Funmileyi O. Awobajo*, Ayodele O. Morakinyo, Titilola A. Samuel, Oluwakemi T. Oyelowo, Abimbola O. Ogunsola, Perpetual U. Onyekwele, Mosunmola E. Okedina and Oluwadamilola O. Ogunbanwo

Dynamics of inflammatory reaction and oxidative stress across maternal serum, placenta and amniotic fluid in laboratory rats and the role played by genistein aglycone

https://doi.org/10.1515/jbcpp-2018-0070
Received April 15, 2018; accepted July 11, 2018; previously published online October 17, 2018

Abstract

Background: Genistein was reported to adversely influence fetal development although this is yet to be fully understood as a mechanism.
Methods: In this study, pregnant rats were divided into control (Cont.) and genistein force-fed (2-mg/kg and 4-mg/kg) groups. Each group was divided further into five subgroups: GD-0, GD-6, GD-13, GD-18, and GD-20 based on the terminal gestational day (GD). On the respective terminal GD, the rats were sacrificed and blood samples and amniotic fluid were carefully collected and separated and placenta homogenates were prepared. These samples were evaluated for oxidative stress and inflammatory reaction. The weights of embryonic implant and placenta tissue were also recorded. Heat shock protein (Hsp) (60 and 90), corticosterone, and oxidative stress biomarkers were determined in all the samples.
Results: Fetal and placental weights in all genistein-exposed groups were significantly decreased. A fluctuation in the level of the Hsp was recorded with a significant decrease recorded in Hsp90 level in the placenta and amniotic fluid towards GD-20 along with a concomitant increase in the corticosterone level in the amniotic fluid in all genistein groups compared to control. Maternal serum at GD-18 and GD -20 recorded a significant increase in antioxidant level (SOD, GSH, CAT) in all genistein-exposed groups. However, these antioxidants were significantly reduced in the placenta and the amniotic fluid compared to control.
Conclusions: Genistein enhances the placenta function in attenuating the risk of oxidative stress in the amniotic fluid and deferentially suppressed inflammatory activities in the placenta during early gestation and towards late gestation period.

Keywords: amniotic fluid; genistein; heat shock protein; inflammatory reaction; placenta; oxidative stress.

***Corresponding author: Funmileyi O. Awobajo,** Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Room 006, Block F, Idi-araba, Surulere, Lagos, Nigeria, Phone: +23408053416937, E-mail: fawobajo@unilag.edu.ng
Ayodele O. Morakinyo, Oluwakemi T. Oyelowo, Perpetual U. Onyekwele, Mosunmola E. Okedina and Oluwadamilola O. Ogunbanwo: Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Lagos, Nigeria
Titilola A. Samuel: Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Lagos, Nigeria
Abimbola O. Ogunsola: Department of Physiology, Ben Carson School of Medicine, Babcock University, Ilisan-Remo, Ogun State, Nigeria

Introduction

Cellular aerobic metabolism is inextricably associated with the generation of reactive oxygen species (ROS) [1], which are capable of inflicting indiscriminate damage to the body's molecular building blocks, causing cell death [2]. Our body thus has an in situ antioxidant system to combat the imbalances in the production and removal of the ROS, which have been implicated in several diseases [1]. The implantation of the blastocyst in the uterus is considered an inflammatory process and, along with pregnancy, will naturally provoke the generation of a large concentration of ROS [3–6]. Endocrine disrupting chemicals can adversely influence blastocyst implantation and embryonic development [7], and substances such as leukocyte inhibitory factor, heparin-binding epidermal growth factor, colony stimulating factor-1, and interleukin-1 have all been implicated in the blastocyst implantation process [8–10]. Genistein, a phytoestrogen, has been reported to precipitate adverse effects on pregnancy via such pathway, such as reducing oocyte fertilization potential, hypothyroidism, altered C-reactive protein, and leptin synthesis; generating free radicals that alter DNA stability [11–13]; and reducing fetal and placental developmental functions.

The primary functions of the placenta include providing the interface for the exchange of nutrients, gases, and waste products between the fetal and maternal blood [14] and the production of hormones and immunoglobulins

essential for sustenance of pregnancy [15]. The placenta also produces ROS, including nitric oxide and carbon monoxide, with adverse consequences leading to trophoblast apoptosis and altered vascular reactivity [1, 16, 17]. Excessive production of ROS has been implicated in pathological pregnancies such as in preeclampsia, especially towards late pregnancy with morbidity outcome [16, 17]. In the first trimester of pregnancy, establishment of blood flow into the intervillous space is associated with a burst of oxidative stress, with the possibility of loss of the pregnancy when the body is unable to effectively curb these increase in ROS [13, 17]. This study is aimed at assessing the influence of genistein with reported antioxidant activities on placenta oxidative status and inflammatory activities in pregnant rats.

Materials and methods

Ethical approval

All protocols used in this study including animal welfare and grouping, humane euthanasia, animal dissection, sample collection, and analysis were approved by the Research and Ethics Committee of College of Medicine, University of Lagos (CM/HREC/11/16/071), and they conformed to the guidelines for care and use of laboratory animals in biomedical research [18].

Genistein chemical and preparation of stock solution

Genistein (purity, 98.2%) was purchased from Chengdu Biopurify Phytochemicals Ltd., China. Genistein was prepared daily by sonication in a predetermined volume of distilled water.

