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Placenta Morphological and Amniotic Fluid Biochemical Changes Associated with Mid-Gestation Single and Twin Pregnancies in Red Sokoto Goats

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ABSTRACT

Fetal fluids and placentas, which are important in feto-maternal homeostasis, could be affected by the number of developing fetuses. Therefore, placenta morphological and amniotic fluid biochemical changes associated with single and twin pregnancies in Red Sokoto goats were studied. Using Richardson's formula, 2.1 × [CRL (cm) + 17], 12 intact gravid uteri (7 single-pregnant and 5 twin-pregnant) from mid-gestation [≈70-100 days gestational age (dGA)] pregnant goats were purposively selected. Feto-maternal biometrics [gravid uterine weight (GUW), total placental fluid volume (TPFV), average placentome diameter (APD), mean crown-rump length (MCRL), mean gestational age (MGA), mean fetal weight (MFW), total placentome number (TPN), and average interplacentomal distance (AID)]; amniotic fluid electrolytes [sodium (Na), potassium (K), chloride (Cl), calcium (Ca) and phosphorus (P)]; total protein; glucose; liver markers (AST and ALT); kidney markers (urea and creatinine), and cortisol concentrations were measured. The histology of the placentomes and the interplacentomal areas was also studied. The twin-pregnant (TP) goats had significantly higher ($P<0.05$) GUW, TPFV, APD, amniotic fluid glucose and cortisol; and lower ($P<0.05$) Ca levels. The rest of the parameters assessed did not differ between the TP and single-pregnant (SP) groups. Placentomes and the interplacentomal areas from the TP group had more diffuse villous inter-digitations and thicker luminal endothelium, respectively. It was concluded that TP Red Sokoto goats regulated placentome morphology, calcium, glucose and cortisol levels in order to achieve optimal conditions for the dams and the fetuses.

Key words: Amniotic fluid, Biochemistry, Goats, Placenta, Twin pregnancy

INTRODUCTION

Fetal membranes fluids and placental vascular network are important for physiologic exchanges and efficient handling of fetal waste products of metabolism between the developing fetus and maternal tissues during gestation (Khadjeh et al. 2007; Fitzsimmons and Bajaj 2019). Biochemical profile of fetal fluids is influenced by the feto-maternal exchange through the placenta, as well as fetal metabolic and secretory activities (Banan Khojasteh et al. 2011; Essawi et al. 2020).

Placentas in ruminants, otherwise known as placentomes, are characterized by discrete areas of intimate attachment between maternal uterine caruncles and fetal chorionic cotyledons (Igwebuike and Ezeasor 2013). Placentomes, formed during implantation, are specialized areas for hemotrophic exchange of nutrients/metabolites between the fetal and maternal blood streams (Guillomot 1995; McGeady et al. 2006). The significance of placental function to fetal growth and development *in utero* has been demonstrated in pre-mating carunclectomized sheep leading to restricted placental growth or blood supply (Robinson et al. 1979). Fetal growth retardation has also been observed in pregnant sheep with uterine artery ligation (Emmanouilides et al. 1968), and heat stress (Galan et al. 1999).

The magnitude of changes associated with gestation is usually influenced by number of developing fetuses (Mackie et al. 2019). The biologic systems of TP goats have the responsibility to make homeostatic adaptation in order to accommodate the expected additional demands of twin pregnancies for the survival of both the dam and the fetuses. Metabolic changes during pregnancy, which could be related to organic structural modifications, are assessed

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by measuring some biochemical markers and comparing with the reference values (Cuckle 2014). Deliberate search of available literatures revealed very scanty information on the feto-maternal characteristics, placental histoarchitecture and metabolic adjustments of twin pregnancies in Red Sokoto goats. The aim of the study was to investigate the feto-maternal and biochemical characteristics of mid-gestation single-pregnant and twin-pregnant goats.

