EFFECT OF OVARIAN STEROIDS DURING EARLY AND MID PREGNANCY ON UTERINE PROTEIN METABOLISM IN MALNOURISHED RATS

Yoshiaki NIYAMA, Kazumi SAKAI, Kyoichi KISHI, and Goro INOUE

Department of Nutrition, School of Medicine, Tokushima University, Tokushima, Japan

Summary The effect of ovarian steroids on the maintenance of pregnancy in rats on an isoleucine-free diet was investigated. A combination of 0.5 μ g of estrone and 4 mg of progesterone or one of these steroids only was injected into the rats for various periods until mid pregnancy. Administration of estrone with progesterone from day 3 to 13 prevented fetal loss completely, and even when injected only from day 9 to 13 about 80% of the rats maintained pregnancy. Pregnant rats fed an inadequate diet without steroids failed to maintain pregnancy. Pregnant rats receiving deficient diets with or without steroids were sacrificed at intervals during early and mid pregnancy and the nucleic acid and protein contents of the uterus and incorporation of 14 C-leucine into uterine proteins were examined. On day 7 of pregnancy, growth of conception products, total RNA, DNA, and protein in the uterus were significantly less in rats that did not receive steroids than in those that did. Incorporation of 14 C-leucine into uterine proteins was the same in both groups until day 11, but on day 14 the incorporation rate rose in the group in which pregnancy was maintained and fell in the group in which it failed. These results suggest that ovarian steroids, particularly progesterone, may act on the uterus to inhibit protein degradation, at least during mid pregnancy. The total amount of estrogen receptor proteins in the uterus was maintained until quite a late stage of pregnancy in rats on an inadequate diet.

Feeding rats a protein-free diet, or a diet deficient in an essential amino acid, from day 1 of pregnancy has been shown to result in fetal wastage (NELSON and EVANS, 1953; NIYAMA *et al.*, 1970). Abortion resulting from a deficient diet can, however, be prevented by injection of estrone with progesterone (NELSON

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and EVANS, 1954; HAZELWOOD and NELSON, 1965; NIYAMA *et al.*, 1970). Thus, one reason for abortion in such rats may be lack of ovarian steroids, due to decreased production or release of pituitary gonadotropin or luteotropin in animals on a deficient diet (SCOTT, 1956; HAYS and KENDALL, 1961; MADHWA RAJ and MOUDGAL, 1970; CALLARD and LEATHEM, 1970). The effect of ovarian steroids on maintenance of pregnancy in underfed rats has been extensively investigated, but little is known of their effect on uterine protein metabolism in these rats. Accordingly, the present study was made to determine (1) the necessity and effective period of steroid administration for successful maintenance of pregnancy in rats fed an isoleucine-free diet, (2) the effects of exogenous hormones on the protein and nucleic acid content of the uterus and the rate of protein synthesis in the uterus during early and mid pregnancy, and (3) the influence of inadequate nutrition on the ability of the uterus to concentrate estrogen into uterine cytosol, measured as the estrogen receptor protein.

MATERIALS AND METHODS

Virgin Sprague-Dawley rats, weighing 175 to 200 g each, were mated. To determine day 1 of pregnancy, vaginal smears were examined daily. Pregnant rats were fed *ad libitum* on an isoleucine-free diet from day 1 of pregnancy, as described previously (NIYAMA *et al.*, 1970). The composition of the L-amino acid mixtures and diet used was based upon those of RANHOTRA and JOHNSON (1965). As an inadequate diet, a diet deficient in isoleucine was used instead of a protein-free diet, because when the exogenous ovarian steroids were not provided, this diet induced complete fetal resorption, whereas with a protein-free diet 10 to 20% of the pregnant rats were able to maintain pregnancy (BERG *et al.*, 1967; KINZEY *et al.*, 1970). Four experiments were carried out, as follows.

Experiment 1. To determine the necessity of ovarian steroids for maintenance of pregnancy, rats were injected daily with 0.5 μ g of estrone and/or 4 mg of progesterone, from day 3 to day 20. On day 21 animals were killed and the maintenance of pregnancy was assessed from the number of viable fetuses.