Research procedure

Ninety-six female Sprague-Dawley (SD) rats with regular estrous cycle and weighing 160–170 g were used for this study. They were housed in clean plastic cages under 12-h light and 12-h darkness, while clean water and phytoestrogen-free rat chow were provided *ad libitum*. They were divided into three major groups (control, genistein 2 mg, and genistein 4 mg) with each group having five sub-groups each, based on the terminal gestational day (GD) at which the animals were sacrificed, i.e. GD-0, GD-6, GD-13, GD-18, and GD-20. They were mated at the ratio of two female rats to one mature male rat. Successful mating was confirmed by the presence of sperm cell in the vagina smear and a persistence diestrous thereafter.

The pregnant rats were randomly distributed to the groups at a population of 30 rats per group and 6 rats per sub-group. The genistein-exposed rats were administered genistein orally at a dose of 2 mg and 4 mg/kg body weight per day until their respective terminal GD, while control received equal of distilled water, the vehicle for the genistein. Earlier reports from our laboratory had shown significant adverse influence on pregnancy and fetal development at 2 mg and 4 mg/kg [11, 12]. There are several reports on the use of wide range of doses of genistein covering between 0.2 mg/kg to 100 mg/kg body weight in rodents [19].

Preparation of samples and assessment of inflammatory reaction

Placenta tissue homogenate (10.0% w/v) was prepared by placing 1.5-g placental tissue pooled from each animal in a homogenizer containing cold 5 mL of cold phosphate buffer. The homogenizer was also placed in an ice bucket. The tissue was homogenized, and the homogenate was centrifuged at 7000 rpm for 30 min at 10 °C. The supernatant was carefully separated with a pipette into Eppendorf tubes and stored at −8 °C until use. The heat shock protein HSP-60 and HSP-90 and corticosterone were assayed from the serum, amniotic fluid, and placenta homogenate samples using ELISA kits (Elab-Science, China) according to the manufacturer's instructions. All samples were prepared in triplicates, and a standard was prepared along with the assay.

Oxidative analysis

As a marker of lipid peroxidation, the level of malondialdehyde (MDA) was measured by using the method of Yagi et al. [20]. The reduced glutathione (GSH) was determined using the method described by Tipple and Rogers [21]. Activity of the SOD enzyme was also determined according to the method described by Kumar et al. [22]. Catalase (CAT) was determined by measuring the exponential disappearance of H_2O_2 at 240 nm and expressed in units/milligrams of protein, as described by Aebi et al. [23], while the absorbance was recorded with a spectrophotometer (UV 160) in all measurements.

Statistical analysis

All results were presented as mean ± SEM, analyzed using ANOVA, and subjected to post hoc test using Duncan's multiple range tests using Graph Pad Prism-5 statistical software. Differences were considered significant at $p \leq 0.05$. Line graphs, bar charts, and tables were used for ease of presentation.

Results

Maternal body weight, placenta, and fetus weight in genistein-treated pregnant rats

Maternal body weight steadily increased with increasing gestational duration in the control and genistein groups (Figure 1). There was a significant increase in the body

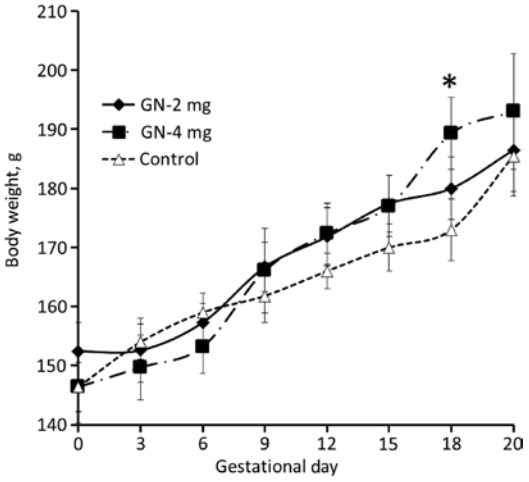

Figure 1: Effect of oral administration of genistein (2 mg/kg and 4 mg/kg) on body weight of pregnant rats from the first day of pregnancy to twentieth day of pregnancy.
*Result significantly different compared with the control result on same gestation day.

weight in the 4-mg/kg group at GD-18 compared with control. There was a significant reduction in the placenta weight at GD-13 in the 2-mg/kg and 4-mg/kg genistein groups when compared with the control. A significant decrease in placenta weight was recorded at GD-18 in the 4-mg/kg group (Table 1). There was a significant increase in the fetal weight at GD-13 in all genistein-treated groups, while a significant decrease in fetal weight was recorded in both 2-mg/kg and 4-mg/kg groups at GD-20 (Table 1).

Oxidative status in the maternal plasma, amniotic fluid, and placenta homogenate

Serum SOD increased towards term after the initial significant decrease at GD-13 in the 2-mg group. In the 4-mg group, serum SOD level was significantly decreased at GD-6 and GD-13 before the significant increase recorded at GD-18 and GD-20. The amniotic level of SOD was not significantly different from control at GD-18 and GD-20 in the 2-mg group, while it was significantly increased and decreased at GD-18 and GD-20, respectively, in the 4-mg group. The placenta tissue level of SOD was significantly reduced at GD-18 and GD-20 in the 4-mg group, while a significant decrease was only recorded at GD-18 in the 2-mg group.

