

## Progestational Agents Prevent Preterm Birth Induced by a Nitric Oxide Synthesis Inhibitor in the Mouse

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**Abstract.** *Background: The ability of the nitric oxide (NO) synthesis inhibitor NG-nitro-L-arginine methyl ester (L-NAME) to induce preterm parturition in the mouse has been previously documented. The present study tested the ability of progestational agents to prevent preterm birth induced by L-NAME. Materials and Methods: L-NAME was administered subcutaneously at 90 mg/kg on gestation day 16. Progesterone, medroxyprogesterone acetate and hydroxyprogesterone caproate were administered subcutaneously at 0 (vehicle), 5 or 10 mg/kg on gestation day 16 one hour before L-NAME and on day 17. Parturition was considered preterm if occurring before gestation day 18. Results: Following treatment with L-NAME alone, 56.5% of the pregnant animals delivered before term. Treatment with progesterone, medroxyprogesterone acetate or hydroxyprogesterone caproate at 5 mg/kg or 10 mg/kg significantly and comparably reduced the rate of preterm birth caused by L-NAME. Conclusion: Progestational agents are able to reduce preterm births induced by nitric oxide synthase (NOS) inhibition.*

Preterm parturition represents a major issue in obstetrics, accounting for 70% of neonatal mortality and 50% of cerebral palsy (1). It is generally accepted that the lack of effective therapeutic measures to prevent preterm birth mostly reflects the limited knowledge of the molecular mechanisms involved in the onset of labor. Several lines of research support the concept that the biomediator nitric oxide (NO) plays a role in blocking myometrial excitatory responsiveness and promoting

the maintenance of pregnancy, and that down-regulation of NO synthesis at term gestation leads to parturition (2). NO synthesis is mediated by three conserved NO synthase (NOS) isozymes: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) (3). NOS activity can be blocked by several inhibitors, including by NG-nitro-L-arginine methyl ester (L-NAME). This competitive and nonselective NOS inhibitor has been instrumental in the identification of many of the physiological actions of NO and the investigation of its role in some pathophysiological processes (4). The treatment of pregnant mice with L-NAME during a gestational phase corresponding to the human third trimester has been found to initiate preterm birth (5, 6). This response appeared to be dependent on NOS inhibition, since preterm birth was prevented by the NO donor sodium nitroprusside (5).

Progestational agents are under evaluation for their potential ability to prevent human preterm birth (7). In the present study, the ability of the progestational agents progesterone (P4), medroxyprogesterone acetate (MPA) and hydroxyprogesterone caproate (17-P) to prevent preterm parturition imposed by L-NAME in the mouse was tested. The study was rationalized by several lines of evidence indicating that P4 may be the main hormonal factor promoting NO synthesis in the pregnant uterus (2). This particularly applies to iNOS, which is the dominant form of the NOS isoform in the pregnant uterus, cervix and placenta (8-11). Studies in rats have shown that while treatment with the P4 antagonist RU-486 promoted preterm labor and was associated with a significant reduction of myometrial iNOS expression, administration of P4 resulted in increased iNOS expression (12). Moreover, exposure to prostaglandin  $F_{2\alpha}$ , which plays a role in preparing the uterus and the cervix for delivery, led to a reduction of iNOS expression that was prevented by treatment with P4 (12). Another study with rats found that P4 was able to increase the expression, not only of iNOS, but also of eNOS in the uterus of ovariectomized rats (13).

Evidence for a key role of P4 in the regulation of uterine NOS is not limited to rats, but is also valid for the mouse. In this species, combined treatment with estrogen and P4

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Table I. Effect of progesterone (P4), medroxyprogesterone acetate (MPA) and hydroxyprogesterone caproate (17-P) on the timing of L-NAME-induced parturition in ICR (CD-1) mice.

