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The Influence of Dehydroepiandrosterone on Early Pregnancy in Mice

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Key Words
Dehydroepiandrosterone · Estrogen · Progesterone · Implantation · Miscarriage

Abstract
The aim of the present report was to study the role of high levels of dehydroepiandrosterone (DHEA) on the ovarian function and embryonic resorption during early pregnancy in BALB/c mice. Pregnant animals were injected with DHEA following both the post-implantatory (DHEA-2) and peri-implantatory (DHEA-6) models. Morphological studies of implantation sites showed 40% of embryonic resorption in the DHEA-2 group while 100% of resorption was observed in the DHEA-6 group. Serum samples of both DHEA-2 and DHEA-6 groups showed higher estradiol levels and a lower progesterone concentration than those of control groups. Ovarian prostaglandin E levels after both DHEA-2 and DHEA-6 treatments increased when compared to control groups. The antioxidant metabolite glutathione diminished during both DHEA treatments. In summary, the data presented here suggest that DHEA treatment during early pregnancy modulates the ovarian function and is responsible for embryonic resorption with different degrees depending on when it is administered.

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Introduction

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are androgen precursors mainly secreted from the adrenal cortex in humans [1]. DHEAS has been suggested to be a precursor and a reservoir of DHEA that, in turn, produces sex steroids. Thus, the serum concentration of DHEAS is higher than that of any other steroids. DHEA is released by the action of endogenous DHEAS sulfatase, which is widely distributed in peripheral tissues [2]. During pregnancy, DHEAS is a major source for estrogen formation in the fetoplacental unit. About one half of total estradiol produced in the placenta originates from maternal DHEAS. Increased levels of serum DHEA have been suggested to suppress immune reactions during pregnancy by means of modifying cytokine levels and thus ensuring the development of gestation [3–5]. Thus, maternal adrenal production rates of DHEA and DHEAS are increased twofold, but the maternal concentration of DHEAS is reduced to between one third and one half of the nonpregnancy levels [6, 7]. The decrease in maternal DHEAS has been suggested to be associated with enhanced estrogen biosynthesis in the placenta. Although DHEA has been demonstrated to possess more biological activity than DHEAS, only a limited number of studies of DHEA in pregnancy have been re-

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ported. While the deletion of P450c17 (the enzyme responsible for the synthesis of DHEA and other steroids) has been found to cause early embryonic lethality [8], abnormally increased levels of DHEA lead to an imbalance of the ovarian function that results in miscarriage [9]. In addition, it has been reported that higher levels of androgens than those of normal controls could be responsible for a detrimental effect on endometrial function, resulting in women with recurrent miscarriages [10].

Luteal regression or luteolysis is a normal and necessary event in the mammalian reproductive cycle. This mechanism has been widely studied in relation to its involvement in early pregnancy dysfunction. Corpus luteum involution has been related to an increased generation of reactive oxygen species [11, 12]. Protection against reactive oxygen species is provided by enzymes (superoxide dismutase, catalase and glutathione peroxidase), metabolites, e.g. glutathione (GSH), or antioxidant vitamins [13–15] and has been suggested to be endocrine regulated [14, 16]. During normal pregnancy, an increased antioxidant status has been reported [17]. Moreover, it has been proposed that the activities of antioxidant enzymes in the corpus luteum are subject to major changes during early pregnancy, suggesting that the corpus luteum of early pregnancy may be rescued from luteolysis through increasing activities of key antioxidant enzymes or metabolites and inhibition of apoptosis [18].

Finally, among the ovarian regulators acting in an autocrine or paracrine fashion, we have to consider prostaglandins (PGs), synthesized from arachidonic acid which, in turn, modulates different ovarian functions, such as the rupture of ovarian follicles associated with ovulation [19, 20] and luteolysis [15, 21]. Particularly, PGE has been studied as an immunosuppressive molecule [22]. Moreover, in a previous report we have found that the addition of PGs to the incubation medium of ovarian tissues resulted in the diminution of ovarian GSH content [15]. We have also demonstrated that the injection of DHEA on prepuberal mice increased ovarian PGE and diminished GSH content [23].