The serum catalase level was significantly increased all through gestation towards term starting from GD-6 in the 2-mg group compared with control. The 4-mg group recorded an initial significant reduction in serum CAT as recorded at GD-6 and GD-13, before the significant

Table 1: Fetal, placenta weight at different gestational days in rats exposed orally to genistein.

Tissue	GD-6			GD-13			GD-18			GD-20		
	Control	2 mg (Gen)	4 mg (Gen)	Control	2 mg (Gen)	4 mg (Gen)	Control	2 mg (Gen)	4 mg (Gen)	Control	2 mg (Gen)	4 mg (Gen)
Placenta weight, g				0.094 ± 0.002	0.086 ± 0.004[a]	0.082 ± 0.005[a]	0.368 ± 0.017	0.334 ± 0.019	0.306 ± 0.020[a]	0.433 ± 0.019	0.438 ± 0.026	0.462 ± 0.016
Fetal weight, g				0.090 ± 0.01	0.110 ± 0.00[b]	0.110 ± 0.00[b]	1.010 ± 0.10	0.990 ± 0.07	0.910 ± 0.03	3.090 ± 0.34	2.460 ± 0.18[a]	2.430 ± 0.06[a]

AF, Amniotic fluid; GD, gestational day; PH, placenta homogenate (n = 8). Results expressed as mean ± SEM. [a]Significant decrease compared with the control on same GD ($p \leq 0.05$ with the control). [b]Significant increase compared with the control on same GD ($p \leq 0.05$ with the control).

increase recorded at GD-18 and GD-20 as the pregnancy progressed towards term. The amniotic fluid level of CAT was significantly reduced at GD-18 and GD-20 in the 2-mg and GD-20 in the 4-mg groups compared with control. Catalase level in the placenta tissue was significantly reduced all through from GD-13 to GD-20 in the 4-mg group, while a significant decrease was only recorded at GD-18 followed by a significant increase at GD-20 in the 2-mg group compared with the control.

Serum MDA was not significantly different at all GDs observed in this study and in all the 2-mg and 4-mg groups compared with control. Serum GSH was significantly lower at GD-6, GD-13, and GD-20, while it was significantly increased at GD-18 in the 4-mg group compared with control. The level of GSH was only significantly reduced at GD-6, while it became significantly increased at GD-13 and 18 in the 2-mg group. Amniotic fluid level of GSH was significantly reduced at GD-20 in both the 2-mg and 4-mg groups. Placenta tissue level of GSH was significantly increased at GD-13 in both the 2-mg and 4-mg groups, while its level was significantly reduced with at GD-18 in both the 2-mg and 4-mg groups. The level remained unchanged when compared with the control at GD-20 in the 4-mg group, while its level was significantly reduced at GD-20 in the 2-mg group.

Corticosterone level in maternal plasma, amniotic fluid, and placenta homogenate

Serum corticosterone level remained unchanged in the 2-mg group at all GDs compared with control. The 4-mg group, however, recorded significant decrease in serum corticosterone level at GD-13 and GD-18, followed by a significant increase in the level at GD-20 compared with the control. The amniotic level of corticosterone was significantly increased at GD-18 and GD-20 in the 2-mg group. The 4-mg group recorded a significant decrease and a significant increase in the level of hormone at GD-18 and GD-20, respectively, compared with the control. While the placenta tissue level of corticosterone remained like that of the control at the various GDs measured, the level was only significantly reduced at GD-18 in the 4-mg group compared with control.

Heat shock protein in maternal plasma, amniotic fluid, and placenta homogenate

In the serum, Hsp60 level was significantly reduced at GD-6 and GD-13 in the 2- and 4-mg groups, while its level was only significantly reduced at GD-18 and GD 20 in the 2-mg and 4-mg groups, respectively. Hsp60 level was, however, significantly increased at GD-18 and GD-20 in the 4-mg and 2-mg groups, respectively. The amniotic level of Hsp 60 was significantly reduced at GD-18 in the 2- and 4-mg groups as well as at GD-20 in the 4-mg group. The amniotic Hsp60 level was, however, significantly increased at GD-20 in the 2-mg/kg group (Figure 2).

There was an initial decrease in the serum Hsp90 towards GD-13 in the 2-mg group, followed by a significant increase that peaked at GD-18 and a further decrease towards parturition as recorded at GD-20 in the 2-mg group. Similar pattern of serum Hsp-90 level was recorded in the 4-mg group, but the initial decrease peaked at GD-6. The decrease in serum Hsp-90 was followed by a significant increase in its level from GD-13 to GD-18 and then a decrease towards parturition as recorded at GD-20. The placenta Hsp90 pattern was similar with a significant increase at GD-13 and, a significant decrease at GD-18 and followed by a significant increase at GD-20 in the 2-mg group. Placenta Hsp90 level in the 4-mg group was significantly higher at GD-13 but remained significantly reduced thereafter towards parturition on GD-20. Amniotic Hsp90 level in the 2-mg group was significantly reduced at GD-18 and GD-20, while the level was significantly increased at GD-18 in the 4-mg group but significantly reduced at GD-20 in the same group (Table 3).

