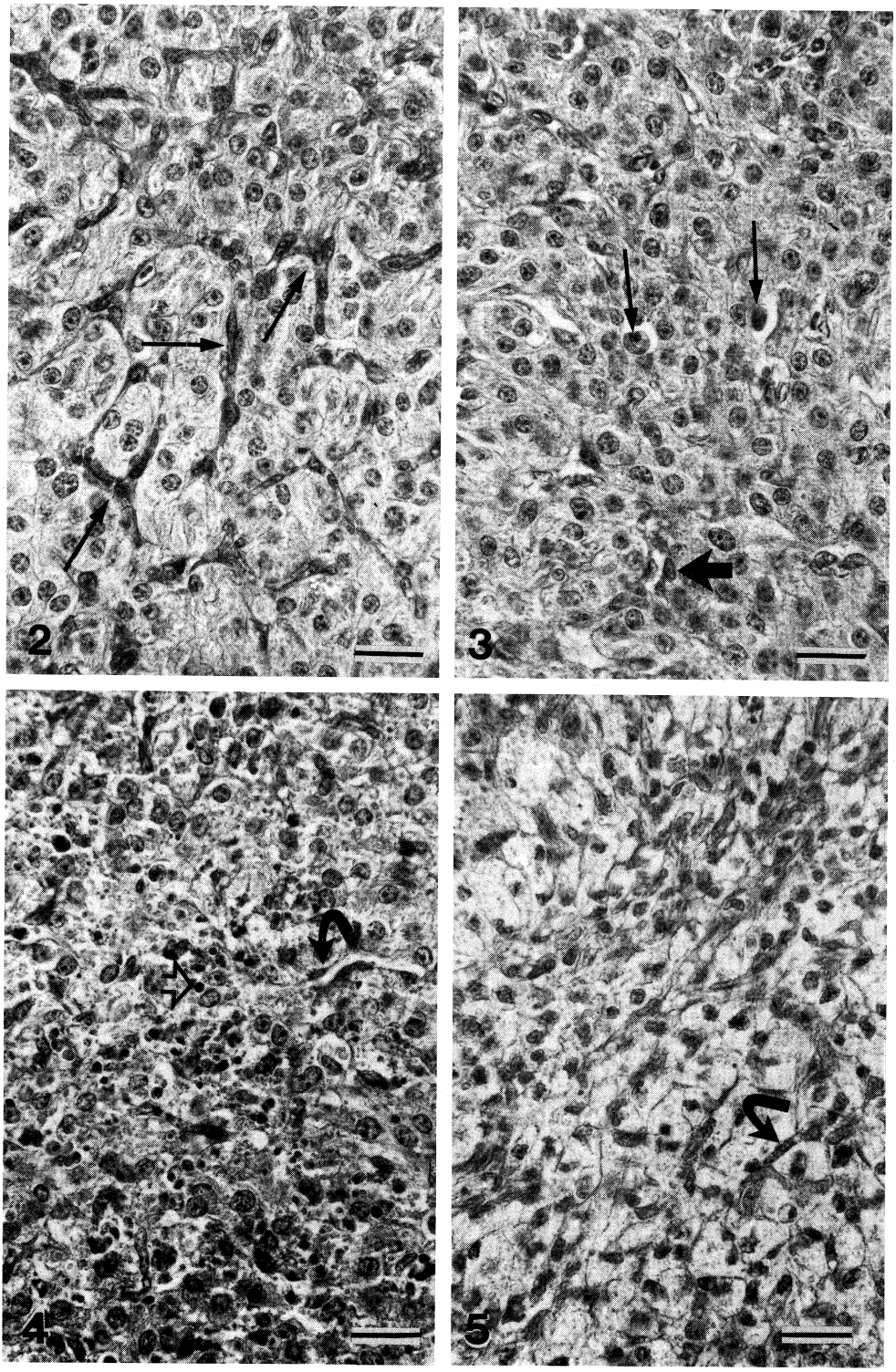
Days after PMSG stimulation	Mean ovarian weight (mg) ± s.d. With $PGF_{2\alpha}$	Without $PGF_{2\alpha}$
4[A]	124·9±14·3	155·7±30·5
5	82·1±11·2	101·4±13·8
6	47·9±4·0[B]	57·3±3·5[B]
7	30·2±2·2[B]	31·5±1·0[B]

[A] Time of killing was at 1100 h (cf. Fig. 1).
[B] $P < 0·001$ versus day 4 after PMSG.

