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Metformin Prevents the Increase of Nitric Oxide and Lipid Peroxidation Induced By Dehydroepiandrosterone in Early Pregnant Mice

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Abstract: The aim of this work was to study the effects of dehydroepiandrosterone (DHEA) and metformin (M) on nitric oxide (NO) system and oxidative stress in embryo implantation sites of early pregnant mice. The biguanide M is used for treating polycystic ovary syndrome but its complete mechanism of action remains unknown. Nitric oxide (NO) has important protective roles during pregnancy, keeping uterine relaxation and vascular function. However, its overproduction leads to nitrative stress by producing reactive nitrogen species. Here we measured NO content by Griess method and the localization of inducible and endothelial nitric oxide synthase (iNOS and eNOS) by immunohistochemistry in implantation sites. Also we measured lipid peroxidation by TBA-RS, glutathion by Ellman's reaction and antioxidant enzymes by enzymatic kinetics in uterine homogenates. We found that the expression of both iNOS and eNOS and the NO content were increased with DHEA ($p<0.001$ for all) and restored to control levels with DHEA+M. Oxidative stress: DHEA increased lipid peroxidation ($p<0.01$) and glutathione (GSH, $p<0.01$). With DHEA+M lipid peroxidation was restored to control levels. The activities of the antioxidant enzymes superoxide dismutase and catalase were not modified. We conclude that hyperandrogenization with DHEA enhances the NO system and lipid peroxidation in implantation sites of early pregnant mice and that M treatment prevents these effects.

Keywords: DHEA, metformin, nitric oxide, oxidative stress, polycystic ovary syndrome, pregnancy.

INTRODUCTION

Nitric oxide (NO) is an essential metabolite involved in vascular function and in numerous physiological processes that maintain homeostasis in mammals. It is a vasodilator agent and it has a biological role to modulate local blood flow and platelet function [1-4]. The production of NO is due to three isoforms of the NO synthase. In healthy vessels endothelial NOS (eNOS) and neuronal NOS (nNOS) constitutively expressed, Ca-dependent enzymes. On the other hand, when the vascular system is diseased and defense mechanisms are activated, the mediators of inflammation induce a NO synthase non-responsive to calcium (iNOS) which produces large quantities of NO in most of the cells of the vessel wall [4-6]. An inadequate production of NO could play a role in many vascular diseases such as hypertension, atherosclerosis, or vascular hyporeactivity associated with septicaemic shock [1].

In pregnancy, NO plays important roles in implantation, decidualization, vasodilatation of decidual, placental and uterine vessels and myometrial relaxation [7]. It has been reported that NO participates in vascular invasion by the trophoblast, and it may play a role in maintaining uterine quiescence during pregnancy [8-11]. However, an overproduction of NO can lead to the increase of reactive nitric oxide species (RNOS, more aggressive oxidant species than reactive oxygen species: ROS) [12]. NO could have a dual action (protective or pro-oxidant) in the corpus luteum development [13, 14]. Peroxynitrite (ONOO-/ONOOH), the product of the reaction of NO with superoxide (O_2^-), leads to nitrative stress, a pro-oxidant and to a pro-inflammatory status, which is associated to pregnancies complicated by preeclampsia, intrauterine growth restriction, pregestational diabetes and miscarriage [13-17]. Besides, it was found that eNOS gene influences the risk of pre-eclampsia and the recurrence of negative pregnancy events [18].

Although NO synthases iNOS and eNOS have been identified in rodent uterus via western blot analysis and immunohistochemistry during implantation and late gestation [19, 20], very few localization and/ or quantification studies of NOS and NO during early pregnancy have been performed.

Dehydroepiandrosterone (DHEA) is the most abundant androgen found in women with polycystic ovary syndrome (PCOS) [21]. This hormone is involved in immune homeostasis and is increased in normal pregnancy contributing to the development of gestation [22]. It has been reported that

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DHEA regulates vascular function, having a protective role [23]. However, abnormally increased DHEA leads to an imbalance in ovarian function and in detrimental effects on endometrial function. This results in low implantation rates and miscarriage [24-27]. Simoncini *et al.* 2003 have reported that DHEA induces NO synthesis by a direct effect on eNOS [28]. We have previously developed a murine model hyperandrogenized with DHEA which reflects endocrine and immune aspects of women with PCOS during early pregnancy [26].