Discussion

In the present study, we investigated the influence of genistein on oxidative stress dynamics and inflammatory reactions across the feto-placenta-maternal serum interphase. Genistein, the major isoflavone component of soybean, has been shown to have mixed effects on the reproductive systems of rodents depending on the sex, time, and dose of exposure [24–26]. We reported the detrimental effect of genistein on female rat's estrous cycle [12], its adverse effects on mating indices, fetal and placenta development, occurrence of hypothyroidism, disruption of the C-reactive protein, and leptin synthesis with increased resumption of embryo [12]. In this study, a significant increase in maternal weight was recorded in the 4-mg group at GD-18, while the placenta and fetal weights were significantly reduced in both 2-mg and 4-mg/kg groups, corroborating a previous report [12]. This adverse effect of genistein on placenta and fetal development suggests that genistein may adversely influence some of the mechanisms that

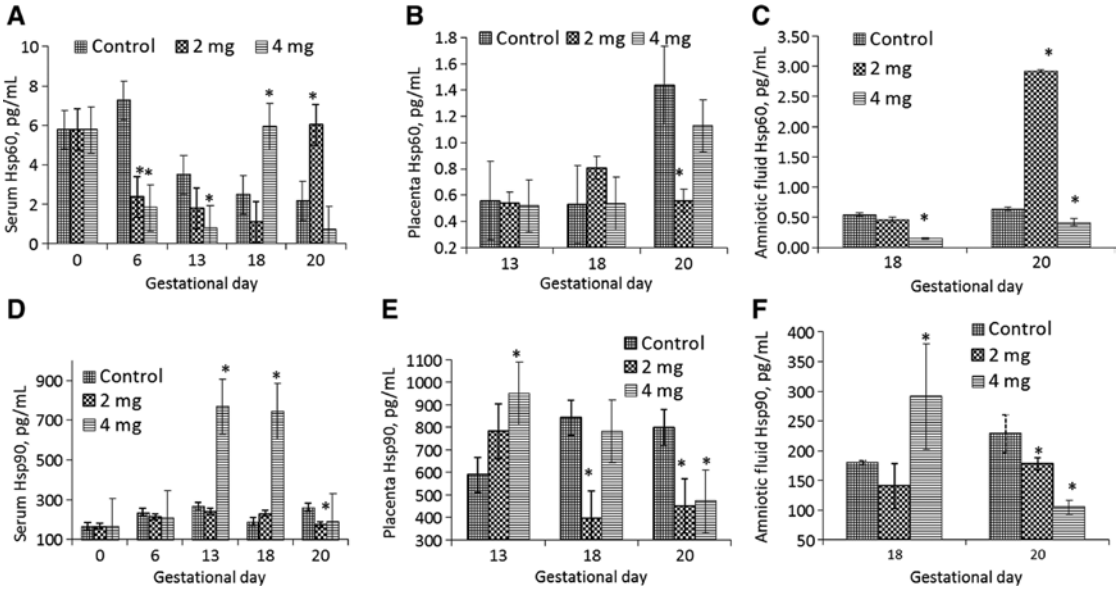

Figure 2: Heat shock protein 60 and 90 (Hsp60, Hsp90) level in serum, amniotic fluid, and placenta homogenate in rats exposed to genistein (Plates A and D are results from the maternal serum. Plates B and E are results from the placenta tissue. Plates C and F are results from the amniotic fluid).
GD (n = 8). Results expressed as mean ± SEM, *significantly increased compared with the control on same GD ($p \leq 0.05$ with the control).

control placental and fetal metabolic processes. Placenta growth is predicated on nutrient availability and the right hormonal milieu to which it also contributes significantly [27]. The primary functions of the placenta are to provide an immunological barrier between fetus and mother; facilitate the transfer of the gases, water, ion, and nutrient; and secrete a vast array of hormones involved in cellular signaling [28]. Fetal nutrition and growth are closely related to placenta functions with increased placenta weight initiating corresponding weight gain in the developing fetus [15]. The reduction in the placenta weight recorded in the genistein groups signals the possibility of the disruption of the cellular components of the placenta, which may explain the consequent reduction in the fetal weights. Placenta and fetal weight are known to have the highest growth rate at a window of growth towards late pregnancy [29].

Oxidative stress occurs in normal pregnancy due to the increasing metabolic activities of the placenta and the reduced scavenging power of the endogenous antioxidants in handling the ROS [17, 30–32]. In this study, oxidative stress level was highest in the maternal serum and lowest in the placenta as the pregnancy progressed towards term in all the genistein-treated groups and control. Furthermore, at GD-20, which is some few hours to delivery, there was a significant increase in oxidative stress as indicated with the significant increase in the serum level of SOD and CAT in all the genistein-exposed groups compared with their levels in the control. This is in addition to the increase recorded in the serum level of GSH in the 2-mg genistein group. A significant increase in placenta CAT was only recorded in the 2-mg group at GD-20. Other authors have also reported increased level of oxidative stress biomarkers in the placenta tissue from early pregnancy [33].

Preeclampsia, which is characterized by poor fetal growth, a resemblance of what we have earlier reported in genistein-treated rats [11], has also been linked to increased oxidative stress [34–36]. Thus, the placenta, which serves as an interphase between the maternal blood and the developing fetus, recorded a reduced oxidative stress in this study and thus shielded the fetus from overexposure to those stressors. Thus, there was a significant decrease in the amniotic level of SOD (4 mg), CAT, and GSH (2 mg and 4 mg) compared with control (Tables 2 and 3).

