The effect of progesterone administration on the expression of metastasis tumor antigens (MTA1 and MTA3) in placentas of normal and dexamethasone-treated rats

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Abstract

Background Dexamethasone (DEX) induces intrauterine growth restriction (IUGR) in pregnant rats. IUGR can occur due to apoptosis of trophoblasts, which is believed to be inhibited by progesterone (P4). A group of genes called MTAs play a role in proliferation and apoptosis. MTA1 upregulates trophoblasts proliferation and differentiation, while MTA3 downregulates proliferation and induces apoptosis. Hence, we hypothesized that during IUGR, placental MTA1 decreases and MTA3 increases and this is reversed by P4 treatment.

Methods Pregnant Sprague–Dawley rats were divided into 4 groups based on daily intraperitoneal injections: control (C, saline), DEX (DEX, 0.2 mg/kg/day), DEX and P4 (DEX + P4, DEX: 0.2 mg/kg/day, P4: 5 mg/kg/day) and P4-treated (P4, 5 mg/kg/day) groups. Injections were started on 15 dg until the day of dissection (19 or 21 dg). Gene and protein expressions of MTA1 and MTA3 were studied in the labyrinth (LZ) and basal (BZ) zones using real-time PCR and Western blotting, respectively.

Results DEX treatment induced 18% reduction in fetal body weight (p < 0.001) and 30% reduction in placental weight (p < 0.01). Maternal P4 level was also significantly lower in DEX treated groups (p < 0.05). MTA1 expression was decreased in the LZ (gene, p < 0.001) and BZ (protein p < 0.01), while MTA3 protein expression was upregulated in the LZ with DEX treatment (p < 0.001). These changes were reversed with P4 treatment.

Conclusion The findings of the present study indicate that DEX induces IUGR through changing the expression of placental MTA1 and MTA3 antigens and P4 improved pregnancy outcome by preventing the changes in MTAs expression.

Keywords Pregnancy · Placental efficiency · Fetal growth restriction · Progesterone · Metastasis tumor antigens

Introduction

Intrauterine growth restriction (IUGR) is used to describe fetal outcomes that fail to reach the full growth potential [1]. IUGR is known to result in postpartum complications after birth and later in life [2]. Several factors can lead to IUGR, most commonly endogenous increase of glucocorticoids (GC) [3] or exogenous exposure to GC as an antenatal treatment [4, 5]. Intrauterine growth restriction has been correlated with lower levels of maternal progesterone (P4) levels in humans and animal models [6, 7].

Progesterone is known for its importance in the initiation and maintenance of pregnancy [8]. During early pregnancy, P4 plays a vital role in blastocyst implantation [9, 10] and it has anti-abortive effect through suppressing the immune system [11, 12]. Progesterone is known for its role in proliferation and differentiation through modulating Wnt signalling
pathway [13, 14]. Progesterone also induces placental angiogenesis through inducing the expression of vascular endothelial growth factor [15] and increasing the blood flow through the release of nitric oxide [16]. In addition, it is known for its anti-apoptotic effect by altering the expression of anti-and pro-apoptotic regulatory proteins [17]- [18, 19]

Lower levels of maternal P4 that was detected in IUGR could be the underlying cause behind less proliferation and angiogenesis and increase apoptosis seen in IUGR placentas [20–22]. Due to the vital role of P4 during pregnancy, several studies evaluated the effect of exogenous P4 administration on pregnancy outcomes. During early pregnancy, P4 treatment in spontaneous preterm delivery improved pregnancy rate by 45% as well as the gestational duration [23–25]. Antenatal P4 treatment in complicated pregnancies, improved postpartum maternal and fetal general wellbeing including fetal weight, less administration to intensive care unit and fetal morbidity [24–28]. The exact mechanism of P4 modulatory effect is unknown. However, one of the mechanisms is believed to be through modulating the apoptotic pathway in the placenta [18].