In order to know the possible effect of androgenization during the process of implantation, we used two pregnant mouse models: peri- and post-implantatory, obtained by daily injection of DHEA of pregnant BALB/c mice.

The periods of time studied, i.e. peri- and post-implantatory, allowed us to study the role of DHEA on both the implantation process and embryonic resorption.

Materials and Methods

Animals and Experimental Protocol

In order to study the effects of high levels of circulating androgens in the development of early pregnancy, we injected DHEA during the peri- and post-implantatory periods. Briefly, BALB/c 8- to 12-week-old virgin female mice were paired with 8- to 12-week-old BALB/c males. The day of appearance of a coital plug was taken as day 0 of pregnancy and the implantation developed during the 5th day of pregnancy. Animals were cared for in accordance with the principles and guidelines of the *Guide for the Care and Use of Laboratory Animals* (US National Research Council, 1996). Mice received food and water ad libitum and were exposed to a 12-hour light,12-hour dark cycle. Pregnant mice were divided into five groups:

(i) post-implantatory group (DHEA-2): pregnant mice were injected subcutaneously with DHEA (6 mg/kg body weight dissolved in 0.10 ml sesame oil) on days 6 and 7 of pregnancy; animals were anesthetized with ether and sacrificed on day 8 of pregnancy by decapitation;

(ii) peri-implantatory group (DHEA-6): pregnant mice were injected subcutaneously with DHEA (6 mg/kg body weight dissolved in 0.10 ml sesame oil) from day 2 to day 7 of pregnancy; animals were anesthetized with ether and sacrificed on day 8 of pregnancy by decapitation;

(iii) controls of the post-implantatory group (control 2): pregnant mice were injected subcutaneously with 0.1 ml of sesame oil on days 6 and 7 of pregnancy and killed on day 8;

(iv) controls of the peri-implantatory group (control 6): pregnant mice were injected subcutaneously with 0.1 ml sesame oil from day 2 to day 7 of pregnancy and killed on day 8;

(v) control pregnant group (control): Pregnant mice without treatment were killed on day 8 of pregnancy; data are shown in figures (serum estradiol and progesterone levels and ovarian GSH content) as the 'control' group.

Twelve animals (n = 12) per group were used, and the experiments were carried out twice. On day 8 of pregnancy, samples were isolated as follows:

(1) blood from the five groups was collected and treated as described below to evaluate progesterone and estradiol levels;

(2) after their removal, uterine tissues were immediately fixed in 4% (w/v) paraformaldehyde to carry out morphological studies to evaluate the resorption rate;

(3) ovarian tissues were removed and immediately frozen at –20°C until used: 12 ovaries for PGE determination and 12 for ovarian GSH content.

Morphological Studies

To study the effect of DHEA on the implantation process in each implantation site, the uterus and decidua were separated and fixed as described above. Tissues were consecutively cut (6 μm/section) and placed on gelatin-coated slides (Biobond; British Biocell International, Cardiff, UK) and air-dried for 2 h before being treated for 5 min in acetone at 4°C. Only sections that passed through the center of the implantation site were selected to be stained with hematoxylin and eosin (Dako Corporation, Carpinteria, Calif., USA).

Estradiol and Progesterone Determination

In order to evaluate the effect of androgenization on the ovarian function, serum progesterone and estradiol levels were analyzed

in the peri- and post-implantatory groups treated with DHEA and compared to control groups. Serum samples were treated as described before [15]: briefly, the blood was allowed to clot, and serum was removed and frozen until either progesterone or estradiol concentrations were determined by radioimmunoassay. Both antisera were provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, Colo., USA). The progesterone antiserum was highly specific for progesterone with low cross-reactivity: <2.0% for 20 α-dihydroprogesterone and deoxycorticosterone, and 1.0% for other steroids normally present in the serum. The sensitivity was 5–10 pg/tube, so 2–5 μl of serum was routinely assayed. The estradiol antiserum showed low cross-reactivity: <1% for progesterone and testosterone, <5% for 17-estradiol and estriol and <10% for estrone. Both results were expressed as nanograms per milliliter serum.