Furthermore, the pattern of the serum level of corticosterone during pregnancy was maintained across the various groups observed in this study. There was a significant decrease in the serum corticosterone level with the advancement in the age of pregnancy, followed by an increase as the pregnancy approached term. The 4-mg group, however, recorded a significant decrease and increase in the serum level of corticosterone at GD-6 and GD-20, respectively (Figure 3), compared with the control group. Cortisol is an effective biological indicator of acute

Table 2: Superoxide dismutase and catalase concentration in the serum, amniotic fluid, and placenta homogenate.

Gestational day	Serum SOD level, mg/mL					Amniotic fluid SOD level, mg/mL		Placental homogenate SOD level, mg/mL		
	0	6	13	18	20	18	20	13	18	20
Control	0.02±0.01	0.02±0.00	0.06±0.01	0.03±0.01	0.07±0.00	0.09±0.00	0.06±0.00	0.01±0.00	0.04±0.01	0.01±0.00
Gen 2 mg	0.02±0.01	0.03±0.01	0.02±0.00[a]	0.21±0.02[b]	0.28±0.04[b]	0.08±0.01	0.07±0.01	0.01±0.00	0.01±0.00[a]	0.01±0.00
Gen 4 mg	0.02±0.01	0.01±0.00[a]	0.03±0.01[a]	0.07±0.01[b]	0.15±0.05[b]	0.18±0.05[b]	0.02±0.00[a]	0.01±0.00	0.01±0.00[a]	0.02±0.00[a]
	Serum CAT, mg/mL					Amniotic fluid CAT, mg/mL		Placentae homogenate CAT, mg/mL		
Control	0.81±0.15	0.77±0.00	0.37±0.10	1.18±0.01	2.01±0.14	2.89±0.01	1.79±0.02	1.21±0.19	1.15±0.01	0.32±0.03
Gen 2 mg	0.81±0.15	0.84±0.30[b]	0.83±0.11[b]	5.79±1.36[b]	8.79±1.29[b]	2.45±0.29[a]	1.30±0.15[a]	0.38±0.05	0.32±0.06[a]	1.67±0.10[b]
Gen 4 mg	0.81±0.15	0.47±0.03[a]	1.03±0.00[a]	2.27±0.00[b]	7.83±3.98[b]	2.53±0.75	1.30±1.04[a]	0.25±0.00[a]	0.18±0.00[a]	0.20±0.00[a]

AF, amniotic fluid; GD, gestational day; PH, placenta homogenate (n=8); CAT, catalase. Results expressed as mean±SEM. [a]Significant lower compared with the control on same GD ($p \leq 0.05$ with the control). [b]Significantly increased compared with the control on same GD ($p \leq 0.05$ with the control).

Table 3: MDA and GSH concentration in the serum, amniotic fluid, and placenta homogenate from rats exposed to genistein at different gestational periods.

Gestation day	Serum MDA, mg/mL					Amniotic fluid MDA, mg/mL		Placentae homogenate MDA, mg/mL		
	0	6	13	18	20	18	20	13	18	20
Control	0.15±0.01	0.14±0.01	0.13±0.01	0.15±0.01	0.16±0.01	0.17±0.00	0.15±0.00	0.12±0.01	0.13±0.01	0.12±0.00
Gen 2 mg	0.15±0.01	0.14±0.01	0.14±0.00	0.16±0.00	0.17±0.01	0.17±0.00	0.14±0.01	0.13±0.00	0.13±0.00	0.12±0.00
Gen 4 mg	0.15±0.01	0.13±0.00	0.14±0.02	0.16±0.00	0.17±0.01	0.17±0.00	0.14±0.01	0.12±0.00	0.12±0.00	0.12±0.00
	Serum GSH, mg/mL					Amniotic fluid GSH, mg/mL		Placentae homogenate GSH, mg/mL		
Control	10.27±0.37	7.77±0.87	3.94±1.09	10.39±1.15	11.44±1.25	14.43±0.12	10.93±0.20	2.32±0.13	17.43±0.85	3.28±0.41
Gen 2 mg	10.27±0.37	4.07±0.24[a]	7.15±0.58[b]	12.79±0.31[b]	15.30±1.37	14.42±0.42	0.14±0.00[a]	3.39±0.00[b]	3.45±0.70[a]	1.68±0.41[a]
Gen 4 mg	10.27±0.37	4.19±0.10[a]	0.14±0.02[a]	13.81±1.00[b]	7.58±0.58[a]	15.09±0.40	0.14±0.00[a]	2.69±0.06[b]	1.82±0.17[a]	2.45±0.47

AF, amniotic fluid; GD, gestational day; PH, placenta homogenate (n=8); MDA, malondialdehyde; GST, glutathione. Results expressed as mean±SEM. [a]Significant reduction compared with the control on same GD ($p \leq 0.05$ with the control). [b]Significant increase compared with the control on same GD ($p \leq 0.05$ with the control).