Results

Experiment 1: Luteolysis and Thrombus Formation

The absolute ovarian weights decreased markedly in the $PGF_{2\alpha}$-treated group and in the control group between days 4 and 7 after PMSG stimulation (Table 1).



Figs 2–5. Luteolysis in immature golden hamsters after superstimulation with PMSG and hCG. No differences appear between the $PGF_{2\alpha}$ group and the control group. For details, see legends on facing page.

There were no statistically significant differences between values in both groups but there was a negative correlation between the absolute ovarian weights and the number of days after PMSG stimulation in each group. If relative ovarian weights for assessing this correlation were calculated, the regression equations were comparable: the $PGF_{2\alpha}$-treated group: $y = 4{\cdot}04 - 0{\cdot}53x$; $r = -0{\cdot}94$; the controls: $y = 3{\cdot}83 - 0{\cdot}50x$; $r = -0{\cdot}90$. In both groups, y was the relative ovarian weight and x the day after PMSG. The histology of corpora lutea was the same in the experimental and the control groups (see legends of Figs 2–5).

Table 2. Number of ovaries with thrombus appearance in immature golden hamsters between days 4 and 7 after PMSG stimulation

500 μg $PGF_{2\alpha}$ were given subcutaneously twice daily between days 4 and 6 after PMSG stimulation. Effects after first injection of $PGF_{2\alpha}$ and without $PGF_{2\alpha}$ are shown

Thrombus size[A]	Time after PMSG stimulation: 4 days, 3 h	4 days, 6 h	5 days	6 days	7 days	4–7 days[B]
			With $PGF_{2\alpha}$			
0	0	1	1	3	4	9
+	0	2	0	1	1	4
++	2	2	2	1	0	7
+++	4	3	3	1	0	11
			Without $PGF_{2\alpha}$			
0	0	2	2	4	5	13
+	3	1	3	1	0	8
++	2	3	2	0	0	7
+++	0	1	1	0	0	2

[A] Semiquantitative evaluation of the amount of thrombus formation in a completely cut ovary: 0, none; +, in 15 sections; ++, in 45 sections; +++, in more than 45 sections. Both ovaries of each animals are compared and that one with the higher number of thrombus-positive sections is recorded.

[B] $P < 0{\cdot}05$: $PGF_{2\alpha}$-treated groups versus the untreated one.