MATERIALS AND METHODS

Sample collection: Twenty-two intact gravid uteri (9 single-pregnant and 13 twin-pregnant) were promptly collected from apparently-healthy pregnant Red Sokoto goats slaughtered at the Nsukka municipal abattoir, Ikpa, Nsukka, Enugu State, Nigeria between March and April, 2019. The samples collected were immediately transported in cold chain to the laboratory for processing. Each gravid uterus was weighed to determine the gravid uterine weight (GUW). The uterine walls were incised along the intercaruncular space on the greater curvature of dorsal surface to expose the underlying fluid-filled chorio-allantois and the fetus. The amniotic fluid was aspirated into calibrated glass measuring cylinder to determine the total placental fluid volume (TPFV). Ten ml each of amniotic fluid were stored in labeled plastic tubes at -20°C until biochemical analyses. Following determination of fetal mean crown-rump length (MCRL) and mean fetal weight (MFW), mean gestational ages (MGA) were deduced using the formula: 2.1 × [CRL (cm) + 17] of Richardson et al. (1976). Ultimately, 12 samples (7 single-pregnant and 5 twin-pregnant) were purposively included in the analysis as mid-gestation pregnancies [≈70-100 days gestational age (dGA)]. By manual counting, the total placentome number (TPN) was determined. The average placentomal diameters (APD) and interplacentomal distances (AID) were measured using vernier calipers (n=10).

Amniotic fluid biochemistry and hormonal assay: Electrolytes: sodium (Na, mmol/L), potassium (K, mmol/L), chloride (Cl, mmol/L), phosphorus (P, mmol/L) and calcium (Ca, mg/dL) were determined by colorimetric method using commercial kit (Agappe Diagnostics, Cham, Switzerland). Kidney markers (urea and creatinine concentrations) were determined by kinetic method using commercial kits (Dialab, Wiener Neudorf, Austria). Liver markers: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by colorimeter method using commercial kits (Dialab, Wiener Neudorf, Austria). The total protein was determined by the direct Biuret method. Serum glucose concentration was estimated using commercial kits (Dialab, Wiener Neudorf, Austria). Cortisol assay was performed using ELISA Kits (Monobind Inc., Lake Forest, California, USA).

Tissue collection and processing for histology: Thick sections (3-5 mm) of the placentomes and interplacentomal areas were fixed overnight in Bouin's fixative composed of saturated aqueous picric acid, formalin (40% formaldehyde) and acetic acid. The tissues were sequentially dehydrated in ascending grades of alcohol; cleared in xylene and embedded in paraffin wax to form hard blocks, which were serially sectioned at 5µm thickness using a rotary microtome (Model 1512; Leitz®, Wetzlar, Germany). The tissue sections were mounted on glass slides coated with 20% albumin, deparaffinized, rehydrated in descending grades of alcohol and stained with hematoxylin and eosin (H&E) according to the methods described previously (Slaoui and Fiette, 2011). The stained slides were examined under the microscope and images captured with a Moticam Camera 1000 (Motic China group Ltd., Xiemen, China).

Statistical analysis: The IBM SPSS statistics version 21.0 for windows (IBM Corp, Armonk, NY, USA) was used for the statistical analyses. Data were analyzed using independent sample student's *t*-test. The means were considered significantly different at P<0.05. The results were presented in tables and graphs as mean±SE.

RESULTS

Feto-maternal biometrics

The results of the feto-maternal biometrics presented in Fig. 1 showed that gravid uterine weight (GUW), total placental fluid volume (TPFV) and average placentome diameter (APD) were significantly higher (P<0.05) in TP goats compared with SP goats. However, there was no significant difference (P>0.05) in the mean crown-rump length (MCRL), mean gestational age (MGA), mean fetal weight (MFW), total placentome number (TPN), and average interplacentomal distance (AID) between SP and TP Red Sokoto goats.

Amniotic fluid electrolytes, total protein and glucose

The results of the amniotic fluid electrolytes, total protein and glucose concentrations are presented in Fig. 2. There was no significant difference (P>0.05) in the amniotic fluid ionic concentrations of sodium, potassium, chloride, calcium, inorganic phosphate and total protein between sinlgeton and TP Red Sokoto goats. However, the amniotic fluids of TP goats had lower (P<0.05) calcium level and higher (P<0.05) glucose level compared with SP goats.

Liver markers, kidney markers and cortisol concentrations: The level of amniotic fluid cortisol in the TP group (1.82±0.43µg/dL) was significantly (P<0.05) higher compared with SP group (0.98±0.17µg/dL). On the other hand, amniotic fluid concentrations of AST, ALT, urea and creatinine did not significantly differ between both groups (Table 1).

Gross and microscopic features of the uterus and the placenta: Observations from the histological sections of the placentomes revealed that there were more diffuse villi formation and inter-digitations between the maternal and fetal tissues in TP goats (Fig. 3D) compared with the SP goats (Fig. 3C). Histology of the interplacentomal areas showed that TP goats had thicker endothelium of the maternal blood vessels (Fig. 3F) compared with the SP goats (Fig. 3E). Moreover, the lumen of the maternal blood vessels in TP goats was also wider (Fig. 3F) compared with the SP goats (Fig. 3E).