Metastasis tumor antigens (MTA) represent a small family of gene products encoded by three distinct genes in humans (MTA1, MTA2, MTA3) [29]. MTAs co-regulate essential components in proliferation, differentiation and apoptosis pathways MTA1 exhibits up-regulation of beta-catenin and cyclin D1, which are indicators of activated Wnt pathway, thus cell proliferation and differentiation [30–32]. On the other hand, MTA3 represses the activity of beta-catenin and thus Wnt pathway, inhibiting cell proliferation and inducing apoptosis [32, 33]. In IUGR placentas, MTA3 was found to be upregulated along with downregulation of beta-catenin, indicating a lower level of trophoblast proliferation and increased placental cell apoptosis in IUGR [22].

Progesterone is an important hormone during pregnancy. Recent evidence showed that pregnancies complicated with IUGR are related to lower levels of maternal P4. In addition, these pregnancies showed decreased placental trophoblast proliferation and increased placental apoptosis manifested by alteration in the level of placental MTA1 and MTA3 expressions. Antenatal P4 proved to show modulatory effect on fetal body weight and wellbeing after birth in IUGR pregnancies. So in this study we aim to evaluate the modulatory effect of P4 on DEX-induced IUGR outcomes and on gene and protein expression of MTA1 and MTA3 in the placental labyrinth and basal zones (LZ and BZ) of IUGR placentas.

Methods

Animals and experimental design

Sprague–Dawley (SD) rats (8–12 weeks old; Animal Resource Center, Faculty of Medicine at Kuwait University; treated according to National Institute of Health (NIH) and Animal Research: Reporting In vivo Experiment (ARRIVE) guidelines and approved by Kuwait University Health Sciences Center Animal Research Ethics Committee). Experimental rats had free access to water and food and maintained on 12 h light/dark cycles, at 22 °C. Rats were mated overnight, the morning at which sperms were detected in the vaginal smear was considered as 0 dg; rats were then caged individually. At 15 dg the rats were divided randomly into 4 treatment groups (n = 6/group) based on daily intraperitoneal (i.p) injections of: saline (control group, C), Dexamethasone (0.2 mg/kg/day, DEX group), Dexamethasone (0.2 mg/kg/day) and Progesterone (5 mg/kg/day, DEX + P4 group) and progesterone (5 mg/Kg/day, P4 group). Treatment dose and duration of DEX were modified from Shoener et al. and Alqaryyan [22, 34], whereas P4 treatment was adopted from Hashimoto [35]. All treatments started from 15 dg until the day of sacrifice (19 dg and 21 dg). No injections were given on the day of dissection.

Tissue collection

On days 19 and 21 gestation, pregnant rats were exposed to CO₂ (1 min) followed by cervical dislocation. The chest was
opened and maternal blood was taken from the right ventricle of the heart in serum collecting tubes with clot activator gel. The tubes were kept on ice for 30 min, followed by centrifugation at 3000 × g for 10 min. The supernatant was stored at −80 °C. A longitudinal abdominal incision was done and the uterus containing the fetuses was removed. Both the fetuses and the placentas (LZ and BZ) were separated on ice and weighed. All fetuses were alive at the time of dissection. The LZ and BZ of placentas of 1 dam were pooled and considered as n = 1. Pregnancies with pups less than 8 or larger than 13 were excluded, since there was an inverse relationship between litter number and fetal body weight independent of any other factor [37, 38]. For RNA analysis placental zones were snap frozen in liquid nitrogen for 5 min and stored at −80 °C; and for protein analysis, the cryoprotective agent dimethylsulfoxide (10% v/v DMSO, Sigma Aldrich Co, USA) was added to samples before freezing at −80 °C.

**Hormone measurement**

Rat progesterone Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cat# R0775, ElabsScience, Texas, United Sates) with sensitivity of 0.2 ng/ml was used to estimate the level of maternal serum P4. The assay procedure was performed according to the manufacturer’s instructions.