Experiment 2. To examine the effective time of steroid administration for maintenance, 0.5 μ g of estrone and 4 mg of progesterone were injected daily for various periods, as indicated in Table 2. The placenta has endocrine functions and secretes gonadotropin, so exogenous ovarian steroids were not necessary for maintenance of pregnancy in rats on a deficient diet after day 12 of pregnancy (FISHER and LEATHEM, 1965; CALLARD and LEATHEM, 1970). Therefore, the effective time of steroid administration in early and mid pregnancy was examined. Rats were autopsied on day 14 and the weight of conception products (*i.e.*, the uterus, placentas, fetuses, and amniotic fluid) was taken as the criterion of whether pregnancy was maintained.

Experiment 3. In this experiment pregnant rats receiving a deficient diet

with or without ovarian steroids were sacrificed on day 3, 5, 7, 9, 11, or 14 and the effects of hormones on the protein and nucleic acid content of the uterus and the rate of ^{14}C -leucine incorporation into uterine proteins were examined. Animals received $4\ \mu\text{Ci}$ ($344\ \text{mCi/mm}$) of ^{14}C -L-leucine (U) intraperitoneally 1 hr before autopsy (around 9.00 A. M.) and ovarian steroids from day 3 to the day before autopsy. In some cases, the free amino acids in the tissues were determined.

Experiment 4. To estimate the capacity of the uterus to concentrate estrogen, deficient rats with or without steroids were killed on day 5, 9, or 14 and the content of estrogen receptor protein in the uterus was determined.

For autopsy, animals were lightly anesthetized with ether and the liver, gastrocnemius muscle, uterus, placenta, and fetuses were removed and weighed. Some of these tissues were used for measurements of nucleic acids, protein, free amino acids, or estrogen receptor proteins.

RNA and DNA were measured spectrophotometrically (WANNEMACHER *et al.*, 1965). Protein was determined by the method of LOWRY *et al.* (1951) on solution obtained by hydrolysis with 0.3N KOH during nucleic acid determination. Free amino acids in tissues were measured by ion-exchange chromatography, using an automatic amino acid analyzer (STEIN and MOORE, 1954) with pooled samples from four to five rats. Radioactivity in tissue protein was measured by scintillation spectrometry in toluene-dioxane-based scintillation fluid (HERBERG, 1960), using an internal standard for quenching correction. The estrogen receptor content of the supernatant of a homogenate of the uterus was determined by equilibrium analysis, according to the method of MÉSTER *et al.* (1970), using $[2, 4, 6, 7\text{-}^3\text{H}]$ estradiol ($100\ \text{Ci/mm}$).

RESULTS

Hormones necessary for maintenance of pregnancy

Table 1 shows the result of Experiment 1. Pregnancy was fully maintained in rats fed the deficient diet with estrone and progesterone, whereas about 75% of the rats receiving only one of the two steroids showed fetal resorption. Moreover, even when pregnancy was maintained in rats receiving one steroid, the litter size was significantly smaller than that in rats receiving both steroids.

Table 2 shows the effect of various periods of steroid administration on maintenance of pregnancy. For complete maintenance injections were necessary from as early as day 3, but about 80% of the pregnant females maintained pregnancy even when injections were given only on days 9–13. Thus, complete maintenance of pregnancy in rats fed an isoleucine-free diet was accomplished by administration of a combination of estrone and progesterone from day 3 of pregnancy.

Table 1. Effect of ovarian steroids on maintenance of pregnancy in rats fed isoleucine-free diet (Experiment 1).

Hormonal treatment ¹	No. of rats maintained (No. /total)	Per cent maintenance	Wt of conception products ^{2,3} (g)	No. of viable fetuses ³	Fetus wt ³ (g)
+ EP	15/15	100	33.6 ± 5.4	11.0 ± 1.6	1.96 ± 0.23
+ E	3/13	23	22.2 ± 10.2 ⁴	6.4 ± 4.0 ⁴	2.47 ± 0.43 ⁴
+ P	3/11	27	20.6 ± 13.7 ⁴	5.7 ± 4.2 ⁴	2.38 ± 0.29 ⁴

¹ 0.5 µg of estrone (E), 4 mg of progesterone (P) or a combination of these steroids (EP) was injected subcutaneously each day from day 3 to day 20.