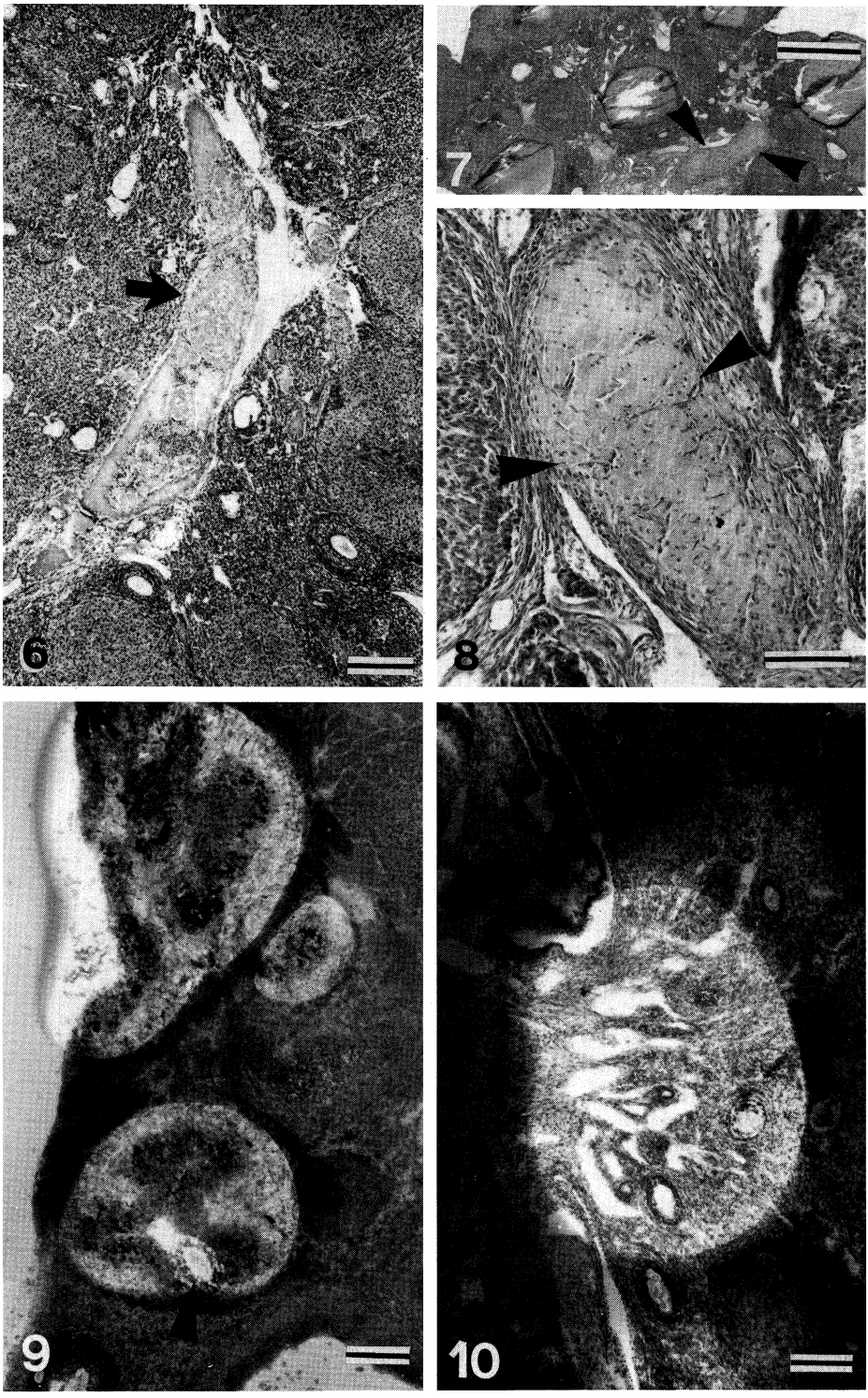
The thrombi appeared before regressive changes took place in the corpora lutea both in $PGF_{2\alpha}$-treated and control animals. Thrombi always developed in veins of the ovarian hilus and the medulla from where they reached into cortical regions (Figs 6–8). Thrombi were fresh on day 4 after PMSG stimulation whereas they were organized on subsequent days. Most of both animal groups showed ovaries with thrombi on days 4 and 5 after PMSG stimulation (Table 2). The number of

Fig. 2. Day 4 after PMSG stimulation. Luteal cells are rich in cytoplasm. They form small groups which are separated by spindle-shaped endothelial cells (arrows). Scale 20 μm.

Fig. 3. Day 5 after PMSG stimulation. Luteal cells have decreased in size and are no longer arranged in small groups. Endothelial cells (large arrow) are reduced in numbers. Scattered necrotic cells (arrows) appear. Scale 20 μm.

Fig. 4. Day 6 after PMSG stimulation. A marked increase in necrotic cells is apparent (open arrow). Luteal cells have little cytoplasm and irregularly shaped, hyperchromatic nuclei. Some capillaries are maintained (solid arrow). The reduction of the capillary bed is evident. Scale 20 μm.

Fig. 5. Day 7 after PMSG stimulation. All necrotic cells have disappeared. Luteal cells show signs of fatty degeneration. Some structures remind one of endothelial cells (arrow). Scale 20 μm.



Figs 6–10. Thrombus formation and localization of fibrinolytic activity in ovaries of immature golden hamsters after superstimulation with PMSG and hCG. No differences are seen with and without treatment of $PGF_{2\alpha}$. For details, see legends on facing page.

ovaries with thrombi seemed to be slightly higher on day 6 after PMSG in the $PGF_{2\alpha}$-treated group than in controls. Ovaries with thrombi were almost absent for both groups on day 7 after PMSG stimulation. The amount of thrombus formation—graded per number of thrombus-positive sections of each ovary—appeared to be higher in the $PGF_{2\alpha}$ group compared with the controls ($P < 0{\cdot}05$) when the amount of thrombus formation was calculated for the whole period of investigation.