**Gene studies using real-time PCR (ReT-PCR)**

Gene expression of MTA1 and MTA3 in placental LZ and BZ were studied using ReT-PCR as described previously [22, 39]. RNA extraction was performed using the TRIzol method (Invitrogen, Massachusetts, USA). RNA purity and quantity were measured using Epoch microplate spectrophotometry (Epoch Tak 3 plate, Biotech Instruments, Vermont, USA) at wavelengths of 260 nm and 280 nm. Samples with A260/A280 ratio > 1.7 were considered pure and used for further analysis. The integrity of samples were checked by agarose gel electrophoresis (1% wt/vol.) stained with ethidium bromide and visualized under UV transilluminator light using Gene Genius Bio Imaging System (Syngene, Cambridge, UK). Only samples with intact RNA were used for further analysis. Genomic DNA was removed from samples (DNase-treated), followed by reverse transcription (reagents purchased either from Invitrogen Incorporation or Applied Biosystems).

TaqlMan assays (Applied Biosystems, California, USA) were used for the housekeeping (Rat actin, assay ID. Rn00667869_m1) and target genes: MTA1 (Assay ID. Rn00574899_m1) MTA3 (Assay ID. Rn0001472640_m1). The PCR reaction was conducted as follows: 2 min at 50 °C (1cycle), 10 min at 95 °C (1cycle), alternate 15 secs at 95 °C (60 cycles) and 1 min at 60 °C. The relative quantitative expression of target gene was done as described in our earlier study [40] based on Livak method for relative gene expression [41]. Cycle threshold (Ct) was used to calculate the relative quantification of the target gene expression compared to the calibrator (19 dg control). The Ct value of the housekeeping gene (actin) was subtracted from the target gene and expressed as ΔΔCt. The ΔΔCt was calculated by subtracting the ΔCt value from that of the calibrator (19 dg control). The normalized gene expression was then determined by using $2^{-\Delta\Delta Ct}$.

**Western blotting analysis**

Protein expression of MTA1 and MTA3 in both placental LZ and BZ was done using Western blotting followed by immunodetection. After washing each of the placental zones twice with ice-cold saline, the samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM pH 7.4 Tris, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% Nadeoxycolate, 0.1% SDS, 2 mM benzamide, phynylmethylsulfonyl fluoride (PMSF) containing a tablet of EDTA-free protease inhibitor. Samples were kept on ice for 1 h then centrifuged (47,000 g for 25 min). The supernatant was collected for protein estimation that was done using Epoch microplate spectrophotometry. Polyacrylamide precast gels (4%–20% mini-protean TGX precast protein gels, Biorad Laboratories, USA) were used for electrophoresis. Samples were prepared by adding NuPage LDS sample buffer (4X) and NuPage sample reducing agent (10X) (Invitrogen, USA) to 70 µg samples before loading. Protein size was determined using rainbow protein marker (ECL rainbow marker-high range, GE Healthcare Lifescience). HEPES running buffer (10X) was prepared as indicated by the manufacturer (Tris 121 g, HEPES 238 g, SDS 10 g in 1 L ddH2O). For gel electrophoresis, a constant voltage of 250 V with a current of 60 mA/gel was applied.

Proteins were then transferred to Polyvinylidene fluoride (PVDF) membrane using transfer buffer (10X) as indicated by the manufacturer (25 mM Tris–HCl, 150 mM glyscience, pH 8.3). After the transfer, membranes were stained with ponceau red stain (0.1% w/v ponceau S, 5% v/v acetic acid) to confirm band transfer. Membranes were then washed out with distilled water several times until the colour disappeared. Membranes then were blocked using 10% non-fat dry milk in TBS-T (20 mM Tris, 137 mM NaCl, pH 7.6, 0.1% v/v Tween) for 1 h at room temperature. After washing the membrane with TBS-T (rinsed twice followed by 20 ml wash for 10 min); membranes were incubated with diluted (5% non-fat dry milk in TBS-T) primary antibodies overnight at 4 °C (Supplementary Table 1). The next morning, after washing the membranes with TBS-T (rinsed twice followed by 3 washes of 20 ml/wash, 10 min each), appropriate secondary antibodies were diluted in milk and incubated for
2 h at room temperature (Supplementary Table 1). Bands were detected using Western blotting luminol reagent kit (Santa Cruz Biotechnology, USA). Then membranes were placed in a cassette and exposed to Kodak films (Sigma Aldrich, Catalogue No. z358495). Membranes were then washed with TBS-T and incubated overnight with actin. Next morning the membrane was washed and incubated with appropriate secondary antibody for actin (Supplementary Table 1). Gene Genius Bio Imaging System was used to measure the optical density (OD) of each sample band and normalized to actin and the positive control ODs.