Treatment	No. of pregnant animals treated	No. of pregnant animals delivering preterm (before gestation day 18)			
		Gestation day			
		16.0-16.5	16.5-17.0	17.0-17.5	17.5-18.0
DMSO + L-NAME	23	0	1 (4.34%)	6 (26.08%)	6 (26.08%)
P4 (5 mg/kg) + L-NAME	18	0	0	1 (5.55%)	2 (11.11%)
P4 (10 mg/kg) + L-NAME	17	0	0	2 (11.80%)	0
MPA (5 mg/kg) + L-NAME	18	0	0	2 (11.11%)	1 (5.55%)
MPA (10 mg/kg) + L-NAME	20	0	0	2 (10.00%)	1 (5.00%)
17-P (5 mg/kg) + L-NAME	20	0	0	2 (10.00%)	2 (10.00%)
17-P (10 mg/kg) + L-NAME	19	0	0	0	3 (15.80%)

P4, MPA and 17-P were administered at a dose of 5 mg/kg or 10 mg/kg subcutaneously on gestation days 16 and 17. L-NAME was injected subcutaneously at 90 mg/kg on gestation day 16 (one hour after the first injection of the progestational agent).

resulted in uterine eNOS and iNOS expression that was higher than levels following estrogen treatment alone (11). Preliminary data have suggested that P4 may up-regulate uterine NO synthesis in human and nonhuman primates (2).

## Materials and Methods

The study was conducted in agreement with the Italian legislation for animal experiments and received the approval of the institutional Ethical Committee. All the chemicals used in the study were purchased from Sigma Chemical (Milan, Italy). In line with our previous investigations assessing the gestational effects of L-NAME (5, 6), sexually mature ICR (CD-1) outbred mice (Harlan Italy, Udine, Italy) were used. The conditions of animal housing and breeding were as described elsewhere (5, 6).

L-NAME was dissolved in sterile and apyrogenic saline solution and administered subcutaneously at 90 mg/kg on gestation day 16. This dosing regimen was previously found to be effective in inducing preterm parturition in ICR (CD-1) mice (6). P4, MPA and 17-P were separately dissolved in dimethylsulfoxide (DMSO) and administered subcutaneously at 0 (vehicle) or 5 or 10 mg/kg on gestation day 16 (one hour before L-NAME injection) and 17 (24 hours after the first injection). Progestin doses were identified on the basis of a preliminary study. The double injection regimen was adopted to compensate for the tendency of a much faster metabolic clearance of progestins displayed by rodents in comparison to humans (14). A control group of animals treated with saline solution and DMSO was also used.

The animals were closely observed to determine the occurrence and timing of preterm delivery and to detect signs of maternal morbidity. Since, in agreement with previous observations (5), control (vehicle) animals delivered between gestation days 18 and 19, parturition (presence of fetuses in the cage) was considered preterm if occurring before gestation day 18. In order to assess the pregnancy status, all the animals were subjected to laparotomy on gestation day 18.

Statistical comparisons were based on the Chi-square test. Differences were considered to be significant when the *p*-value was <0.05.

## Results

The impact of the various treatment regimens on pregnancy duration is presented in Figure 1. L-NAME caused 56.5% of animals to deliver before term. Treatment with P4, MPA or 17-P at 5 or 10 mg/kg significantly (*p*<0.05) reduced, with comparable efficacy, the frequency of L-NAME-induced preterm parturition. Treatment with P4, MPA or 17-P at 5 mg/kg reduced preterm birth rates to 16.6%, 16.6% and 20.0%, respectively. The ability of the progestational agents to prevent preterm birth did not increase with increment of the dosage level, with percentages of preterm births resulting from treatment with P4, MPA, or 17-P at 10 mg/kg being 11.8%, 15.0% and 15.8%, respectively. L-NAME given with vehicle (DMSO) or in combination with progestational agents was not associated with detectable signs of maternal toxicity and no maternal deaths occurred during the study (not shown). Table I details the timing of preterm deliveries. In the majority of instances, preterm deliveries occurred on gestation day 17. As expected, all the control (vehicle) pregnant animals delivered on gestation day 18.5-19.0 (not shown). Maternal laparotomies carried out on gestation day 18 showed the majority of fetuses were viable and that preterm parturition gave birth to the entire litter (not shown).