² Conception products consist of the uterus, placentas, fetuses, and amniotic fluid.

³ Values represent means of values in rats where pregnancy was maintained ± S.D.

⁴ Significantly different from value for rats with EP ($P < 0.05$).

Table 2. Effect of time of steroid administration on maintenance of pregnancy (Experiment 2).

Day of injection ¹	No. of rats maintained ² (No./total)	Per cent maintenance	Wt of conception products ³ (g)
3-13	10/10	100	4.55±0.76
4-13	8/10	80	4.70±0.90
5-13	8/10	80	4.16±0.64
6-13	7/10	70	3.85±0.79
7-13	5/7	71	3.90±0.44
9-13	19/24	79	4.19±0.70
11-13	1/6	17	2.41

¹ 0.5 µg of estrone and 4 mg of progesterone.² The weight of conception products on day 14 was taken as a criterion whether pregnancy was maintained.³ Values represent means of values in rats in which pregnancy was maintained ± S.D.

Table 3. Changes in food intake, body weight, and weight of conception products in pregnant rats receiving isoleucine-free diet with or without ovarian steroids (Experiment 3).

Day of autopsy	Hormonal treatment ¹	No. of rats	Food intake (g)		Body wt loss ^{2,3} (g)	Wt of conception products (g)
			Total ²	Daily		
3	— EP	5	12± 1 ⁴	6.2	— 9±2 ⁴	0.23±0.04 ⁴
5	— EP	5	19± 3	4.7	—19±4	0.31±0.05
	+ EP	5	18± 4	4.5	—22±2	0.33±0.02
7	— EP	8	29± 5	4.8	—25±3	0.35±0.07 ⁵
	+ EP	7	32± 4	5.4	—26±3	0.42±0.03
9	— EP	8	34± 2	4.3	—37±4	0.42±0.05 ⁵
	+ EP	7	39± 5	4.9	—35±3	0.50±0.07
11	— EP	5	49± 5	4.9	—38±5	0.36±0.12 ⁶
	+ EP	7	52± 5	5.2	—35±4	1.06±0.24
14	— EP	7	70±10	5.4	—47±4	0.24±0.07 ⁶
	+ EP	7	68±16	5.2	—47±3	4.34±0.68

¹ A combination of 0.5 µg of estrone and 4 mg of progesterone was given from day 3 to the day before autopsy.² Daily differences in the food intake and body weight loss in rats with and without steroids were insignificant.³ Difference between the body weights on day 1 and the day of autopsy.⁴ Values represent means ± S.D.⁵ Significant difference between rats with and without steroids ($P < 0.05$).⁶ Significant difference between rats with and without steroids ($P < 0.01$).

Effect of ovarian steroids on growth of conceptuses

Changes in the food intake, body weight, and weight of conception products in rats with or without steroids are shown in Table 3. All the rats ate about 5 g of the deficient diet daily and showed marked loss of body weight regardless of whether or not hormones were administered. In rats not receiving steroids, the weight of conception products increased slowly until day 9 and then decreased, indicating that fetal resorption occurred on about day 9. On the contrary, in groups receiving steroids the weight of conception products continued to increase, particularly after day 11, despite the inadequate diet. The difference in the weight of conceptuses in the groups with and without steroids became significant after day 7. The food intake and loss of body weight in the two groups were identical, so this finding suggests that ovarian steroids may act favorably on fetus formation, probably by improving the efficiency of transfer of nutrients from the mother to the embryos.

Effect of ovarian steroids on uterine nucleic acids and protein

In the group in which pregnancy was maintained, the RNA concentration in the uterus tended to increase during pregnancy, whereas that of DNA decreased. Consequently, the RNA/DNA ratio in this group rose during pregnancy. In the rats not receiving steroids, the RNA concentration increased during early pregnancy and then remained elevated. The DNA concentration did not show any appreciable change until day 11, and on day 14 it increased remarkably. Thus, the RNA/DNA ratio increased slightly till day 7 and then decreased. The total amounts of RNA and DNA in rats receiving steroids rose progressively

Table 4. Effect of ovarian steroids on total nucleic acids and protein in the uterus of pregnant rats fed isoleucine-free diet (Experiment 3).