The insulin-sensitizing drug metformin (M) is widely used in the treatment of type II diabetes mellitus [29, 30] and PCOS [31, 32]. These conditions are accomplished with a decreased antioxidant capacity that could contribute to the known increased risk of cardiovascular diseases as atherosclerosis or hypertension in patients with diabetes and PCOS [33, 34]. The biguanide M is a non-hormonal treatment that has shown to reduce oxidative stress in numerous systems. Apparently it exerts its antioxidant effects directly scavenging ROS or indirectly modulating the intracellular ROS production [35-40]. Moreover, NO has been proposed as an intermediate of the M action [41]. In women with PCOS, it has been seen that M treatment decreases androgen levels, improves frequency of menstrual cycle and ovulation [42-46] and prevents abortions in early pregnant women with PCOS [47-50]. Considering that there are an increasing number of women with PCOS who became pregnant after M treatment it is expected that the use of this drug around and during the time of pregnancy will increase. However, the clinical practice is ahead of the knowledge of the mechanism involved, which remains partially unknown.

In recent works using our DHEA-treated-early pregnancy model we have shown that M prevented some endocrine and immune alterations induced by DHEA: embryo resorption, glucose, progesterone, progesterone-induced blocking factor (PIBF) and cyclooxygenase2 (COX2) in the implantation sites, cytokine production, ovarian oxidative stress and uterine NOS activity [51, 52].

In the present report we focused on determining the localization of iNOS and eNOS, as well as the NO production and oxidative stress at the implantation sites during early pregnancy. We also were interested in knowing the local effects of DHEA and M on these parameters. In this way, we are expanding our previous findings on the mechanisms by which DHEA induces and M prevents embryo resorption on early pregnant BALB/c mice.

MATERIALS AND METHODS

Animals

All procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (The National Academies Press, Washington, D.C., 2011 [53]). The studies were approved by the Independent Committee on Ethics in Research of the School of Medicine of the University of Buenos Aires. The mice were housed under controlled temperature (22 º C) and illumination (14 h-light, 10-h darkness; lights on at 05:00) and allowed free access to Purina Rat Chow and water. A total of 86 virgin female BALB/c mice (8-12-week-old) were paired with BALB/c males (8-12-week-old). The animal model used has been previously described [52]. Briefly, the day of appearance of a coital plug was taken as day 0 of pregnancy. Implantation occurs in the morning of the 5th day, therefore, on 6th and 7th days (post-implantation) animals were divided in 4 groups: Control (supplied with vehicles), M (treated orally by canulla with 240 mg/kg of metformin in 0.1 ml of water), DHEA (injected s.c. with 60 mg/kg of dehydroepiandrosterone in 0.1 ml of sesame oil) and DHEA+M (treated under the same conditions with DHEA and M). On day 8 of pregnancy (bodyweight 20 ± 2 g), animals were euthanized by cervical dislocation. After embryos were removed, uterine tissues from 14 animals per group were divided as follows: 7 uteri of each group were immediately homogenized in buffer Tris-Base 20 mM, pH=7, 6 to determine oxidant-antioxidant parameters (superoxide dismutase and catalase activity, lipid peroxidation, glutathione and nitrites concentration). The remaining 7 uteri were immediately fixed in 4 % (w/v) paraformaldehyde to carry out immunohistochemical determination of eNOS and iNOS.

In order to test any long-term adverse effect of the treatments, 3 additional groups of mice (10 animals each) including Control, M and DHEA+M, were allowed to proceed to parturition. These animals had a normal pregnancy with normal number and morphology of pups (8- 12 pups/litter).

Experimental Procedures

Immunohistochemical Localization of iNOS and eNOS

The immunolocalization of iNOS and eNOS was performed on implantation sites. Uterine sections were stained with immunoperoxidase staining kit CSA/ HRP (Dako). Briefly, uterine tissues fixed in 4% paraformaldehyde were embedded in paraffin wax, consecutively cut (6-µm-thick sections) and placed on silanized glass slides (Biobond; British Biocell International, Cardiff). Only sections that passed through the center of the implantation sites were selected.