## Discussion

As a novel finding, this study showed that progestational agents, namely P4, MPA and 17-P, could prevent the preterm parturition induced by the NOS inhibitor L-NAME in the mouse. The tested agents showed comparable efficacy, reducing the frequency of L-NAME-mediated preterm birth by three- to four-fold. There was no apparent dose dependency, since treatment with progestational agents at 5

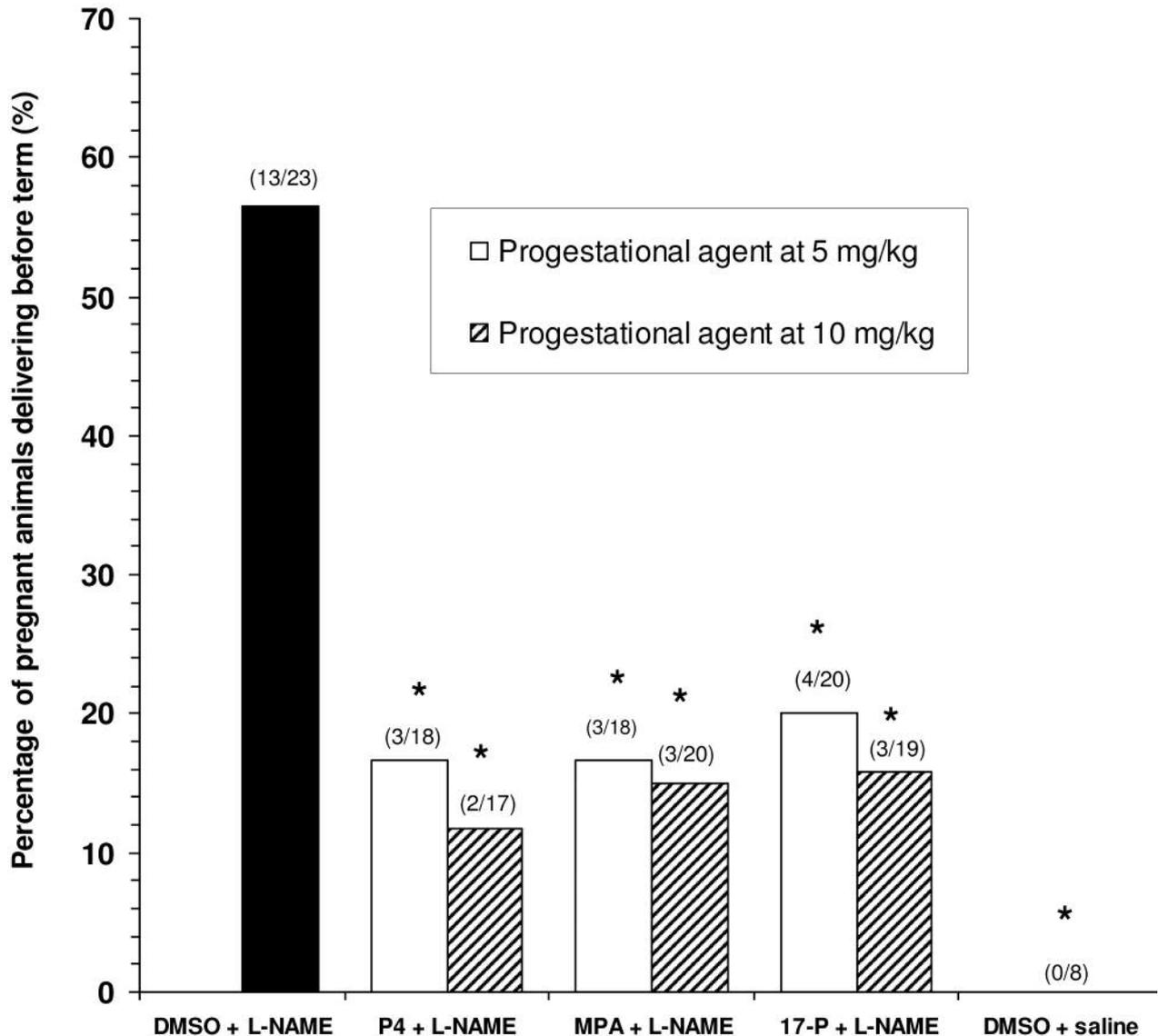


Figure 1. Effect of progesterone (P4), medroxyprogesterone acetate (MPA) and hydroxyprogesterone caproate (17-P) on L-NAME-induced preterm parturition. Progesterational agents were injected subcutaneously at 0 (vehicle), 5 mg/kg, or 10 mg/kg on gestation days 16 and 17. L-NAME was injected subcutaneously on gestation day 16 at 90 mg/kg, one hour after the first administration of the progesterational agent. The vehicle group was administered appropriate doses of dimethylsulfoxide (DMSO) (on gestation days 16 and 17) and saline solution (on gestation day 16 one hour after the first administration of DMSO). Parturition was considered preterm if occurring before gestation day 18. The numbers in parentheses indicate the proportion of pregnant animals delivering preterm to total animals treated. \*Statistically significant vs. DMSO-L-NAME group ( $p < 0.05$ , Chi-square test).

or 10 mg/kg resulted in comparable inhibition of L-NAME-mediated preterm birth.

When an animal model reveals that a therapeutic agent elicits a beneficial or an adverse effect, the relationship of the doses tested in the animal to those used in human therapy must be addressed. With 17-P, the progesterational agent upon which most of the scientific interest in the prevention of preterm parturition has recently been focused, it appears that

the lower dose (5 mg/kg) used in this study is not far from the human therapeutic dosage: if 17-P is administered at 250 mg weekly (7), the dose corresponds to 3.6 mg/kg (assuming a pregnant women weighs 70 kg).

Accumulated evidence indicates that in the mouse perturbations of the NO system can interrupt uterine quiescence and lead to preterm birth. Premature emptying of the uterus was induced in the mouse not only by the NO