Day of autopsy	Hormonal treatment	Uterus wt (g)	RNA (mg)	DNA (mg)	RNA/DNA	Protein (mg)
3	— EP	0.23±0.04 ¹	0.85±0.10 ¹	1.72±0.27 ¹	0.51±0.15 ¹	38± 3 ¹
5	— EP	0.31±0.05	1.08±0.12	1.71±0.30	0.63±0.15	39± 7
	+ EP	0.33±0.02	0.96±0.18	1.71±0.39	0.56±0.04	38± 8
7	— EP	0.35±0.07 ²	1.33±0.33 ²	1.91±0.38 ³	0.73±0.09	47± 8 ³
	+ EP	0.42±0.03	1.82±0.36	2.54±0.29	0.72±0.14	64± 7
9	— EP	0.32±0.03 ²	1.07±0.32 ²	1.72±0.37 ²	0.64±0.18	45± 8
	+ EP	0.43±0.08	1.52±0.35	2.32±0.50	0.64±0.08	53±10
11	— EP	0.36±0.12 ²	1.11±0.30 ²	1.85±0.57 ²	0.61±0.11	46±16 ³
	+ EP	0.51±0.08	1.97±0.48	2.65±0.41	0.75±0.18	68±16
14	— EP	0.24±0.07 ³	0.93±0.48 ³	1.78±0.52 ³	0.51±0.12 ³	34±14 ³
	+ EP	0.97±0.12	3.91±0.78	4.17±0.79	0.95±0.17	118±23

¹ Values represent means ± S.D.

² Significant difference between rats with and without steroids ($P < 0.05$).

³ Significant difference between rats with and without steroids ($P < 0.01$).

and were significantly higher than those in animals not receiving steroids after day 7, due mainly to the greater weight of the uterus (Table 4). Changes in total protein were similar to those in total RNA or DNA. Thus, significant differences were observed between the groups with and without steroids in the growth of conceptuses, total nucleic acids, and protein after day 7 of pregnancy.

Rate of ^{14}C -leucine incorporation into tissue protein

Table 5 shows the rate of ^{14}C -leucine incorporation into tissue protein. The specific and total radioactivities in the uterus in both groups had increased slightly by day 5 and then remained constant until day 11. This suggests that protein synthesis in the uterus was not influenced by exogenously administered ovarian steroids, at least until day 11. However, the radioactivities in rats receiving steroids increased markedly on day 14, while those in rats not receiving steroids decreased.

Table 5. Incorporation of ^{14}C -leucine into tissue proteins in malnourished pregnant rats with and without ovarian steroids (Experiment 3).

Day of autopsy	Hormonal treatment	Radioactivity ¹					
		Uterus		Muscle ²		Liver	
		S.A. ³ (dpm/mg protein)	Total (dpm)	S.A. ³ (dpm/mg protein)	Total (dpm)	S.A. ³ (dpm/mg protein)	Total (dpm)
3	— EP	39	1,470	4	940	14	18,410
5	— EP	207	7,180	24	6,370	197	225,300
	+ EP	214	9,430	17	4,610	353	352,670
7	— EP	185	9,730	19	4,620	355	378,300
	+ EP	220	14,300	30	6,630	357	337,900
9	— EP	117	4,400	7	1,630	209	212,130
	+ EP	118	7,250	23	4,800	224	258,880
11	— EP	335	17,000	32	6,690	418	368,700
	+ EP	278	19,060	28	5,440	314	341,600
14	— EP	86	2,760	35	7,670	212	212,400
	+ EP	1,880	203,240 ⁴	22	4,370	370	375,300

¹ The SE for each mean is within $\pm 10\%$ of the mean.

² Gastrocnemius muscle.

³ Specific activity.

⁴ Significantly different from the value in rats without ovarian steroids ($P < 0.01$).