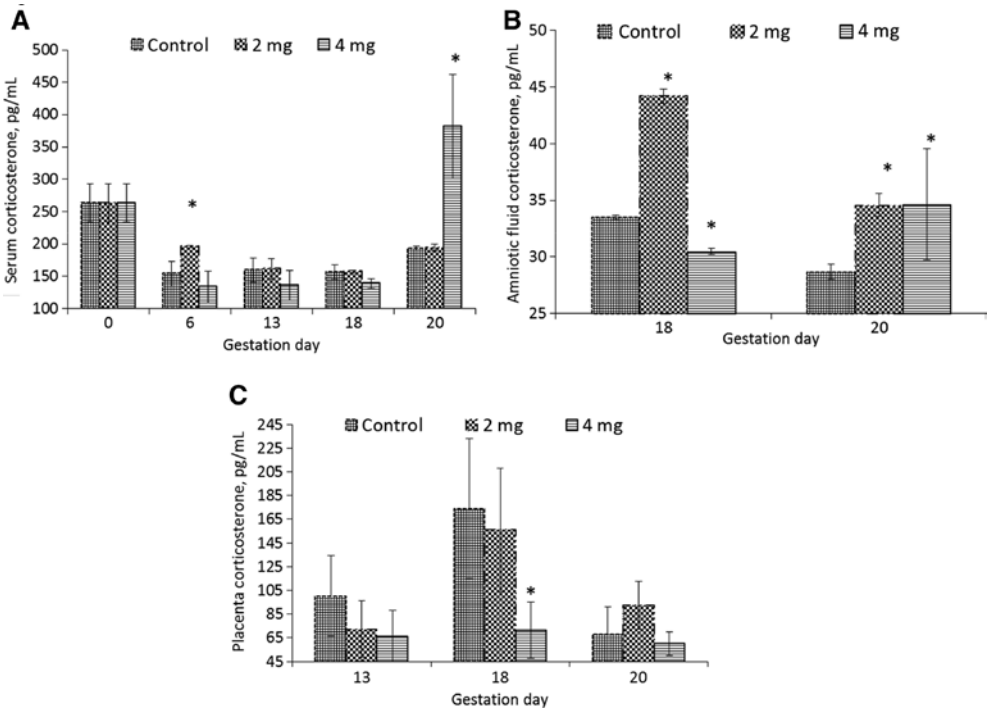

Figure 3: Corticosterone level in (A) serum, (B) amniotic fluid, and (C) placenta homogenate from rats exposed to genistein at different gestational periods.
*Result significantly different compared with the control result on same gestation day.

stress response [37], and a high concentration in maternal blood can negatively influence fetal development with adverse consequents on pregnancy outcome and postnatal development [38, 39].

Although the developing fetus is partially shielded from the direct impact of maternal cortisol by the placental enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the enzyme that oxidizes cortisol to its inactive form cortisone, some amount of cortisol still finds their way through the placenta to the fetus, as recorded in this study and as reported by other authors under different conditions [40]. The level of cortisol may rise when the placenta integrity is further compromised by increased oxidative stress. The amniotic level of corticosterone was thus significantly increased at GD-18 in the 2-mg group and GD-20 in both the 2-mg and 4-mg groups compared with the control in this study. There are several reports with evidence indicating that increased exposure of the fetus to glucocorticoids especially at mid- to late pregnancy will result in adverse outcomes including intrauterine growth restriction (IUGR) [41–44], several metabolic diseases, and cardiovascular-related diseases [45, 46]. Therefore, the significant increase in corticosterone level recorded in the amniotic fluid of all the genistein-exposed rats in this study may partly explain the mechanism by which genistein precipitated a significant reduction in growth of the placenta and the developing fetus [11, 12], a resemblance of IUGR.

The recognition of oxidative stress across the maternal circulation, placenta, and amniotic fluid compartments prompted us to evaluate the inflammatory activities across the maternal circulation cum placenta-amniotic fluid. Our previous report indicated a significant decrease and increase of C-reactive protein [12], a biomarker of inflammation at the early stage of pregnancy and towards term, respectively, in the maternal plasma. Placentation was signaled by the invasion of the uterine wall by the syncytiotrophoblast [16, 47], and the remodeling of the maternal spiral arteries, with the invasion of the decidua and myometrium of the uterine wall by the cytotrophoblasts, in which the placental epithelial cells are in a pro-inflammatory phase [7].

In this study, the serum level of the heat shock protein-60 (Hsp60) was significantly lower in the 2-mg and 4-mg genistein-treated groups compared to control at the early stage of pregnancy, signifying a reduction in the inflammatory activities in the genistein-treated groups. Hsp60 is a highly immunogenic protein whose synthesis has been reported to be greatly upregulated under non-physiological conditions [48]. The Hsp proteins are involved with body immune responses [49] and the folding and translocation of newly synthesized

proteins, protecting cell from structural defects. However, increased serum level of Hsp90 especially around GD-6 towards GD-20 was more pronounced than that of Hsp60, which recorded a significant surge from GD-13. In addition, all the samples analyzed in this study recorded a higher concentration of Hsp90 in multiples of tens in the serum and hundreds both in the amniotic fluid and the placenta, respectively. This gave an indication that Hsp90 is a better biomarker of inflammatory activities than Hsp60 in this study. The 4-mg/kg group, however, recorded a similar surge in serum Hsp60 and Hsp90 between GD-13 and GD-18, giving an indication of increased inflammatory activities around this period of pregnancy in genistein-exposed rats.