PG Radioimmunoassay

The tissue (i.e. each ovary) was weighed and incubated in Krebs-Ringer bicarbonate with glucose (11.0 mmol/l) as external substrate (pH = 7.0) for 1 h in a Dubnoff metabolic shaker under an atmosphere of 5% CO_2 in 95% O_2 at 37°C. At the end of the incubation period, the tissue was removed and the solution was acidified to pH 3.0 with 1 *M* HCl and extracted for PG determination 3 times with 1 volume of ethyl acetate. Pooled ethyl acetate extracts were dried under an atmosphere of N_2 and stored at –20°C until PG radioimmunoassay was performed. PGE was quantified using rabbit antiserum from Sigma Chemical Co. (St. Louis, Mo., USA). Sensitivity was 10 pg/tube, and cross-reactivity was 100% PGE and <0.1% with other PGs. Results were expressed as picograms PGE per milligram protein. Ovarian protein content was determined by the Bradford method. Six ovaries from each of the five groups described above were employed.

Ovarian Glutathione Content

The glutathione assay was carried out as previously described [15]. The reduced form of glutathione (GSH) comprises the bulk of cellular protein sulfhydryl groups. Thus, measurement of acid-soluble thiol is commonly used for the estimation of GSH content in tissue extracts. Briefly, 300 μl of homogenates obtained from pooled tissues (3 ovaries from different animals for each point) in 0.5% v/v trichloroacetic acid were incubated with 1.75 *M* Tris buffer (pH = 7.4) containing NADPH and glutathione reductase. The reaction involves the enzymatic reduction of the oxidized form to GSH. When Ellman's reagent (a sulfhydryl reagent, 5,5-dithiobis-2-nitrobenzoic acid; Sigma) is added to the incubation medium, the chromophoric product resulting from this reaction develops a molar absorption at 412 nm that is linear to the first beyond 6 min; after this, the reaction remains constant. Results were expressed as micromoles GSH per gram ovarian tissue. Twelve ovaries from each of the five groups described above were employed.

Statistical Analysis

Statistical analyses were carried out using the Instat program (Graph Pad software, San Diego, Calif., USA). One-way ANOVA and nonparametric tests (Newman-Keuls multiple comparison) that compare all the pairs of columns were used. A p value <0.05 was considered significant.

Results

Effect of DHEA on Early Pregnancy: Role in the Implantation Process

The embryo, trophoblast and decidua with abundant lacunae were observed in control animals on day 8 of pregnancy (fig. 1). In contrast, the analysis of implantation sites from different animals showed that DHEA treatment during the post-implantatory period (days 6 and 7 of pregnancy: DHEA-2) yielded 40% of embryo resorption. It was also observed that the decidua near the embryo was totally necrotized with some cellular debris (fig. 2). When DHEA was injected during the peri-implantatory period (from day 2 to day 7 of pregnancy: DHEA-6) 100% of embryonic resorption was found as can be concluded from the examination of uterine morphology after hematoxylin and eosin staining. These results were confirmed by serum progesterone determination that diminished to nonpregnant values (data not shown). The uterus presented a morphological appearance as of a nonpregnant mouse (fig. 3). The experiments were repeated until 36 sections from each group were analyzed. Table 1 summarizes the data obtained.

Serum Estradiol and Progesterone Levels during DHEA-Induced Embryonic Resorption

The DHEA injection was able to increase significantly serum estradiol levels during both post- (DHEA-2) and peri-implantatory (DHEA-6) periods of treatment (fig. 3: c vs. b and e vs. d, $p < 0.001$). Moreover, the effect of DHEA was strongly marked when it was injected during the longer administration (DHEA-6; fig. 4: e vs. c, $p < 0.001$). No significant differences between controls, i.e. pregnancy, control 2 and control 6, were found.