Table 1: Amniotic fluid liver enzymes (AST & ALT), kidney metabolites (urea & creatinine), and cortisol levels in single-pregnant and twin-pregnant Red Sokoto goats

GROUP	AST (IU/L)	ALT (IU/L)	Urea (mg/dL)	Creatinine (mg/dL)	Cortisol (µg/dL)
SINGLE	11.31±2.15	9.18±1.06	61.07±5.98	3.41±0.97	0.98±0.17^{a}
TWIN	12.88±3	11.41±2.12	61.37±7	3.1±0.79	1.82±0.43^{b}
P-value	0.684	0.382	0.975	0.816	0.025

Means with different superscripts in a column are significantly (P<0.05) different.

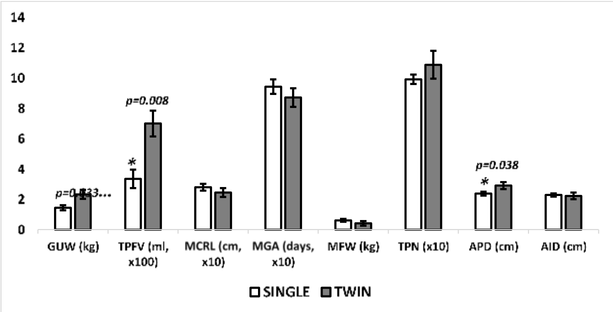

Fig. 1: Feto-maternal biometrics of single-pregnant and twin-pregnant Red Sokoto goats. Gravid uterine weight (GUW), total placental fluid volume (TPFV), mean crown-rump length (MCRL), mean gestational age (MGA), mean fetal weight (MFW), total placentome number (TPN), average placentome diameter (APD) and average interplacentomal distance (AID). Single-pregnant (n=7), Twin-pregnant (n=5); *significant differences (P<0.05).

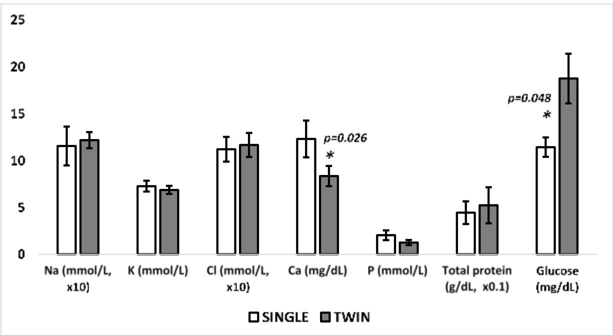

Fig. 2: Amniotic fluid electrolytes, total protein and glucose levels in single-pregnant and twin-pregnant Red Sokoto goats. Sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphate (P) and total protein. Single-pregnant (n=7), Twin-pregnant (n=5); *significant differences (P<0.05).

DISCUSSION

During pregnancy, remarkable changes are known to occur in the developing fetus and the dam (Narelle 2017). The knowledge of feto-maternal biometrics is essential to predict parturition, prepare the fetus and dam nutritionally and otherwise, and in handling some fetal developmental/ infertility problems (Kumar et al. 2004). The GUW and TPFV which were higher in TP goats could be a direct reflection of the number of fetuses *in utero*. The summation of two fetuses in the uteri of TP doe-goats, against one fetus in SP goats resulted in higher weights of the gravid uteri. Consistent with our finding, a greater uterine weight had been reported in TP ewes compared with SP ewes (Grazul-Bilska et al. 2006). More placental fluid is both required and secreted in twin pregnancy in order to provide the enabling environment for the developing fetuses (Hill et al. 2000). The greater space created by the twin fetuses also helps to accommodate the fluid secreted (Ippolito et al. 2014).

