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Suppressive Effect of Neonatal Rat Serum on PMSG-induced Ovulation in Immature Rats

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Summary

Neonatal rat serum (NS) was confirmed to have a remarkably higher potency to bind estrogen *in vitro* than adult one. The NS was injected intravenously into PMSG-primed immature rats in order to examine whether it can suppress the gonadotropin induced ovulation.

0.7 ml of NS suppressed PMSG-induced ovulation in immature rats when injected 0–24 h after PMSG but not 27 h or later.

The suppressive effect was canceled with LHRH at 53 h or with estrogen at 6 h after PMSG.

It is suggested that the NS may bind and inactivate endogenous estrogen in PMSG-primed rats, resulting in suppression of PMSG-induced ovulation via blockade of LHRH release.

Perinatal rat serum is known to have a strong affinity for estrogen(1–4). The estrogen binding component of the serum has been identified as α-fetoprotein (5). The protein seems to modulate to lessen the free estrogen levels by binding during neonatal 2 weeks when the whole blood estrogen are remarkably high (6, 7).

Perinatal serum, however, has a further binding capacity with exogenous estrogen (3) and therefore, it could be available for a blocking agent of estrogen action *in vivo*. PMSG-induced ovulation in immature rats was suppressed by antibody of estrogen (8) or by anti-estrogen, clomiphene (9–11).

The present study is concerned with whether or not the neonatal serum (NS) can suppress PMSG-induced ovulation in immature rats.

Materials and Methods

Animals and treatment of sera

Wistar rats inbred in our laboratory were used. Animals were kept in an air-conditioned room (24±2°C) with a controled illumination of 12 h light and 12 h darkness and provided food and water *ad libitum*.

Neonatal rats of 0–2 days of age and adult female rats in late diestrus were decapitated to collect the blood into the centirifugation tube. Sera were obtained and kept at −70°C after centrifugation at 4°C. When each serum was collected to approximately 100 ml, it was thawed to subdivide into 1 ml units and kept again in the freezer until used.

Estrogen binding capacity of serum

Estimating the capacity was performed in the light of that of Germain *et al* (3) with a small revision. Either of neonatal serum (NS) or adult serum (AS) which was diluted to 250 μl (final volume) with Tris-HCl buffer (pH 7.4), was mixed with 10 μl of 0.1 μCi ^{3}H-estradiol 17β (6–7 ^{3}H, NEN S.A. 50 Ci/m mol) in a test tube and incubated at 37°C for 1 h. After that, 75 μl of charcoal mixture (10 mg/ml buffer, Norite:American Norite Co.) was added to absorb free ^{3}H-estradiol. After setting the tubes in the cold water for 0.5 h, the whole mixture was centrifuged with 3000 rpm for 10 minutes. Fifty μl of the supernatant was added into the scintilation vial with 5 ml toluene scintilator (Dotite POPOP 25 μg, Dotite PPO 20 mg) and counted with a liquid scintilation counter (Packard Co.). The count of supernatant of NS or AS was compared with the total count without charcoal and the estrogen binding capacity was represented as percent.

Effect of NS on PMSG-induced ovulation

Female rats of 25 days of age (b.w. 59±0.6 (S.E.)g, n=123) were injected subcutaneously with 3 IU PMSG (P-mex: Sankyo-Zoki) per 0.1 ml saline at 10:00 h and killed by decapitation 72 h later. Ampulla was examined for ova and number of ova was counted under a dissecting microscope by gently pressing the oviduct between two slides. At autopsy, ovaries and uterus after removing the inner fluid were weighed using a torsion balance.

Dose and time of treatment of NS

Either of 0.5 ml or 0.7 ml of NS thawed just before administration was injected into femoral vein of rats under a light ether anesthesia at 6 or 24 h after PMSG. Inactivated NS which was boiled for 20 minutes and centrifuged to remove precipitations was also injected to some rats post PMSG. Since ovulation was blocked by 0.7 ml NS (as described in the result) this amount of NS or AS was injected to rats at the various time after PMSG as shown in Table 3 to compare the effect on the ovulation.

Some rats given NS at 6 h were killed at 96 h after PMSG to examine delayed ovulation.

LHRH or estrogen treatment

LHRH decapeptide (Takeda) dissolved in 0.1 ml of saline was injected subcutaneously at 53 h after PMSG at varying dose per rat which had been given PMSG and 0.7 ml NS 6 h after PMSG.

Estradiol-17β (Tokyo Kasei) disolved in 0.2 ml sesame oil was injected subcutaneously at 6 h after PMSG at varying dose per rat simultaneously with 0.7 ml NS.

Statistics

The Fisher's exact probability test was used to assess the difference in the ovulation rate in each group and Student's t test was used to compare the number of ova and the weight of organs in each group.