Figure 5 represents the effects of DHEA on serum progesterone levels from pregnant mice. The DHEA treatment significantly diminished serum progesterone concentration when it was injected during the post-implantatory or during the peri-implantatory time (fig. 5: c vs. b and e vs. d, $p < 0.001$). As in the case of estradiol, the effect was enhanced with the longer time of DHEA treatment (fig. 5: e vs. c, $p < 0.001$).

Effect of DHEA on Ovarian PGE Levels during Early Pregnancy

The DHEA treatment during both the post- and peri-implantatory period significantly increased ovarian PGE levels (fig. 6: c vs. b and e vs. d, $p < 0.001$) compared to control groups. During the peri-implantatory time (DHEA-6), ovarian PGE increased with the DHEA injec-

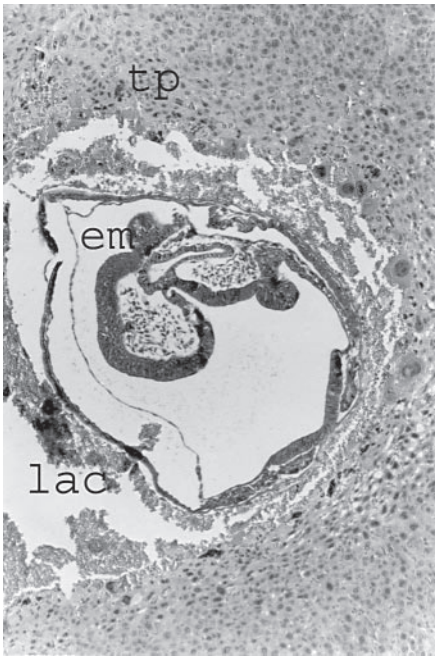

Fig. 1. Implantation site from pregnant control mice. A representative section of implantation sites from pregnant BALB/c mice on day 8 of gestation or injected with vehicle (oil) as was described in Materials and Methods (control 2 and control 6). Tissues were stained with hematoxylin-eosin. em = Embryo; tp = trophoblast; lac = decidual lacunas.

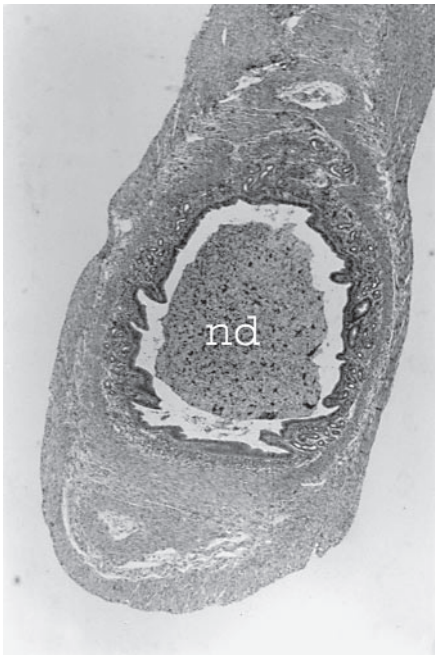

Fig. 2. Effect of DHEA on early pregnancy. A representative section of implantation sites with advanced grade of resorption in DHEA-2-treated mice. BALB/c pregnant mice were injected with DHEA (6 mg/kg body weight dissolved in 0.10 ml sesame oil) on days 6 and 7 of pregnancy (the post-implantatory group) and killed on day 8. nd = Necrotic deciduas.