Although the amniotic fluid level of Hsp60 was only significantly increased in the 2-mg/kg group, from GD-18 towards GD-20 in this study, the level of Hsp90 was both significantly increased and reduced at GD-18 and GD20, respectively, compared with control. Placenta level of Hsp60 was, however, significantly lower at GD-20 compared to control, while Hsp90 level was significantly reduced in the placenta tissuess samples at GD-20 in both 2-mg and 4-mg groups. The detection of Hsp60 and Hsp70 antibody complexes in the placenta has been suggested to contribute to the induction of preterm birth resulting in adverse pregnancy outcome [50]. Thus, the presence of Hsp60 and Hsp90 in the placenta as recorded in this study will partly explain the earlier reported increased resorption of embryonic implants in female rats exposed to 2-mg and 4-mg genistein [11, 12].

In conclusion, despite the increased oxidative stress in maternal blood, genistein antioxidant activities enhanced the placenta functions in reducing the transfer of the stressor across into the amniotic fluid. However, the inflammatory activities across the placenta in relation to both maternal blood and amniotic fluid were adversely affected, leading to increased corticosterone delivery through the placenta to the amniotic fluid especially in late pregnancy. These are suggestive of mechanistic pathways for genistein-mediated adverse effects on both placenta and fetus in rodents.

Acknowledgments: The authors appreciate the technical assistance of Mr. Folorunsho Adekunle and Mr. Dada Kayode in animal handling and sample collection. This research work was carried out at the Department of Physiology, College of Medicine of the University of Lagos, Lagos, Nigeria, with funding support through the University of Lagos CRC grant (CRC-2015/13).

Author contributions: The research work was conceptualized by Dr. F.O. Awobajo. It was jointly designed by Dr. F.O. Awobajo (FOA), Dr. A.O. Morakinyo, Dr. T.A. Samuel, and Dr. O.T. Oyelowo. Investigation and acquisition of experimental data were undertaken by all authors. Manuscript was originally drafted by FOA and reviewed, edited, and approved by all authors.

Research funding: University of Lagos, Nigeria (CRC No 201303 Code No 11-004-5120).

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Borras C, Gambin J, Lopez-Grueso R, Pallardo FV, Vina J. Direct antioxidant and protective effect of estradiol on isolated mitochondria. BBA-Mol Basis of Dis 2010;1802:205–21.
2. Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ, et al. Onset of maternal arterial blood flow and placental oxidative stress a possible factor in human early pregnancy failure. Am J Pathol 2000;157:2111–22.
3. Guerin P, El-Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 2001; 7:175–89.
4. Harvey AJ, Kind KL. Thompson JG. REDOX regulation of early embryo development. Reproduction 2002;123:479–86.
5. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reproductive Biol Endocrinology 2005;3:28–37.
6. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy; the role of the immune system at the implantation site. Ann NY Acad Sci 2011;1221:80–7.
7. Eckert JJ, Fleming, TP. The effect of nutrition and environment on the preimplantation embryo. Obstet Gynaecol 2011;13:43–8.
8. Pampfer S, Donnay I. Apoptosis at the time of embryo implantation in mouse and rat. Cell Death Differentiation 1999;6:533–45.
9. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 1992;359:76–9.
10. Simon C, Gutierrez A, Vidal A, de-los-Santos MJ, Tarin JJ, Remohí J, et al. Outcome of patients with endometriosis in assisted reproduction: results from in-vitro fertilization and oocyte donation. Hum Reprod 1994;9:725–9.
11. Awobajo FO, Nandedkar TD, Balasinor NH. Genistein alters oestrous cyclicity, oocyte fertilization and implantation process in rats. Nig Q J Hosp Med 2013;23:188–93.
12. Awobajo FO, Onokpite BO, Ali YM, Babaleye TA, Uzor PO, Tijani KO. Genistein precipitated hypothyroidism, altered leptin and C-reactive protein synthesis in pregnant rats. Niger J Physiol Sci 2015;20;30:79–85.
13. Patravale VB, Pandit NT. Design optimization of a novel method for extraction of genistein. Indian J Pharma Sci 2011;73:184–92.