Result

Estrogen-binding capacity of NS

The binding capacities of NS were more than 80 and 70% in 1 and 1/2 dilutions, respectively. Even if the dilution was increased to 1/10, it was still 34%. When the dilution advanced to 1/50, the capacity decreased to 1.3%.

On the other hand, AS did not have any estrogen-binding capacity (Table 1).

TABLE 1. *Binding Capacity of NS or AS for ^{3}H-estradiol.*

Binding rate of ^{3}H-estradiol	Dilution rate of serum			
	1	1/2	1/10	1/50
NS	86.3	71.7	34.0	1.3
AS	1.3	0	—	—

Sera were incubated at 37°C with the indicated concentration of ^{3}H-estradiol (S.A. 50 Ci/mol).

After 1 h, the percentage of the ^{3}H-estradiol found to sera was determined by the charcoal absorption method.

Effect of NS on PMSG-induced ovulation

Dose of NS

Treatment of 0.5 ml NS hardly suppressed ovulation, while 0.7 ml NS did at 6 or 24 hours after PMSG (Table 2).

TABLE 2. *Dose of NS of Rats to Suppress the Ovulation in PMSG-primed Immature Rats.*

Time of injection	Dose (ml)	Ovulation rate
6 h after PMSG	0.5	5/6
	0.7	2/12*
	0.7 (inactivated)	5/5
24 h after PMSG	0.5	5/5
	0.7	1/7**

PMSG (3 IU) was injected to the rat (25 days of age) at 10:00 h after which NS was injected i.v.. Rats were killed 72 h after PMSG to examine ova in ampulla. *$p<0.05$, **$p<0.01$ compared with 0.5 ml treated group (Fisher's exact probability test).

Ovulation rate was significantly low in each of 0.7 ml groups compared with that of 0.5 ml groups at 6 h ($P<0.05$) or 24 h ($P<0.01$). But inactivated NS did not block ovulation at all.

Time of NS treatment

Ovulation rate was low in the group which was given NS at 0–24 h after PMSG. On the other hand, ovulation was not blocked in the AS group (Table 3). The suppression of ovulation was almost nil when NS was given to the rat at 27 h and later after PMSG. The ovulation rate between groups given NS at 24 and 27 h was significantly different ($P<0.05$). All the rats given NS at 6 h and killed 96 h post PMSG ovulated, indicating the presence of ova in ampulla (3 rats) and in ithmus (2 rats).

TABLE 3. *Effect of NS at Various Times on the Ovulation with PMSG Priming in Immature Rats*

Time after PMSG	NS		AS	
	Ovulation rate	Number of ova	Ovulation rate	Number of ova
0	0/5**	0	7/8	5.7±1.0
6	2/12*	7.0±1.0♯	5/6	7.4±0.7
24	1/7**	6	7/8	7.4±1.0
27	6/7	7.0±0.6		
30	5/6	7.4±0.8	5/6	7.0±0.9
48	7/8	8.3±0.5	8/8	7.5±0.5
6†	3/5††	8.7±0.7		

PMSG (3 IU) was injected to the rat (25 days of age) at 10:00 h after which NS or AS (0.7 ml) was injected i.v. at various times.

Rats were killed 72 h after PMSG to examine ova in ampulla.

♯ Mean±S.E.

*$P<0.05$, **$P<0.01$ compared with the corresponding AS group (Fisher's exact probability test)

† Rats were killed at 96 h after PMSG.

†† Ova were found in ithmus in remaining 2 rats.

Number of ova

Number of ova per ovulated rat was at the range of 5–11 in the NS and AS groups (Table 3). When combined the records of 0–48 h after PMSG, mean numbers of ova were 7.2±0.3 (S.E.) (n=21) and 6.9±0.4 (n=32) in NS and AS, respectively. But these values were not statistically significant.

Weight of ovaries and uterus

Ovarian weights were 25.6±1.2 (S.E.) (n=21) and 16.2±0.4 (n=24) mg in rats with and without ovulation at autopsy, respectively and uterine weights were 98.7±4.2 (S.E.) and 157±5.0 mg in the same category when the records of the NS group were combined throughout the treatment of 0–48 h after PMSG. The above values were significantly different on the ovaries ($P<0.01$) and the uterus

($P<0.01$). All the uteri of the rat without ovulation were balooned with the inner fluid. In the AS group, most of rats ovulated and the weights of ovaries and uterus in these animals were 24.8 ± 0.8 (n=32) and 115.1 ± 2.6 mg, respectively. These values were not significant, compared with those of rats with ovulation in the NS group.

Effect of LHRH on ovulation

Treatment of 0.02 μg LHRH did not cancel the suppressing effect of NS on the ovulation but 0.2 or more LH–RH did perfectly (Table 4). Ovulation rate was significantly different between the groups given 0.02 μg and those given 0.2 μg or more ($P<0.01$).