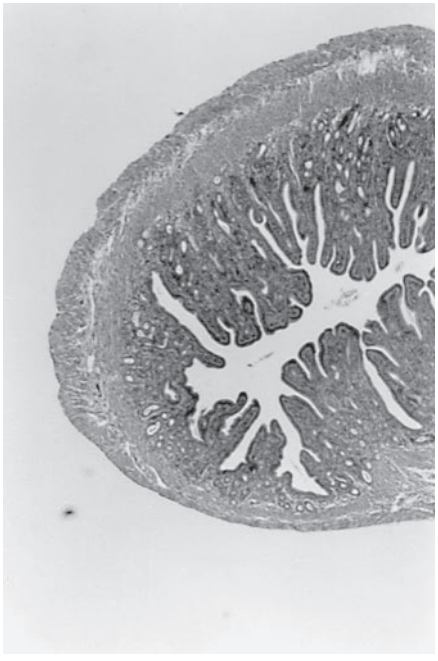

Fig. 3. Effect of DHEA on early pregnancy. A representative section of implantation sites with advanced grade of resorption in DHEA-6-treated mice. BALB/c pregnant mice were injected with DHEA (6 mg/kg body weight dissolved in 0.10 ml sesame oil) from day 2 to day 7 of pregnancy (the peri-implantatory group) and killed on day 8. The aspect of uterine tissue was similar to that of nonpregnant animals.

Table 1. Effect of DHEA on embryonic resorption

Treatment	Animals	Resorption rate, %
Control pregnancy	0/36	0
Control DHEA-2	0/36	0
DHEA-2	15/36	40
Control DHEA-6	0/36	0
DHEA-6	36/36	100

Animals: number of animals that expulsed embryos on day 8 of pregnancy per total number of animals. Effect of DHEA on embryonic resorption rates, which were expressed as a percentage of embryonic resorption over a total of 36 animals studied for each group.

tion. However, this effect was less effective than in the case of the post-implantatory treatment (DHEA-2; fig. 6: e vs. c, $p < 0.001$) and thus, contrary to estradiol and progesterone, the longest DHEA treatment was less successful to increase PGE production.

Effect of DHEA on Ovarian Total GSH Content during Early Pregnancy

In order to analyze the relationship between miscarriage and alterations in the protective role that involves GSH during DHEA treatment, total GSH was evaluated on ovaries from DHEA-2 and DHEA-6 groups and compared to their respective controls. DHEA treatment was able to diminish significantly (fig. 7: b vs. a, d vs. c, $p < 0.001$) ovarian GSH content not only during the post-implantatory, but also during the peri-implantatory period.

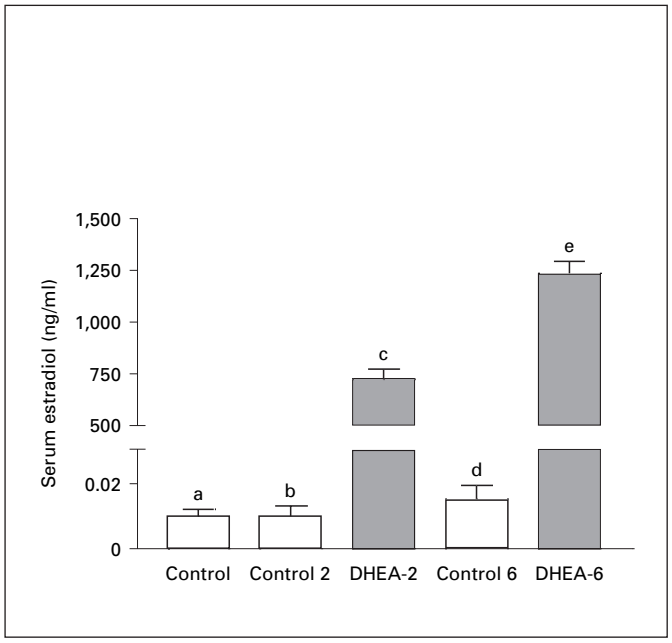

Fig. 4. Serum estradiol levels during DHEA-induced embryonic resorption. Each column represents the mean ± SEM of 12 measurements from different animals. One-way ANOVA and nonparametric tests (Newman-Keuls multiple comparison) that compare all the pairs of columns were used. A p value <0.05 was considered significant. The assay was carried out twice. Control: pregnant animals killed on day 8 of pregnancy; control 2: pregnant animals injected with vehicle on days 6 and 7 of pregnancy and killed on day 8; control 6: pregnant animals injected with vehicle from day 2 to day 7 of pregnancy and killed on day 8; DHEA-2: pregnant animals injected with DHEA (6 mg/kg body weight dissolved in 0.10 ml sesame oil) on days 6 and 7 of pregnancy and killed on day 8; DHEA-6: pregnant animals injected with DHEA from day 2 to day 7 of pregnancy and killed on day 8. c versus b: $p < 0.001$; e versus d: $p < 0.001$; e versus c: $p < 0.001$.