Estrogen receptors in the uterus during pregnancy

The amounts of estrogen receptors in the uterus on days 5, 9 and 14 of pregnancy are shown in Table 6. The binding capacity per milligram wet weight of uterus in both groups was lower on day 9 than on day 5 or 14. There were no significant differences in the concentrations and total amounts of receptors in

the groups with and without steroids on day 5 or 9. On day 14 the concentration in rats not receiving steroids was significantly higher than in rats receiving steroids, but the total amounts in the latter were about 3 times greater than in animals in which pregnancy failed ($P < 0.05$), because of the increased weight of the uterus. These results show that the binding capacity of uterine cytosol in malnourished pregnant rats was maintained until relatively late in pregnancy.

Table 6. Changes in the estrogen receptor content of the supernatant fraction of the uterus (Experiment 4).¹

Day of autopsy	Hormonal treatment	No. of rats	Uterus wt (mg)	Estrogen receptor content		
				Concentration ($\times 10^{-3} \mu\text{M}/\text{mg}$ tissue)	Specific activity (pM/mg protein)	Total (μM)
5	— EP	4	305 $\pm$ 28	16.22 $\pm$ 7.2	0.168 $\pm$ 0.072	5.06 $\pm$ 2.57
	+ EP	6	280 $\pm$ 39	16.50 $\pm$ 5.0	0.149 $\pm$ 0.046	4.62 $\pm$ 1.69
9	— EP	5	300 $\pm$ 55	9.13 $\pm$ 4.9	0.093 $\pm$ 0.041	2.54 $\pm$ 0.35
	+ EP	5	387 $\pm$ 56	7.40 $\pm$ 0.1	0.069 $\pm$ 0.014	2.89 $\pm$ 0.72
14	— EP	4	216 $\pm$ 23	15.76 $\pm$ 4.3	0.147 $\pm$ 0.036	3.30 $\pm$ 0.88
	+ EP	6	949 $\pm$ 167 ²	10.69 $\pm$ 2.0 ²	0.087 $\pm$ 0.025 ²	10.38 $\pm$ 2.64 ²

¹ Values represent means $\pm$ S.D.

² Significantly different from the value in rats without steroids ($P < 0.05$).

DISCUSSION

In the present experiments fetal wastage in pregnant rats fed an isoleucine-free diet was completely prevented by daily administration of 0.5 μg of estrone and 4 mg of progesterone from day 3 of pregnancy. Injection of either one of these steroids alone was less effective. NELSON (1959) reported that daily injection of 0.5 μg of estrone, 4 mg of progesterone, and a combination of these steroids into protein-deficient rats resulted in maintenance of pregnancy in 30%, 70 to 80%, and 100% of the animals, respectively. Similar results were obtained by other investigators (LEATHEM, 1966; KINZEY *et al.*, 1970). The effective period of steroid administration of maintenance of pregnancy has been determined in rats on protein-deficient diets. LEATHEM (1966) and CALLARD and LEATHEM (1970) found that injection of hormones on days 3–12 resulted in successful maintenance of pregnancy in 100% of rats tested, while their injection on days 7–12 and 7–9 resulted in 91% and 36% maintenance, respectively. KINZEY and SREBNIK (1963) observed that injection of hormones on days 6–10 was sufficient to maintain pregnancy. In the present study on isoleucine-deficient rats, ovarian steroids were required from day 3 for 100% maintenance of pregnancy. The strain of rats and diet used may influence the effective timing of hormone injection and particularly the time of its start, as CALLARD and LEATHEM (1971) suggested that maintenance of pregnancy in the absence of the ovary and supplemented steroids may

vary in different species.

Even when the period of hormone injection was shortened to between days 9 and 13, about 80% of experimental animals maintained pregnancy, but on steroid injection between days 11 and 13 only 17% of the animals maintained pregnancy (Table 2). This indicates that day 9 or 10 is a critical time for maintenance of pregnancy in malnourished rats. There are many reports indicating that nutritionally induced fetal wastage is due mainly to impairment of function of the hypophysis. Thus, this finding suggests that hypophyseal functions related to reproduction are preserved until quite a late stage of pregnancy in malnourished rats. In this connection it is interesting to note that total content of estrogen receptors in the uterus of rats receiving steroids was comparable to that in animals not receiving steroids till day 9 of pregnancy, though not on day 14. Thus, about day 9 of pregnancy also seems to be a critical time for the capacity of the uterus to bind estrogen.