Then, tissue sections were deparaffinized, rehydrated through a series of graded alcohols and washed in PBS. Endogenous peroxidase activity was blocked with 0.1 % (v/v) hydrogen peroxide for 50 min. Non-specific binding sites were blocked by treating tissues with TNB blocking reagent (NEN Life Science Products, Boston, MA, USA). Then, sections were incubated overnight at 4º C with rabbit polyclonal anti- human eNOS and iNOS antibodies respectively (Cayman Chemical Company, Ann Arbor, MI, USA) diluted 1:200 in blocking buffer. Control sections were processed without primary antibody. Sections were then incubated for 30 min with biotinylated anti-rabbit antibody, washed in PBS and treated with streptavidin–biotin complex for 30 min. The reaction was visualized by diaminobenzidine (DAB staining kit, Dako; Hamburg, Germany) and sections were counterstained with haematoxylin and covered with DPX (Sigma-Aldrich, St Louis, MO, USA).

Immune mark was analyzed using Image-Pro Plus version 4.1 (Media Cybernetics, Silver Spring, MD). It was determined marked area/ total area and expressed as a percentage.

Nitric Oxide Concentration

Nitrites concentration was determined by the Griess reaction, a colorimetric assay based on the reaction of $NaNO_2$

with sulfanilic acid that produces color readable at 540 nm [54]. Samples were incubated with sulfanilic acid and values were referred to a $NaNO_2$ standard curve. NO_2 concentration was expressed as nmol NO_2/mg protein.

Oxidative Stress-Related Parameters

Uterine tissues without embryos were homogenized in buffer Tris-Base 20 mM, pH=7,6 with a Teflon-glass homogenizer on ice. The suspension was centrifuged at 9000 ×*g* for 10 min at 4°C. The pellet was discarded and the supernatant was saved and stored at –20º C for the oxidative stress determinations that are described ahead and for the protein content determination by Bradford method.

Superoxide Dismutase (SOD)

SODs are a group of metalloenzymes that detoxify ROS through the conversion of $O_2^{\bullet-}$ to hydrogen peroxide and molecular oxygen. Total SOD activity was assayed by a spectrophotometric method based on the inhibition of a superoxide-induced epinephrine oxidation [55]. Briefly, 30 mM epinephrine in 0.05% v/v acetic acid was added to 50 mM glycine buffer (pH = 10,2) with uterine homogenates. SOD activity was measured at 480 nm for 4 min. One unit (U) is equivalent to the amount of enzyme that inhibited the oxidation of epinephrine by 50%. It was expressed as USOD/ mg protein.

Catalase

The enzyme catalase promotes the conversion of hydrogen peroxide (H_2O_2) into water and oxygen ($H_2O + O_2$). The consumption of H_2O_2 was monitored by spectrophotometer in a reaction medium consisting of 50 mM phosphate buffer, pH 7,2 and 3 mM H_2O_2 every 10 sec during 1 min at 240 nm [56]. Results were expressed as nmol catalase/ mg protein.

Lipid Peroxidation

The amount of malondialdehyde (MDA), product of the breakdown of polyunsaturated fatty acids, may be taken as an index of peroxidation reaction. The TBA reactive species (TBA-RS) method was previously described [57] and quantifies MDA, that reacts with trichloroacetic acid (TCA) 15% (w/v)- thiobarbituric acid (TBA) 0.375% (w/v)- HCI 0.25M, yielding a red compound that absorbs at 535 nm. Homogenates were treated with TCA-TBA-HCl and heated for 15 min in water at 100°C. After cooling, the flocculent precipitate was removed by centrifugation at 1000 ×*g* for 10 min. The absorbance of samples was determined at 535 nm. Results were expressed as nmol MDA/ mg protein.

Glutathione Content

The GSH assay was previously described [58]. The reduced form of GSH comprises the bulk of cellular protein sulphydryl groups. Thus, measurement of acid-soluble thiol is used for estimation of GSH content in tissue extracts. Briefly, 300 µl of uterine homogenates were incubated with buffer Tris 1.75 M (pH= 7, 4) containing NADPH and GSH reductase. The reaction involves the enzymatic reduction of the oxidized form (GSSG) to GSH. When Ellman's reagent (a sulphydryl reagent 5, 5-dithiobis-2 nitrobenzoic acid; Sigma-Aldrich, St Louis, MO, USA) is added to the incubation medium, the chromophoric reaction product absorbs at 412 nm with a linear increase during 6 min; after this, the reaction remains constant. Results were expressed as mmol GSH/mg protein.