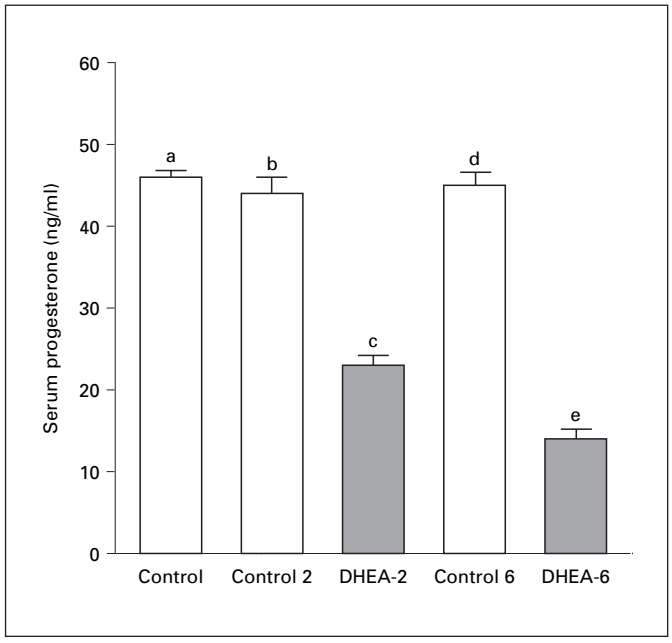

Fig. 5. Serum progesterone levels during DHEA-induced embryonic resorption. Each column represents the mean ± SEM of 12 measurements from different animals. One-way ANOVA and nonparametric tests (Newman-Keuls multiple comparison) that compare all the pairs of columns were used. A p value <0.05 was considered significant. The assay was carried out twice. Control: pregnant animals killed on day 8 of pregnancy; control 2: pregnant animals injected with vehicle on days 6 and 7 of pregnancy and killed on day 8; control 6: pregnant animals injected with vehicle from day 2 to day 7 of pregnancy and killed on day 8; DHEA-2: pregnant animals injected with DHEA (6 mg/kg body weight dissolved in 0.10 ml sesame oil) on days 6 and 7 of pregnancy and killed on day 8; DHEA-6: pregnant animals injected with DHEA from day 2 to day 7 of pregnancy and killed on day 8. c versus b: $p < 0.001$; e versus d: $p < 0.001$; e versus c: $p < 0.001$.

Discussion

DHEA is a C19 adrenal steroid secreted by adrenal glands, gonadal tissue and cells in the central nervous system [24, 25]. DHEA is a biosynthetic precursor of virtually all steroid hormones, including testosterone and estradiol [26, 27]. In the plasma, DHEA is predominantly present as DHEAS, the stored form. DHEAS is converted to the active form DHEA by intracellular sulfatases that are expressed in a number of cell types [2]. Although DHEA/DHEAS is one of the most abundant steroid hormones in the circulation, its physiological role remains unclear. We had found that DHEA was able to modulate ovarian function of BALB/c mice such as PG production, oxidative stress and progesterone and estradiol synthesis. These results correlated with a direct action of DHEA on T cell phenotype expression [23]. During pregnancy, DHEA and DHEAS are absolutely necessary for estrogen formation in the fetoplacental unit, to suppress the maternal immune system and modify cytokine levels, thus ensuring the development of gestation [3]. However, abnormally increased levels of DHEA result in miscarriage [9]. Therefore, the present report was conducted to analyze the effect of DHEA on the ovarian function during early pregnancy in mice and was designed to compare its action before and after the implantation process.