**Statistical analysis**

Statistical differences between groups were evaluated using package for statistical analysis (SPSS). The homogeneity of variances were evaluated followed by evaluating the significance using two way analysis of variance (ANOVA). Least significant difference (LSD) post hoc analysis was used if the variance was homogeneous, whereas Games-Howell post hoc analysis was used when variances of homogeneity was absent. A p value of < 0.05 was considered significant. All data were expressed as mean ± standard error of the mean (SEM). All figures were created using Graphpad Priem Software.

**Results**

**Dexamethasone induces IUGR and decreases maternal progesterone level**

Fetal body weight increased significantly with gestation from 19 to 21 dg in all experimental groups (p < 0.001; Fig. 2a). On 21 dg, treatment with DEX resulted in a reduction (18%) in fetal body weights compared to the C and P4 groups (p < 0.001 and p < 0.05, respectively; Fig. 2a), indicating a clear IUGR caused by DEX treatment. Co-administration of DEX and P4 prevented the reduction in fetal body weight seen with DEX treatment. At 19 dg, the maternal serum levels of P4 in the DEX + P4 group was significantly higher compared to all other groups (p < 0.01–p < 0.001; Fig. 2b). Towards the end of pregnancy, by 21 dg, maternal serum levels of P4 dropped significantly in all the groups compared to their corresponding groups at 19 dg (p < 0.01–p < 0.001, Fig. 2b). At 21 dg, DEX treatment resulted in 36% reduction in maternal P4 level compared to the C group (p < 0.05, Fig. 2b).

**Dexamethasone restricts placental growth**

Placental weight did not change from 19 to 21 dg. At 21 dg, DEX-induced IUGR resulted in lower weight of whole placentas, LZ and BZ compared to C group (30%, 31% and 38% respectively, p < 0.05; Table 1). Progesterone co-treatment with DEX prevented the reduction in whole placental weight as well as in the weight of the basal zone (p < 0.05, Table 1) compared to DEX-treated placentas. Placental efficiency evaluates the placental function in fetal formation, measured as fetal weight divided by placental weight. Placental efficiency increased significantly with the progression of gestation in all groups compared to 19 dg (p < 0.001, Table 1). At 21 dg, DEX treatment resulted in increased placental efficiency compared to C and P4 groups (p < 0.05, Table 1). The efficiency was improved furthermore when DEX was co-treated with progesterone at 21 dg (p < 0.001, Table 1).
Table 1  Placental weight, LZ weight, BZ weight and placental efficiency at 19 and 21 dg; DEX treatment resulted in reduced whole placental weight and the weight of LZ and BZ. P4 co-treatment prevented the reduction in placental and BZ weights. Data are represented as mean ± SEM (n = 6).

| Parameter                  | 19 dg          | 21 dg          |
|---------------------------|----------------|----------------|
|                           | Control (C)    | Dexamethasone (DEX) | Dexamethasone and progesterone (DEX + P4) | Progesterone (P4) |
| Placental Weight (g)      | 0.53 ± 0.02    | 0.45 ± 0.05    | 0.32 ± 0.02    | 0.35 ± 0.04    |
| Labyrinth Zone Weight (g) | 0.32 ± 0.01    | 0.32 ± 0.02    | 0.23 ± 0.01    | 0.22 ± 0.01    |
| Basal Zone Weight (g)     | 0.20 ± 0.01    | 0.19 ± 0.01    | 0.13 ± 0.01    | 0.18 ± 0.01    |
| Placental Efficiency      | 7.95 ± 0.36    | 9.08 ± 0.42    | 9.35 ± 0.42    | 6.72 ± 0.65    |