Statistical Analysis

Statistical analyses were carried out using the Instat program (GraphPAD software, San Diego, CA, USA). One way ANOVA test was used (Tukey post-test Multiple Comparison that compares all the pairs of columns). A P value < 0.05 was considered significant. Results are presented as mean ± SEM.

RESULTS

Expression of iNOS and eNOS in Implantation Sites

The immunohistochemical analysis of iNOS and eNOS in implantation sites showed that in Control and DHEA+M these enzymes are expressed in trophoblastic and maternal blood cells (Fig. **1A** and **C** for iNOS, **2A** and **C**, for eNOS: tr, bc). The expression in M treated mice showed an appearance similar to Control (picture not shown). In DHEA treated mice these proteins showed an increased expression on decidual matrix when compared to Control (Fig. **1B** and **2B**: md and amd). Quantification of marked area/ total area by Image Pro Plus revealed that DHEA increased the protein expression of iNOS (Fig. **1D**, One way ANOVA *** $p<0.001$) and eNOS (Fig. **2D**, One way ANOVA *** $p<0.001$). Treatment with metformin (DHEA+M) partially avoided this increase for iNOS while eNOS expression was significantly lower than Control (Fig. **2D**, One way ANOVA *** $p<0.001$).

Nitric Oxide Concentration

Nitric oxide (NO) concentration in uterine homogenates without embryos was increased by DHEA respect to Control (Fig. **3**, One way ANOVA *** $p<0.001$). This increase was avoided by the treatment with metformin (DHEA+M).

Oxidant/ Antioxidant Status

Four parameters of the oxidant/ antioxidant status were measured in uterine homogenates without embryos: superoxide dismutase and catalase, lipid peroxidation and glutathione. Superoxide dismutase and catalase showed no differences between treatments (Fig. **4A** and Fig. **4B** respectively, One way ANOVA $p>0.05$). Lipid peroxidation suffered an increase in hyperandrogenized mice (DHEA) respect to Control (Fig. **4C**, One way ANOVA ** $p<0.01$) and metformin treatment prevented this effect (DHEA+M). The antioxidant metabolite glutathione was increased both with DHEA and with DHEA+M (Fig. **4D**, One way ANOVA ** $p<0.01$).

DISCUSSION

DHEA- Hyperandrogenization

The present murine model resembles the endocrine and immune profile of early pregnant women with PCOS treated with metformin [51, 52]. High concentrations of DHEA induce a pro-inflammatory environment and triggers the synthesis of nitric oxide (NO) [52,59,60]. Besides, this induces the loss of luteal function and embryo resorption [26,51,61]. The present findings show the increase in NOS expression and in NO production after hyperandrogenization. We propose