Our data demonstrated that after DHEA treatment (both before and after implantation) serum progesterone levels diminished, a fact that was in agreement with pre-

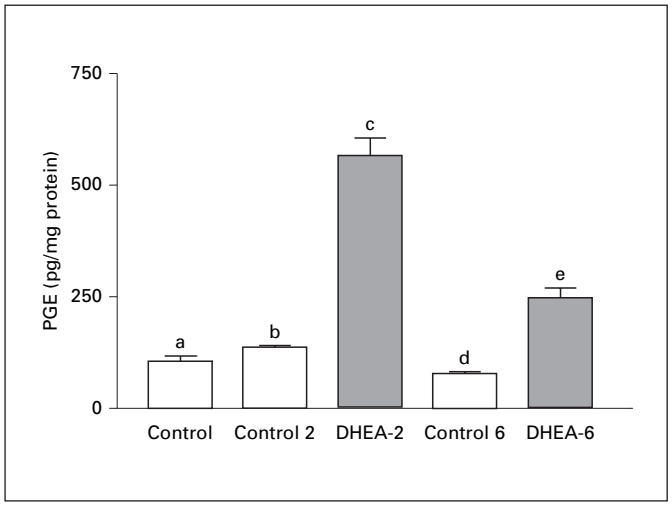

Fig. 6. Effect of DHEA on ovarian PGE production during early pregnancy. Ovarian PGE production of control (oil-injected) and DHEA-treated mice. Each column represents the mean ± SEM of 6 measurements from different animals. One-way ANOVA and nonparametric tests (Newman-Keuls multiple comparison) that compare all the pairs of columns were used. A p value <0.05 was considered significant. The assay was carried out twice. Control: pregnant animals killed on day 8 of pregnancy; control 2: pregnant animals injected with vehicle on days 6 and 7 of pregnancy and killed on day 8; control 6: pregnant animals injected with vehicle from day 2 to day 7 of pregnancy and killed on day 8; DHEA-2: pregnant animals injected with DHEA (6 mg/kg body weight dissolved in 0.10 ml sesame oil) on days 6 and 7 of pregnancy and killed on day 8; DHEA-6: pregnant animals injected with DHEA from day 2 to day 7 of pregnancy and killed on day 8. c versus b: $p < 0.001$; e versus d: $p < 0.001$.

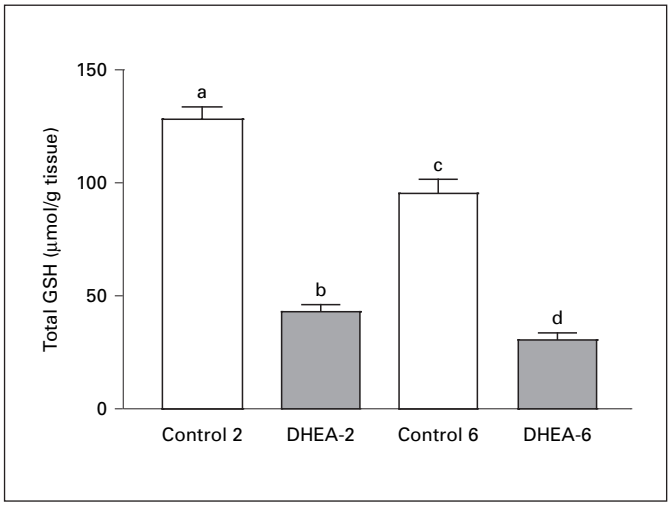

Fig. 7. Effect of DHEA on ovarian GSH content during early pregnancy. Ovarian GSH content of control (oil-injected) and DHEA-treated mice. Each column represents the mean ± SEM of 6 measurements from different animals. One-way ANOVA and nonparametric tests (Newman-Keuls multiple comparison) that compare all the pairs of columns were used. A p value <0.05 was considered significant. The assay was carried out twice. Control 2: pregnant animals injected with vehicle on days 6 and 7 of pregnancy and killed on day 8; control 6: pregnant animals injected with vehicle from day 2 to day 7 of pregnancy and killed on day 8; DHEA-2: pregnant animals injected with DHEA (6 mg/kg body weight dissolved in 0.10 ml sesame oil) on days 6 and 7 of pregnancy and killed on day 8; DHEA-6: pregnant animals injected with DHEA from day 2 to day 7 of pregnancy and killed on day 8. b versus a: $p < 0.001$; d versus c: $p < 0.001$.

vious reports [10]. We also found that DHEA treatment (DHEA-2: after implantation and DHEA-6: during implantation) significantly increased serum estradiol levels when compared to control healthy pregnant mice.