14. Guide MN, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res 2004;114:397–407.
15. Sanin LE, López SR, Olivares ET, Terrazas MC, Silva MA, Carrillo ML. Relationship between birth weight and placenta weight. Neonatology 2001;80:113–17.
16. Jauniaux E, Poston L, Burton GJ. Placental-related diseases of pregnancy: involvement of oxidative stress and implications in human evolution. Hum Reprod Update 2006;12:747–55.
17. Negi R, Pande D, Karki K, Khanna RS, Khanna HD. Oxidative stress and preeclampsia. Adv Life Sci 2011;1:20–3.
18. National Research Council (NRC). Division of earth and Life Studies. Institute for Laboratory Animal Research, Committee: guide for the care and use of laboratory animals, 8th edition. Washington, D.C.: National Academy Press, 1996: 11–124.
19. Dinsdale EC, Ward EE. Early exposure to soy isoflavones and effects on reproductive health: a review of human and animal studies. Nutrients 2010;2:1156–87.
20. Yagi K. Simple assay for the level of total lipid peroxides in serum or plasma. In: Armstrong D, editor. Free radical and antioxidant protocols, methods in molecular biology. Humana Press, 1998;108:101–6.
21. Tipple TE, Rogers LK. Methods for the determination of plasma or tissue glutathione levels. Methods Mol Biol 2012;889:315–24.
22. Kumar A, Dutt S, Bagler G, Ahuja PS, Kumar S. Engineering a thermo-stable superoxide dismutase functional at subzero to >50 °C, which also tolerates autoclaving. Sci Rep 2012;2:387.
23. Aebi H. Catalase in vitro. In Meth Enzymol 1984;8:121–6.
24. Levy J, Faber K, Ayyash L, Hughes C. The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. Proc Soc Exp Biol Med 1995;208:60–6.
25. Wisniewski AB, Klein SL, Lakshmanan Y, Gearhart JP. Exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats. J Urol 2003;169:1582–6.
26. Jefferson WN, Patisaul HB, Williams CJ. Reproductive consequences of developmental phytoestrogen exposure. Reproduction 2012;143:247–60.
27. Lumey LH. Compensatory placental growth after restricted maternal nutrition in early pregnancy. Placenta 1998; 19:105–11.
28. Jansson T, Powell TL. Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. Clin Sci (Lond) 2007;113:1–13.
29. Witlin AG, Li ZY, Wimalawansa SJ, Grady JJ, Grafe MR, Yallampalli C. Placental and fetal growth and development in late rat gestation is dependent on adrenomedullin1. Biol Reprod 2002;67:1025–31.
30. Wisdom SJ, Wilson R, McKillop JH, Walker JJ. Antioxidant systems in normal pregnancy and in pregnancy-induced hypertension. Am J Obstet Gynecol 1991;165:1701–4.
31. Leal CA, Schetinger MR, Leal DB, Morsch VM, da Silva AS, Rezer JF, et al. Oxidative stress and antioxidant defences in pregnant women. Redox Rep 2011;16:230–6.
32. Kurlak LO, Green A, Loughna P, Pipkin FB. Oxidative stress markers in hypertensive states of pregnancy: preterm and term disease. Front Physiol 2014;5:1–8.
33. Burton GJ, Jauniaux E. Placental oxidative stress: from miscarriage to preeclampsia. J Soc Gynecol Investig 2004;11:342–52.
34. Many A, Hubel CA, Fisher SJ, Roberts JM, Zhou Y. Invasive cytotrophoblasts manifest evidence of oxidative stress in preeclampsia. Am J Pathol 2000;156:321–31.
35. Raijmakers MT, Peters WH, Steegers EA, Poston L. Amino thiols, detoxification and oxidative stress in preeclampsia and other disorders of pregnancy. Curr Pharm Des 2005;11:711–34.
36. Lee DY, Kim E, Choi MH. Technical and clinical aspects of cortisol as a biochemical marker of chronic stress. BMB Rep 2015;48:209–16.
37. Burton GJ, Yung HW, Cindrova-Davies T, Charnock-Jones DS. Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. Placenta 2009;30(A):S43–8.
38. LeWinn KJ, Stroud LR, Molnar BE, Ware JH, Koenen KC, Buka SL. Elevated maternal cortisol levels during pregnancy are associated with reduced childhood IQ. Int J Epidemiol 2009;38:1700–10.
39. Davis EP, Sandman CA. The timing of prenatal exposure to maternal cortisol and psychosocial stress is associated with human infant cognitive development. Child Dev 2010;81:131–48.
40. Benediktsson R, Calder AA, Edwards CR, Seckl JR. Placental 11 betahydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. Clin Endocrinol (Oxf) 1997;46:161–6.
41. Levitt NS, Lindsay RS, Holmes MC, Seckl JR. Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. Neuroendocrinology 1996;64:412–8.
42. Nyirenda MJ, Seck JR. Intrauterine events and the programming of dulthood disease: the role of fetal glucocorticoid exposure. Int J Mol Med 1998;2:607–14.
43. Bloom SL, Sheffield JS, McIntire DD, Leveno KJ. Antenatal dexamethasone and decreased birth weight. Obstet Gynecol 2001;97:485–90.
44. Langdown ML, Sugden MC. Enhanced placental GLUT1 and GLUT3 expression in dexamethasone-induced fetal growth retardation. Mol Cell Endocrinol 2001;185:109–17.
45. Barker DJ. Fetal nutrition and cardiovascular disease in later life. Br Med Bull 1997;53:96–108.
46. Sugden MC, Langdown ML, Munns MJ, Holness MJ. Maternal glucocorticoid treatment modulates placental leptin and leptin receptor expression and materno-fetal leptin physiology during late pregnancy, and elicits hypertension associated with hyperleptinaemia in the early-growth-retarded adult offspring. Eur J Endocrinol 2001;145:529–39.
47. Pijnenborg R, Dixon G, Robertson WB, Brosens I. Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. Placenta 1980;1:3–19.
48. Belhia F, Gremlich S, Muller-Brochut AC, Damnon F, Hhlfeld P, Witkin SS, et al. Anti-60-kDa heat shock protein antibodies in fetal serum: a biomarker for unexplained small for gestational age foetuses. Gynecol Obstet Invest 2010;70:229–305.
49. Zugel U, Kaufmann SH. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev 1999;12:319–91.
50. Ziegert M, Witkin SS, Sziller I, Alexander H, Brylla E, Hartig W. Heat shock proteins and heat shock protein antibody complexes in placenta tissue. Infectious Dis Obstetrics Gynecol 1999;7:180–5.