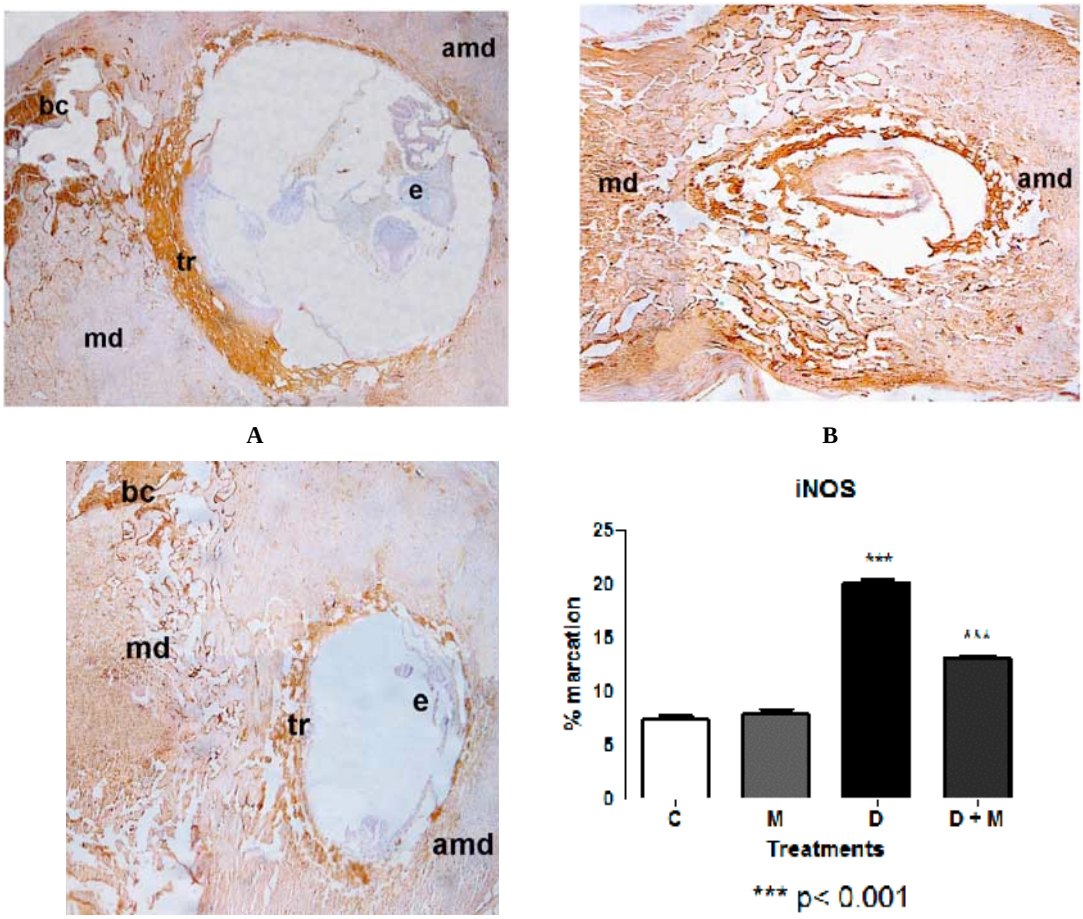
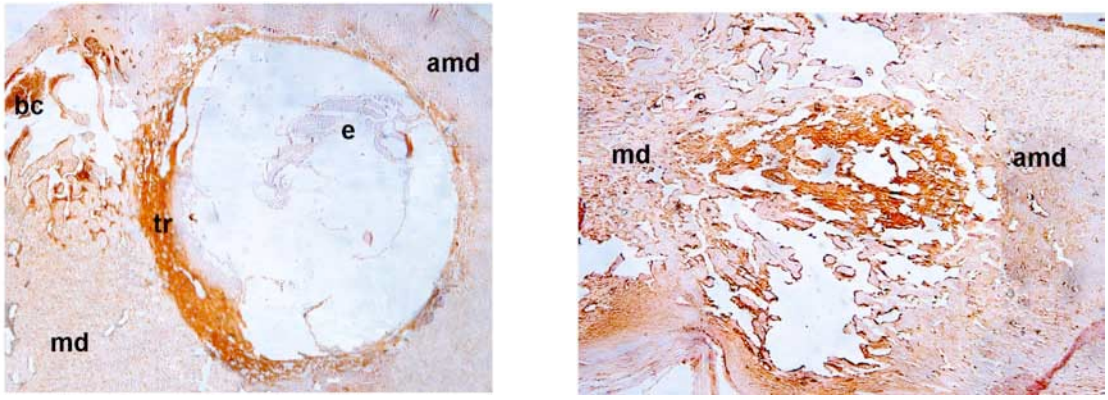
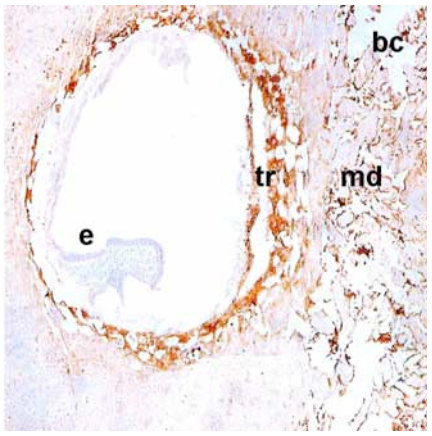
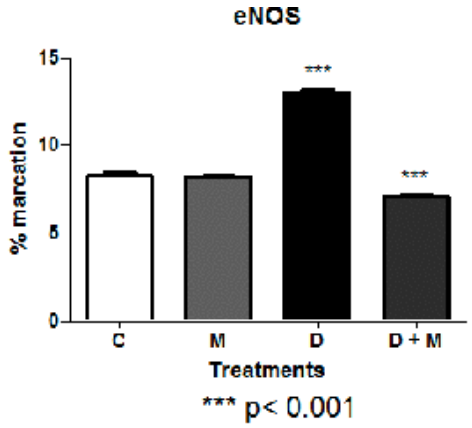