blocker L-NAME (5, 6), but also by methylene blue (15). This agent is a well-known inhibitor of soluble guanylate cyclase, the enzyme which mediates most of the biological actions of NO by means of its catalytic product guanosine 3', 5'-cyclic monophosphate (cGMP). The contribution of the NO system in controlling uterine quiescence may vary with species. This may explain why L-NAME treatment alone failed to trigger preterm parturition in rats, although it was able to potentiate the capacity of the antiprogesterone onapristone to induce preterm labor (16). An issue that should be addressed before the differential response displayed by mice and rats to L-NAME can be attributed to species-specific factors concerns the role of the experimental conditions. While in the rat study (16) the animals were exposed to L-NAME by way of osmotic minipumps, enabling the continuous infusion of the agent for the selected period of exposure, in the present study the mice were treated by injections, possibly resulting in a shorter exposure, but in a more drastic NOS inhibition.

P4 is known to maintain pregnancy by inducing myometrial quiescence and relaxation by mechanisms which have not yet been fully clarified and which can significantly differ across species (17). In rodents, parturition is preceded by a decrease of maternal P4 levels secondary to regression of the corpus luteum. Currently the mechanism(s) for the protective influence elicited by progestational agents on L-NAME-mediated parturition are not known. The existing evidence for a molecular interplay between P4 and the uterine nitrenergic system, with specific reference to the ability of P4 to up-regulate iNOS expression and production in the pregnant uterus, supports the following hypothesis. A possible sequence underlying our current observations may include: inhibition of NO synthesis, mediated by acute exposure to L-NAME, initiates preterm labor; treatment with progestational agents up-regulates iNOS, which is able to generate large amounts of NO (about 1000-fold larger than the constitutive NOS isoforms); the catalytic activity of iNOS partially compensates for the NO deprivation thereby blocking myometrial excitatory responsiveness and allowing the maintenance of pregnancy.