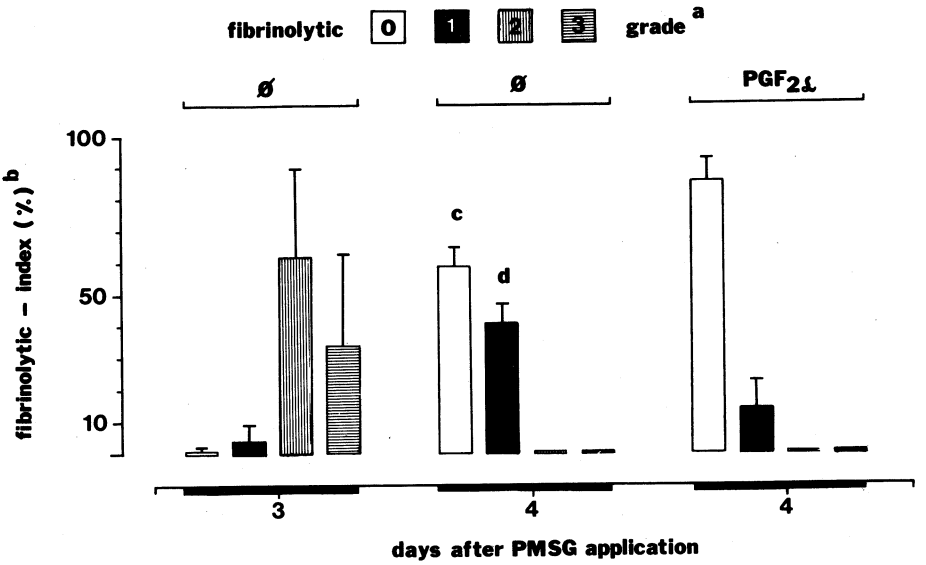


Fig. 11. Fibrinolytic activity in superstimulated ovaries of immature golden hamsters, 6 h after subcutaneous injection of 500 μg $PGF_{2\alpha}$ on day 4 after PMSG stimulation and compared with $PGF_{2\alpha}$-untreated controls from days 3 or 4 after PMSG stimulation. [a], Fibrinolytic grade in one section set: 0, no lytic areas 3 h after incubation; 1, lytic areas <2 mm in diameter 3 h after incubation; 2, lytic areas <2 mm in diameter 2 h after incubation and large confluent areas covering two-thirds of the section 3 h after incubation; 3, lytic areas <2 mm in diameter 1 h after incubation and large confluent areas covering two-thirds of the section 2 h after incubation. [b], Percentage of section sets from a completely cut ovary with different fibrinolytic grades. Data (means ± s.d.) are derived from both ovaries. [c], $P < 0{\cdot}001$ versus fibrinolytic grade 0 of days 3 and 4 after PMSG stimulation. [d], $P < 0{\cdot}001$ versus fibrinolytic grade 1 of days 3 and 4 after PMSG stimulation.

Experiment 2: Fibrinolytic Activity

FA (Figs 9 and 10) was demonstrated in unruptured and ruptured follicles, some of which were in an early stage of corpus luteum formation. This follicular

Fig. 6. Day 4 after PMSG stimulation. A large fresh venous thrombus is shown in the ovarian medulla (arrow). Many intact corpora lutea have developed. Scale 200 μm.

Fig. 7. Day 5 after PMSG stimulation. A large organized thrombus appears in the medulla (arrowheads). The cortex demonstrates many corpora lutea and some cystic follicles. Scale 1000 μm.

Fig. 8. Similar to Fig. 6. The ingrowth of granulation tissue into a thrombus is shown (arrowheads). Scale 100 μm.

Fig. 9. Day 3 after PMSG stimulation. Fibrinolytic activity appears in a ruptured follicle transforming into a corpus luteum as well as in an unruptured and luteinized follicle. The retained oocyte is marked (arrowhead). Fibrin slide method. 2 h (!) incubation. Scale 200 μm.

Fig. 10. Day 4 after PMSG stimulation. Fibrinolytic activity is confined to vessels of the ovarian medulla. Fibrin slide method. 3 h (!) incubation. Scale 200 μm.

FA appeared 2 h after incubation and only on day 3 after PMSG stimulation (Fig. 9). Some fibrinolytic zones were seen in the area of the surface epithelium with or without growing follicles or corpora lutea in its vicinity. FA was observed in arterial, venous or lymphatic vessels of the ovarian hilus and medulla. The FA in the surface epithelium as well as in the vessels developed 3 h after incubation and only on day 4 after PMSG. Thrombi occurred on day 4 after PMSG, not on day 3 after PMSG.

The extent of FA was high in control animals on day 3 after PMSG (Fig. 11). FA was found in almost every set of serial sections (approximately 100%), though the degree of FA changed between grades 2 and 3 from one set of sections to the other. Already in control animals, there was a distinct decline in FA on day 4 after PMSG in contrast to that on day 3 after PMSG. In the former group (day 4 after PMSG), roughly 50% of the serial section sets showed FA of the low fibrinolytic grade 1. The decline of FA was enhanced after treatment with $PGF_{2\alpha}$. Thrombi in the $PGF_{2\alpha}$-treated and control animals on day 4 after PMSG were more likely to appear in FA-free areas.