Fig. (1). immunolocalization for iNOS in implantation sites from 8 days pregnant mice. Immunohistochemistry was performed in uterine sections incubated with anti-iNOS antibody and stained with an immunoperoxidase staining kit. The intense brown color indicates positive staining. **A**: Control (40 X). iNOS immunoreactivity is present in trophoblastic (see **tr**) and in maternal blood cells (see **bc**). **B**: DHEA (40 X). The positive staining is extended to the entire decidual matrix (see **md** and **amd**). In this slide it is possible to see a remaining trophoblastic tissue that is also positive for iNOS. **C**: DHEA+M (40 X). The localization of iNOS is similar to Control, but the decidual matrix shows a more intense staining than this group. **D**: Quantification of marked area / total area by Image Pro Plus. iNOS expression was increased in DHEA treated mice. M treatment partially prevented this effect. Error bars represent the mean $\pm$SEM, one way ANOVA *** $p<0.001$, N=7/ group.

e: embryo. **tr**: trophoblast. **md**: mesometrial decidua. **amd**: antimesometrial decidua. **bc**: maternal blood cells.







C **D**

Fig. (2). immunolocalization for eNOS in implantation sites from 8 days pregnant mice. Immunohistochemistry was performed in uterine sections incubated with anti-eNOS antibody and stained with an immunoperoxidase staining kit. The intense brown color indicates positive staining. **A**: Control (40 X). eNOS immunoreactivity is present in the trophoblastic (see **tr**) and in maternal blood cells (see **bc**). **B**: DHEA (40 X). The positive staining is extended to the entire decidual matrix (see **md** and **amd**). In this slide it is possible to see a remaining trophoblastic tissue that is also positive for eNOS. **C**: DHEA+M (40 X). The localization of eNOS is similar to Control. **D**: Quantification of marked area / total area by Image Pro Plus. eNOS expression was increased in DHEA treated mice. eNOS expression in DHEA+M was significantly lower than in Control. Error bars represent the mean ±SEM, one way ANOVA *** p<0.001, N=7/ group.

e: embryo. **tr**: trophoblast. **md**: mesometrial decidua. **amd**: antimesometrial decidua. **bc**: maternal blood cells.

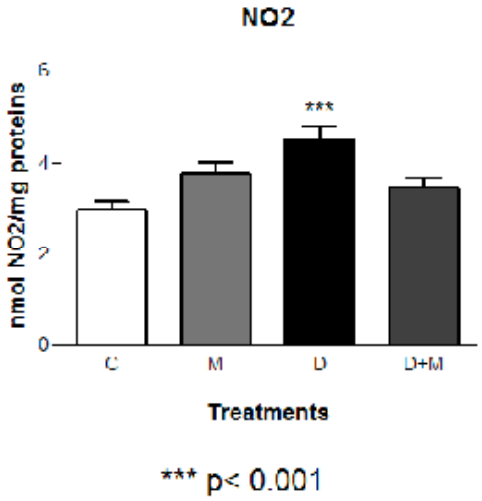
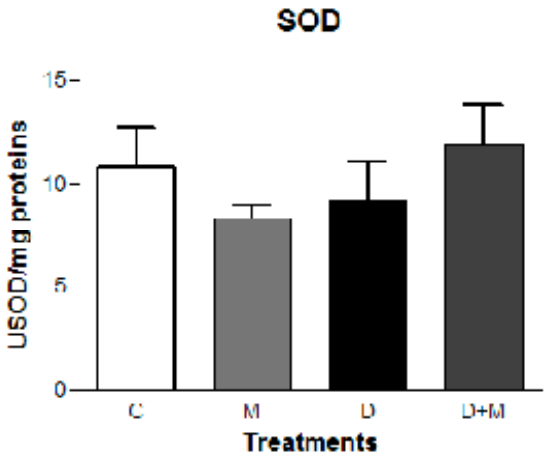
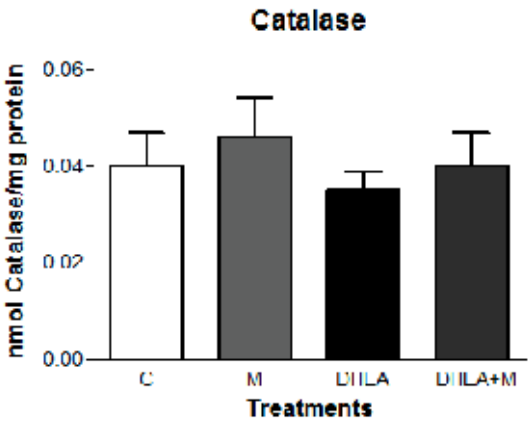