Therefore, when DHEA was injected during the peri-implantatory period, 100% of embryonic resorption (or no implantation) was observed against 40% of the rate when it was administered after implantation. This difference may reflect that the abnormally higher levels of DHEA during the implantatory window would completely avoid the implantation of embryos. Moreover, both the highest diminution of serum progesterone and increase in serum estradiol correlated with the longer DHEA administration (the peri-implantation group, DHEA-6). It has been determined that progesterone production and low levels of estradiol are required to maintain a pregnancy [28], but although estradiol is also necessary to induce and maintain the expression of progesterone receptors [29], increased levels of this steroid hormone during the implantation window have been reported to induce embryonic resorption [30]. Moreover, fetal death in mice lacking the 5α-reductase type I gene caused by estrogen excess has also been reported [31].

Considering that it has been reported that elevated androgen levels have been associated with early pregnancy loss [32] and poor oocyte quality [33], but that testosterone itself is not thought to be an embryonic toxin [31], we could infer that DHEA itself could be responsible for embryonic resorption observed in the present report. In fact, Du et al. [4] have recently reported that DHEA favors Th2 immune response in vitro by modulating cytokine production during interaction of T cells with antigen-presenting cells, and Tagawa et al. [5] have demonstrated a physiological immunosuppressive role of DHEA on the maternal immune system to ensure pregnancy development. However, abnormally increased levels of

DHEA could lead to miscarriage as we observed and as it has been previously reported in women with recurrent miscarriages [9, 10].

In view of the role of PGs in the mechanism of corpus luteum regression [15], we were also interested in studying the ovarian PGE levels during both DHEA-2 and DHEA-6 treatments. We observed that both groups presented higher levels of PGE than control groups. Contrary to serum progesterone and estradiol levels, the longer DHEA treatment (DHEA-6) showed the lowest effect on PGE production. This could be explained by the fact that in previous studies, using a Western blot assay, we found that the longer DHEA treatment produced downregulation of cyclooxygenase expression, the main enzyme involved in the PG production from arachidonic acid (data not shown).

Glutathione (γ-glutamylcysteinylglycine) is a ubiquitous tripeptide thiol in animal cells. It is the most prevalent low-molecular-weight peptide present in cells. It participates in many cellular functions, including DNA and protein synthesis, regulation of enzyme activity, both inter- and intracellular transport, and cellular protection as a major antioxidant. By providing a reducing milieu to the cell, it directly and indirectly protects against oxidative damage, free radical damage and other types of toxicity caused by both endogenous and exogenous compounds [34–38]. In previous studies [23], we had found a significant diminution of ovarian GSH content after DHEA treatment in prepuberal mice. Data presented here are in agreement with those previous findings and show that both DHEA-2 and DHEA-6 treatments significantly diminished ovarian GSH production. The fact that both ovarian PGE and GSH content were altered with respect to control healthy pregnant mice could indicate that DHEA treatment during early pregnancy would yield a dysfunctional status of the ovarian tissue that could lead to the premature regression of the corpus luteum, manifested by the diminution of serum progesterone. Thus, the role of DHEA on embryonic resorption could be focused not only on the action of the androgen in the ovarian tissue, but also on its influence in the immune system. We have previously demonstrated that DHEA is able to modulate T lymphocyte expression of ovarian tissue and lymph nodes and thus regulate cytokine production [23]. In agreement with this, the immunomodulatory properties of DHEAS and its metabolites have been proposed as the key players in the andropause [39] and androgen-dependent skin disorders [40].

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