## References

- Smith R: Parturition. *N Engl J Med* 356: 271-283, 2007.
- Maul H, Longo M, Saade GR and Garfield RE: Nitric oxide and its role during pregnancy: from ovulation to delivery. *Curr Pharm Des* 9: 359-380, 2003.
- Moncada S, Higgs A and Furchgott R: International Union of Pharmacology Nomenclature in Nitric Oxide Research. *Pharmacol Rev* 49: 137-142, 1997.
- Hobbs AJ, Higgs A and Moncada S: Inhibition of nitric oxide synthase as a potential therapeutic target. *Ann Rev Pharmacol Toxicol* 39: 191-220, 1999.
- Tiboni GM and Giampietro F: Inhibition of nitric oxide synthesis causes preterm delivery in the mouse. *Human Reprod* 15: 1838-1842, 2000.
- Tiboni GM, Giampietro F and Di Giulio C: The nitric oxide synthesis inhibitor *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) causes limb defects in mouse fetuses: protective effect of acute hyperoxia. *Pediatr Res* 54: 69-76, 2003.
- Mackenzie R, Walker M, Armson A and Hannah ME: Progesterone for the prevention of preterm birth among women at increased risk: a systematic review and meta-analysis of randomized controlled trials. *Am J Obstet Gynecol* 194: 1234-1242, 2006.
- Buhimschi I, Ali M, Jain V, Chwalisz K and Garfield RE: Differential regulation of nitric oxide in the rat uterus and cervix during pregnancy and labour. *Hum Reprod* 11: 1755-1766, 1996.
- Purcell TL, Buhimschi IA, Given R, Chwalisz K and Garfield RE: Inducible nitric oxide synthase is present in the rat placenta at the fetal-maternal interface and decreases prior to labour. *Mol Hum Reprod* 3: 485-491, 1997.
- Ali M, Buhimschi I, Chwalisz K and Garfield RE: Changes in expression of the nitric oxide synthase isoforms in rat uterus and cervix during pregnancy and parturition. *Mol Hum Reprod* 3: 995-1003, 1997.
- Huang J, Roby KF, Pace JL, Russell SW and Hunt JS: Cellular localization and hormonal regulation of inducible nitric oxide synthase in cycling mouse uterus. *J Leukoc Biol* 57: 27-35, 1995.
- Dong YL, Fang L, Gangula PR and Yallampalli C: Regulation of inducible nitric oxide synthase messenger ribonucleic acid expression in pregnant rat uterus. *Biol Reprod* 59: 933-940, 1998.
- Ogando D, Farina M, Ribeiro ML, Perez Martinez S, Cella M, Rettori V and Franchi A: Steroid hormones augment nitric oxide synthase activity and expression in rat uterus. *Reprod Fert Dev* 15: 269-274, 2003.
- Little B, Billiar RB, Rahman SS, Johnson WA, Takaoka Y and White RJ: *In vivo* aspects of progesterone distribution and metabolism. *Am J Obstet Gynecol* 123: 527-534, 1975.
- Tiboni GM, Giampietro F and Lamonaca D: The soluble guanylate cyclase inhibitor methylene blue evokes preterm delivery and fetal growth restriction in a mouse model. *In Vivo* 15: 333-337, 2001.
- Yallampalli C, Buhimschi I, Chwalisz K, Garfield RE and Dong YL: Preterm birth in rats produced by the synergistic action of a nitric oxide inhibitor (NG-nitro-L-arginine methyl ester) and an antiprogesterin (onapristone). *Am J Obstet Gynecol* 175: 207-212, 1996.
- Mesiano S: Myometrial progesterone responsiveness and the control of human parturition. *J Soc Gynecol Investig* 11: 193-202, 2004.

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