Fig. (3). Nitrites concentration was determined by the colorimetric Griess method in uterine homogenates without embryos from 8 days pregnant mice. The hyperandrogenized group (DHEA) showed a significant increase in nitrites respect to Control that was avoided by the simultaneous treatment with metformin (DHEA+M). Error bars represent the mean± SEM, one way ANOVA *** p<0.001, N=7/ group.





A **B**

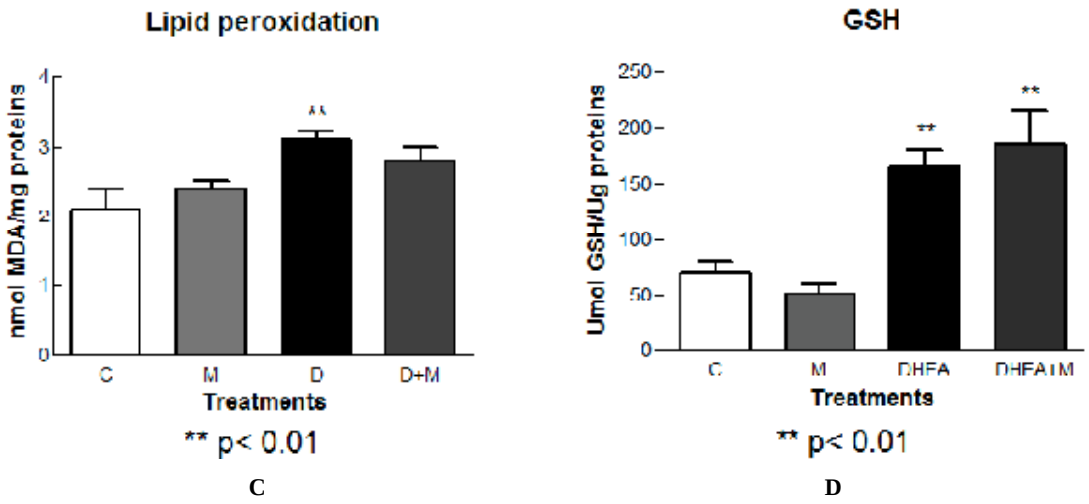


Fig. (4). oxidative stress in uterine homogenates without embryos from 8 days pregnant mice.

A: the concentration of the antioxidant enzyme superoxide dismutase (SOD) was determined by a colorimetric method that measures epinochrome disappearance. This enzyme showed no differences between treatments. Error bars represent the mean± SEM, one way ANOVA $p>0.05$, N=7/ group.

B: the concentration of the antioxidant enzyme catalase was determined by a colorimetric method that measures epinephrine disappearance. This enzyme showed no differences between treatments. Error bars represent the mean± SEM, one way ANOVA $p>0.05$, N=7/ group.

C: the product of lipid peroxidation malondialdehyde (MDA) was detected by TBA-RS. Hyperandrogenized mice (DHEA) suffered an increase in lipid peroxidation respect to Control and M treatment prevented this effect (DHEA+M). Error bars represent the mean±SEM, one way ANOVA ** $p<0.01$, N=7/ group.

D: the concentration of the antioxidant metabolite GSH was measured by a colorimetric method that detects the GSH reductase activity. Hyperandrogenization increased GSH. The treatment with metformin (DHEA+M) showed no differences with DHEA and was different from Control. Error bars represent the mean±SEM, one way ANOVA ** $p<0.01$, N=7/ group.

that NO has an important role in the cascade of events triggered by high concentrations of DHEA that leads to embryo resorption. We base this idea on the increased levels of NO, iNOS and eNOS in DHEA-hyperandrogenized mice, with an increased rate of embryo resorption and on previous reports showing a direct effect of DHEA on NO production modulating iNOS and eNOS expression [28, 51]. In a previous work of our group using the same mouse model of hyperandrogenization with DHEA, we have observed a decrease in NOS activity [51]. The increase in the expression and the decrease in the activity of NOS seem contradictory; nevertheless, a possible explanation could be that NOS is increased by high levels of DHEA leading to an overproduction of NO that could down-regulate the activity of NOS. This mechanism of regulation of NO has been seen in other experimental models [62, 63].