The effect of different treatments on placental MTA1 gene and protein expression

MTA1 gene expression in the LZ was significantly reduced (p < 0.001) in all experimental groups compared to C group at 19 dg (p < 0.001; Fig. 3a). With progression of pregnancy, at 21 dg, all groups showed a significant increase in the level of MTA1 gene expression compared to their corresponding groups at 19 dg (p < 0.05–p < 0.001; Fig. 3a). A single ~ 80 kDa band was detected for MTA1 and unlike the gene expression, the protein expression in the LZ showed a significant reduction in MTA1 level at 21 dg in all groups compared to their corresponding groups at 19 dg (p < 0.05–p < 0.01; Fig. 3b). No significant differences in protein levels were detected among the groups in this zone at any of the gestational ages (Fig. 3b).

MTA1 gene expression in the BZ was significantly higher in P4 treated group at 19 dg compared to all other groups at this age (p < 0.01, Fig. 3c). The significant increase in MTA1 gene expression in P4 disappeared at 21 dg (p < 0.01, Supplementary Fig. 5) due to significant reduction in gene expression in P4 group compared to 19 dg (p < 0.001, Supplementary Fig. 5). At the protein level, both DEX treated groups (DEX and DEX + P4) showed significant lower expression of MTA1 compared to C and P4 groups at 19 dg (p < 0.001; Fig. 3d). The effect of DEX on MTA1 expression in the basal zone disappeared with progression of gestation (21 dg) since the level of protein in C and P4 groups decreased significantly at 21 dg compared to 19 dg (p < 0.001; Fig. 3d).

MTA3 gene expression did not show any significant difference between groups at any gestational age (Fig. 4a). At the protein level, a single ~ 60 kDa band was detected for MTA3 in both placental zones. In the LZ, DEX treatment resulted in a significant increase in protein level of MTA3 compared to all experimental groups at 19 dg (p < 0.01–p < 0.001; Fig. 4b). With advanced gestation (at 21 dg), MTA3 protein level in the LZ showed significantly lower level of expression in all groups compared to their corresponding groups at 19 dg (p < 0.05–p < 0.001; Fig. 4b) with no difference in the expression between groups.

In the BZ, MTA3 gene levels were lower in all the groups at 21 dg compared to their corresponding groups at 19 dg, except DEX-treated group (p < 0.05–p < 0.01; Fig. 4c). The protein expression of MTA3 in the BZ showed significant increase with P4 treatment compared C group (p < 0.05; Fig. 4d). However, with advanced gestation the level of MTA3 expression with P4 decreased compared to 19 dg (p<0.05; Fig. 4d) and the difference in the expression diminished between the groups.
Discussion

Dexamethasone effect on the extent of IUGR development depends greatly on the dose and the route of administration [42]. The regimen of DEX treatment in the present study was based on literature review in inducing moderate IUGR [22, 34]. Dexamethasone in the present model induced a clear IUGR with 18% reduction in fetal body weight and 30% reduction in placental weight. Dexamethasone also induced a reduction in the detected level of maternal P4 as reported previously [6, 7].

The reduction in fetal and placental weights with DEX treatment were associated with lower levels of maternal P4 with DEX treatment as detected in our study.

The regimen of P4 treatment in the present study was modified from previous studies on the effect of P4 on pregnancy outcomes [35, 45]. Progesterone administered did not cause a significant increase in maternal P4 levels compared to C group possibly due to increased excretion of P4 metabolite that was seen when P4 levels increases [46]. However, at 19 dg, maternal P4 levels increased significantly when P4 was co-treated with DEX, possibly due to the lack of DEX inhibitory effect on placental endocrine function after a short DEX exposure (15–18 dg). Another possibility is the effect of DEX on reducing the activity of P4 catabolic enzymes at this gestational age [47].