There are intimate metabolic relations between branched-chain amino acids in mammals, such as leucine-isoleucine antagonism observed on administration of excess leucine (HARPER, 1964). Thus, the question arises whether it is suitable to use labeled leucine as an indicator of protein synthesis in isoleucine deficiency. In our work the free amino acids in the tissues of pregnant rats fed an isoleucine-free diet were measured (Table 7). The concentrations and patterns of branched-chain amino acids in the tissues of deficient animals were similar to those in control animals and did not change on hormone administration. Thus, labeled leucine seems adequate as a tracer in isoleucine deficiency.

In malnourished rats with or without injected steroids, uptake of ^{14}C -leucine into uterine protein increased on day 5 of pregnancy and remained elevated until day 11. In the group receiving steroids, it increased further on day 14, while it decreased in rats not receiving steroids. These changes in the rate of protein synthesis were in accord with changes in the RNA/DNA ratio in the uterus. Similar changes in ^3H -leucine incorporation into uterine protein during normal pregnancy were reported by REID and HEALD (1970), suggesting that the patterns of protein synthesis in the uterus in normal and malnourished pregnant animals are almost identical when pregnancy is maintained.

Differences between rats with and without steroids in the growth of conceptuses, total RNA, DNA, and protein in the uterus became evident as early as day 7 of pregnancy, whereas the effect of steroid administration on protein synthesis did not become apparent until day 14. OKA and SCHIMKE (1969) investigated the effects and interaction of estrogen and progesterone in immature chick oviduct. They found that concomitant administration of progesterone with estrogen increased the wet weight, total nucleic acids, and protein. However, a combination of both steroids resulted in the same degree of increase in uptake of ^{14}C - α -aminoisobutyric acid into oviduct proteins as with estrogen alone. This and the present results strongly suggest that exogenously administered ovarian

Table 7. Effects of isoleucine deficiency and ovarian steroids on free amino acids in tissues of pregnant rats (on day 14).¹

Steroids	Plasma ²		Muscle ^{2,3}		Liver ²		Uterus ²	
	Complete	Ile-free	Complete	Ile-free	Complete	Ile-free	Complete	Ile-free
	—	+	—	+	—	+	—	+
Leu	109	173	0.16	0.24	0.17	0.26	0.39	1.10
Ile	68	79	0.10	0.14	0.12	0.17	0.22	0.60
Val	155	187	0.19	0.20	0.39	0.27	0.39	1.01
							1.14	0.86

¹ Free amino acids were measured on pooled samples from four or five rats, using an automatic amino acid analyzer (Yanaco, LS 50).² $\mu\text{M/liter}$ or g.³ Gastrocnemius muscle.

steroids, particularly progesterone, caused uterine or oviduct hyperplasia by inhibiting protein degradation. In fact, MULLER *et al.* (1970) stated that the effects of progesterone on protein turnover in chicken oviduct cannot be explained in terms of increase in protein synthesis but are due to an action on protein degradation.

Abortion due to malnutrition is probably not caused by the insensitivity of the uterus to steroids, because pregnancy was maintained on injection of estrogen and progesterone into rats fed diets deficient in protein or an essential amino acid. However, OSLAPAS and LEATHEM (1965) suggested that protein deficiency interfered with the normal uterine response to estrogen. Accordingly, in this work the sensitivity of the uterus to estrogen in rats on an inadequate diet was examined in terms of the receptor protein content of the uterine cytosol, because the interaction of estrogen with cytoplasmic protein in the uterus is an important step in concentrating circulating estrogen. Changes in the concentration and total amount of receptor protein in malnourished rats receiving steroids were similar to those observed in normal pregnant rats by FEHERTY *et al.* (1970). Moreover, the amounts of receptor protein in rats not receiving steroids were comparable to those in animals receiving steroids on day 5 or 9, although on day 14 the content was less than in the latter animals. This suggests that uterine sensitivity to steroids in malnourished animals remains normal for quite a long time during pregnancy.

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