TABLE 4. *Effect of LHRH on the Ovulation in Immature Rats with Pretreatment of PMSG-NS.*

Dose of LHRH (μh)	Ovulation rate
0.02	0/5
0.2	5/5**
5	6/6**

PMSG (3 IU) was injected to the rat (25 days of age) at 10:00 h after which NS (0.7 ml) was injected i.v. at 6 h and LHRH was injected at 53 h.

Rats were killed 72 h after PMSG to examine ova in ampulla.

**$P<0.01$ compared with 0.02 μg LHRH (Fisher's exact probability test).

Effect of estrogen on ovulation

The blockade of ovulation was not negated at all with vehicle alone. But estrogen treatments exerted the full effect for the negation of the suppressed ovulation (Table 5).

TABLE 5. *Effect of Estradiol-17B on the Ovulation in Immature Rats with Pretreatment of PMSG-NS.*

Dose of estradiol (μg)	Ovulation rate
0 (vehicle)	0/4
0.5	6/6**
5	6/6**

PMSG (3 IU) was injected to the rat (25 days of age) at 10:00 h after which NS (0.7 ml) was injected i.v. at 6 h and estradiol was injected at the same time.

Rats were killed 72 h after PMSG to examine ova in ampulla.

**$P<0.01$ compared with the vehicle group (Fisher's exact probability test)

The ovulation rate was significantly different between the groups with or without estrogen treatment ($P<0.01$).

Discussion

Binding capacity to ^{3}H-estradiol was clearly shown in NS but not in AS in rats.

This indicates a good consistency with earlier reports reflecting that NS has the full extra binding capacity to estrogen even when the levels of the endogenous estrogen is remarkably high during neonatal period (1–3).

It was probable that NS might suppress ovulation *in vivo* via the estrogen binding action.

Our data show that the NS, not AS, has the capacity to supreess PMSG-induced ovulation and that heated NS does not exert the suppressive effect at all. This suggests that the ovulation inhibiting factor of NS originates from protein (probably, α-fetoprotein).

The time zone to suppress the ovulation was found to be 0–24 h after PMSG.

The zone agrees well with that of suppressed ovulation with the lower dose (500 μg/100 g b.w.) of clomiphene which is one inhibitor of the estrogen receptor (10). Ferin *et al.* (8), by using antibody to estrogen and Umezu (10), by using clomiphene (5 mg/100 g b.w.) observed that these agents suppressed the ovulation at 0–34 or 0–36 h after PMSG. The ability for NS to negate the estrogenic action seems to be weaker than the antibody or the higher dose of the receptor inhibitor.

The ovulation following a single injection of PMSG to immature rat is due to the discharge of ovulatory hormone (LH and FSH) from the animals own pituitary (12–14) and the critical time of excitation of the central nervous system (CNS) (15, 16) for the discharge is from 13:00 to 15:00 h, which corresponds with 51–53 h after PMSG in this experiment.

Thus, the time zone of the suppressed ovulation with these anti-estrogenic agents including NS is more than 15 h (8) or 24 h (10) before the central excitation. It is unlikely that the early event with estrogen to the subsequent discharge of the ovulatory hormone needs an elevation of blood estrogen, because Parker *et al* and Barh and Ben-Jonathan (17, 18) observed that the elevated levels of estrogen were in 31–55 h and 48–57 h, respectively, but not within 24 h after PMSG.

In rats with the suppressed ovulation after PMSG-NS treatment, the uterus was fully balooned with the increased weight. It was not the case with clomiphene treatment where uterine growth was suppressed through competition of estrogen receptor (10, 11). The potency for NS to negate estrogenic action may be moderate compared with other anti-estrogenic agents.

The state of the uterus was known to be in the category of the proestrus which was followed the next day by ovulation in the PMSG-primed rat (13, 14, 19). Also, ova in ampulla was detected in most of rats ovulated when they were killed at 96 h after PMSG.

Therefore, the blockade of ovulation with NS after PMSG was due to the delay of the ovulation for 24 h.

The blocked ovulation with NS after PMSG was negated with LHRH treatment, which was the same case with clomiphene (19). This suggests that the competence with NS on the action of the endogenous estrogen extends to the suppression of LHRH release on the day preceeding ovulation.

The fact that the exogenous administration of estrogen negated the inhibited ovulation in the rat with PMSG-NS priming supports the proposition that this effect was derived from the anti-estrogenic action of NS.

The dose of 0.5 μg was enough to negate the anti-estrogenic action and was comparable with the dose in which inhibited ovulation was canceled after PMSG-clomiphene priming (11).

Thus, just like other anti-estrogenic agents, NS which possesses extra binding capacity seems to bind the estrogen secreted from ovaries after PMSG, and in turn, suppress LHRH release, resulting in the suppression of the ovulation.

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