The reduction in fetal and placental weights with DEX treatment were associated with lower levels of P4 detected in maternal serum. P4 co-treatment with DEX prevented the reduction in fetal weight. This could be explained by

![MTA1 gene expression and protein expressions in the LZ at 19 and 21 dg](image1)

![MTA1 gene expression and protein expressions in the BZ at 19 and 21 dg](image2)

![MTA1 gene expression and protein expressions in the LZ at 19 and 21 dg](image3)

![MTA1 gene expression and protein expressions in the BZ at 19 and 21 dg](image4)
maintaining the weight of placenta and preserving placental function seen with P4 treatment. Placental efficiency, which indicates the placental capability in providing the fetus, was improved with DEX treatment. This may imply that, as a compensatory mechanism, the placenta is preserving fetal weight at the expense of its own growth. The efficiency improved further when DEX was cotreated with P4.

Metastasis tumor antigens are well known for their involvement in the pathway of cell proliferation and apoptosis. MTA1, 2 and 3 have been detected in human [32] and rat placentas [22, 39]. In the present study, MTA2 was not evaluated because it lacked to prove its significance in placental development and IUGR in DEX-induced IUGR model [22]. MTA1 is known for its role in proliferation and DNA damage repair through upregulating beta-catenin and cyclin D1 [30–32]. Placental growth ceases by 19 dg, indicated by no change in placental weight between 19 and 21 dg seen in the present study and previous studies [22, 36]. Furthermore, placental apoptosis is known to take place towards the end of pregnancy[43]. This is associated to lower level of MTA1 expression detected in the present study in both placental zones by 19 dg only. Since placental levels of beta-catenin was found to be reduced in DEX-induced IUGR model [22], it is believed that DEX inhibits the effect of MTA1 by decreasing the expression of beta-catenin and Wnt pathway leading to decreased proliferation seen in IUGR placentas. The basal zone is the zone responsible for hormonal production, any reduction in the size of this zone, as seen with DEX treatment in the present study, would directly affect hormonal production noticed as reduction in P4 level and fetal development.

On the other hand, MTA3 is known for its apoptotic effect by inhibiting beta-catenin and blunting Wnt pathway [32, 38]. MTA3 protein expression increased significantly with DEX treatment at 19 dg. At 21 dg the level of MTA3 protein expression decreased in all experimental groups compared to their corresponding groups at 19 dg. c MTA3 gene expression and b protein expression in the BZ at 19 and 21 dg; MTA3 gene expression decreased significantly in all experimental groups between 19 and 21 dg, except DEX-treated group. Protein level of MTA3 increased significantly with P4 treatment at 19 dg. A finding that disappeared by 21 dg due to significant reduction in protein level in P4 at this gestational age compared to 19 dg. *Significant differences between groups at the same gestational age (*p<0.05, **p<0.01, ***p<0.001); # Significant differences between 19 dg and the corresponding group on 21 dg ( #p<0.05, ##p<0.01, ###p<0.001). Control (C), dexamethasone (DEX), dexamethasone and progesterone (DEX + P4), and progesterone (P4) treated groups. Data are represented as mean±SEM (n = 6).
Conclusion

DEX treatment induced clear IUGR indicated by reduction in fetal and placental growth. The restriction in pregnancy outcomes seen with DEX treatment is believed to be through decreasing the expression of placental proliferative antigen MTA1 and increasing the expression of MTA3 that supresses proliferation and induces apoptosis. P4 treatment prevented the increase in MTA3 levels detected with DEX treatment. Co-administration of P4 with DEX proved to prevent the reduction in fetal body weight and placental weight seen with DEX treatment through modulating the expression of MTA1 and MTA3. However, the downstream signalling pathways underlying P4 modulatory effect need to be further explored. One of the limitations of this study is the lack of immunohistochemical data due to limited time and resources.

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Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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