Discussion

There was a marked decrease in ovarian weights between days 4 and 7 after PMSG stimulation in immature superovulated golden hamsters which has not been observed in superstimulated rats (Horikoshi and Wiest 1971). Using the light microscope, this decrease is supported by the large-scale appearance of necrotic cells in corpora lutea on day 6 after PMSG and the complete disappearance of these cells on day 7 after PMSG stimulation. It is possible that luteal and endothelial cells contribute to such cell necrosis as has been confirmed morphometrically in sheep (Nett *et al.* 1976), but not in guinea pigs in which cell necrosis is thought to be confined to endothelial cells (Hossain and O'Shea 1981). It cannot be explained satisfactorily at the moment how necrotic cells are removed within 24 h in corpora lutea of superstimulated golden hamsters. One assumes for luteolysis in guinea pigs that endothelial cells are intravascularly discharged and subsequently dissolved (Azmi and O'Shea 1984). A similar way of removal may exist in white-footed mice where luteal cell complexes are found to protrude into the vascular system (Spanel-Borowski *et al.* 1983).

The new finding in our study concerns the thrombus formation which occurs frequently on day 4 after PMSG stimulation independent of a treatment with or without $PGF_{2\alpha}$. Part of this problem is that in our animal model spontaneous regression seems to begin soon after the corpora lutea become established. One could therefore argue that the processes induced by exogenous $PGF_{2\alpha}$ do not differ in principle from those induced by endogenous $PGF_{2\alpha}$. There is, however, one 'minor' argument against this assumption. The amount of thrombus formation has been higher in the experimental than in the control group, not for each day after PMSG stimulation, but for days 4–7 after PMSG.

Thrombus formation cannot be considered as a terminal event since organized thrombi were seen on days 5 and 6 after PMSG stimulation. Furthermore, the occurrence of thrombi in the experimental as well as the control group excludes a shock-like process induced by $PGF_{2\alpha}$. One may suggest that thrombus formation contributes to the onset of luteolysis by a temporary ischemia. No permanent damage is caused by these venous thrombi as the ovarian medulla is rich in vascular

anastomoses (Ellinwood *et al.* 1978). Most of the fresh thrombi will impede blood flow for only a short time. Either the thrombi retract for the blood to pass by, are reorganized and new channels formed (Florey 1970), or the thrombi are dissolved by localized FA. The FA stimulation by thrombotic components has been recently demonstrated in endothelial cells (Loskutoff *et al.* 1982).

Thrombus formation is correlated with a marked loss in FA between days 3 and 4 after PMSG stimulation. On the day after ovulation (day 3 after PMSG), FA was high and localized in antral follicles and in developing corpora lutea. This certainly reflects FA by the presence of plasminogen activator in unruptured and ruptured Graafian follicles (Beers *et al.* 1975). Yet on day 4 after PMSG stimulation FA was low, and only apparent in the area of the surface epithelium and in vessels of the medullary region. Probably, this localization of FA has been present on day 3 after PMSG. It escaped detection, however, since on day 3 after PMSG stimulation large confluent fibrinolytic areas covered the whole section. An imbalance between the fibrinolytic and the coagulative process in favour of the latter will have caused thrombus formation. Whether the FA has been more suppressed in the experimental group than in controls, cannot be stated with certainty as the fibrin slide method does not allow a true quantitative evaluation. In this context, numerous aggregated platelets are seen in pregnant hamsters during $PGF_{2\alpha}$-induced luteolysis (Bagwell *et al.* 1976).

Thrombus formation during luteolysis does not resolve the controversy whether vascular constriction (Gutknecht *et al.* 1970; Pharriss *et al.* 1970; Nett *et al.* 1976) or functional changes in luteal cells (Behrman *et al.* 1971; Baird 1974; Janson 1975; and Hossain *et al.* 1979) appear first during luteolysis. In addition, the relevance of our findings to naturally occurring luteolysis in adult hamsters cannot be firmly established because immature ovaries have been treated by a high dose of gonadotrophins and a high multiple does rate of exogenous $PGF_{2\alpha}$. Further experiments with adult animals are necessary to investigate the applicability of our observations.

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