The increased concentrations of NO are accomplished by oxidative stress in the uterine tissue. This is reflected in the increase in lipid peroxidation, index of the oxidative damage to the tissue. Regarding the antioxidant defenses, the enzymes measured here are not modified by any of the treatments but the antioxidant metabolite GSH is increased by DHEA treatment, possibly as a reaction to the oxidation. The association between elevated NO and oxidative stress agrees with the previous knowledge about the pro-oxidant effect of high levels of NO in corpus luteum development [13, 16-18].

In this study we localized for the first time the enzymes iNOS and eNOS in murine implantation sites during early pregnancy: these enzymes are expressed in the trophoblastic and maternal blood cells. This localization could be explained because of the very dynamic remodeling of maternal vessels in implantation sites during early pregnancy [8-11].

Treatment with Metformin

In previous reports we have seen that the treatment with metformin prevents most of the effects induced by DHEA in mice having protective effects on ovaries and uterus. [51, 52, 64]. In the present work we demonstrate that M totally prevents the increase in NO induced by DHEA. This prevention is not due to an effect on the NOS activity (as we have seen in a previous work [51]), but to an effect on the NOS expression. Regarding the expression of iNOS, this treatment reverts partially the increase induced by DHEA, whereas with regard to the eNOS M reverts its increase and even we observe a decrease in DHEA+M respect to Control that we cannot explain.

M is known to have an antioxidant role [35-40]. Our present results are in agreement with this because M prevents the increase in lipid peroxidation induced by DHEA. GSH is also elevated in DHEA+M. The increase in GSH could have a role in the prevention of lipid peroxidation because it is an instantaneous antioxidant defense.

The treatment with metformin seems to be a good option for maintaining pregnancy to term in patients with PCOS. Regulation of nitric oxide by M probably favors the normal vascular function in these patients. It could contribute to decreasing the risk of cardiovascular diseases [33, 34]. Besides, M is a non-hormonal treatment and did not show to be teratogenic, making it a good choice for patients with PCOS [50, 64].

There remain some aspects to be studied, including long time effects of this treatment in pregnancy. It would be interesting to focus on the offspring born under metformin treatment, its glucose metabolism, its endocrine regulation and reproductive performance.

CONCLUSION

On the basis of the present results, we conclude that DHEA induces and metformin prevents a detrimental increase in NOS and NO and lipid peroxidation in implantation sites during early pregnancy in mice. This work contributes to the knowledge and explanation of the etiology of PCOS during early pregnancy. It investigates the mechanism of action of DHEA, one of the principal androgens present in PCOS, and of metformin, a promising drug for treatment of PCOS. Moreover, this study deepens the knowledge of some important factors that are decisive for pregnancy, like NOS-NO system and oxidant/antioxidant balance. These factors contribute to pregnancy success or fail and lead to miscarriage.

ABBREVIATIONS

COX2	=	cyclooxygenase 2
DHEA	=	dehydroepiandrosterone
eNOS	=	endothelial nitric oxide synthase
g	=	times gravity
GSH	=	Glutathione
h	=	hours
HCI	=	hydrochloric acid
iNOS	=	inducible nitric oxide synthase
Kg	=	kilograms
M	=	metformin
MDA	=	malondialdehyde
mg	=	milligrams
min	=	minutes
µl	=	microliters
mM	=	millimolar
NADPH	=	Nicotinamide adenine dinucleotide phosphate
nm	=	nanometers
NO	=	Nitric oxide
PCOS	=	polycystic ovary syndrome
PIBF	=	progesterone-induced blocking factor
pmol	=	picomoles
RNOS	=	reactive nitric oxide species
ROS	=	reactive oxygen species
sc	=	subcutaneously
sec	=	seconds
SOD	=	Superoxide dismutase
TBA	=	thiobarbituric acid
TCA	=	trichloroacetic acid
w/v	=	weight/volume

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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