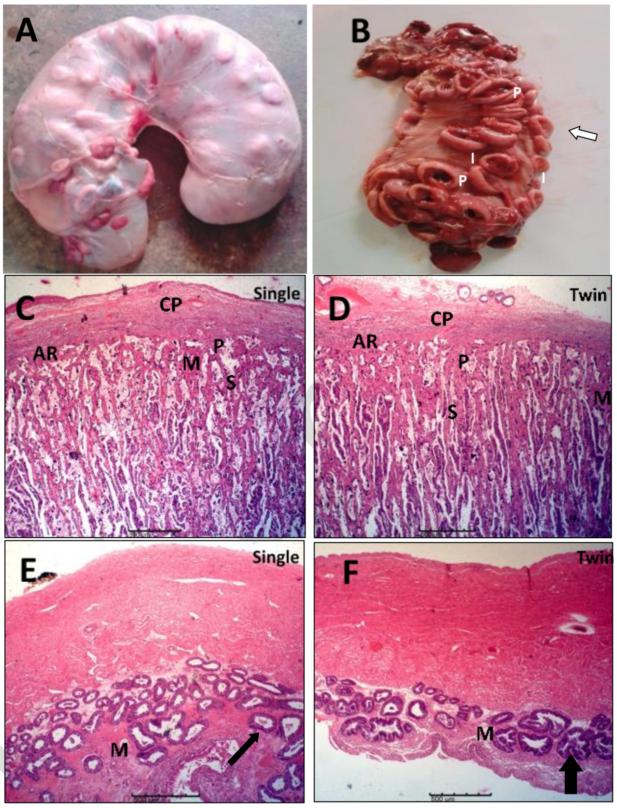

Fig. 3: Placental characteristics of single-pregnant and twin-pregnant Red Sokoto goats. Photograph of intact gravid uterus (Fig. 3A), and uterus incised to expose the placentomes and the interplacentomal areas (Fig. 3B) showing round or oval placentomes (P), interplacentomal areas (I), and chorionic plate (arrow) that formed the concave face of the placentome typical of cotyledonary placenta. Micrographs of the placentomes from SP goats (Fig. 3C) and TP goats (Fig. 3D) showing primary chorionic villi (P) arising from the chorionic plate (CP), secondary chorionic villi (S), and maternal tissue (M), H&E stain, X100. Micrographs of the interplacentomal areas of the SP goats (Fig 3E) and TP goats showing maternal connective tissue (M) and maternal blood capillaries (white arrows, thicker in TP goats). Note the wider lumen and thicker endothelium of the capillaries in Fig. 3F. H&E stain, X100.

There was no significant difference in the MCRL, MFW, TPN and AID between SP and TP Red Sokoto goats. Except for the pathological cases of teratogenicity and other genetic abnornalities, each fetus at a given age should have a specified weight and crown-rump length (Gabr et al. 2015), hence the no variation in the MCRL and MFW was observed in the study between SP and TP groups. The number of placentomes counted in this study was consistent with the reports of previous studies on pregnant goats (Abd-Elnaeim 2008; Igwebuike and Ezeasor 2013). The average number of placentomes in domestic ruminants, which is not dependent on the number of implanted fetuses, have been estimated to be between 75 and 125 (Senger 2003). It was reported that the available placentomes was distributed among the fetus(es). Even if it was only one fetus, the feto-maternal units from both the ipsi-lateral and contra-lateral horn would serve the fetus (Cerdeira et al. 2020). However, the functional demands on individual placentomes may vary and cause structural adjustments, as the number of fetuses increases. This therefore explains the increased APD observed in TP goats. Previous researchers have also reported that there was no variation in the placentome diameter between SP and TP ewes (Kaşikçi et al. 2011).

The observed decrease in amniotic fluid concentrations of calcium in TP goats compared with the SP goat could be related to increased utilization/uptake of calcium by the twin fetuses in the process of bone formation (Jo et al. 2015). In cattle, a similar decrease in amniotic fluid concentration of calcium was equally reported as the fetuses developed from mid gestation to late gestation (Essawi et al. 2020). Most biological processes that require energy expenditure, such as muscle contraction, blood coagulation, bone formation and cardiac control, directly need calcium, thereby reducing the calcium available in the amniotic fluid (Suttle 2010). The clinical implication of this is that TP goats may require calcium supplementation in the last stages of gestation in order to avoid calcium deficiency-related diseases like parturient paresis (Kojouri 2003). No differences were detected in amniotic fluid concentrations of sodium, potassium, chloride and inorganic phosphate in the present study. This suggested that the amount of these minerals required for the growth of the fetuses and pregnancy tissues could be sufficiently obtained from the diet, and optimized by a decrease in endogenous excretion (Härter et al. 2015). This is consistent with the previous findings, where there was no variation in the potassium, sodium and phosphates (Banan Khojasteh et al. 2011). The similarity in the total protein levels could be due to the fact that proteins are not filtered through the fetal nephrons into fetal urine during fetal excretion, hence the number of developing fetuses is inconsequential. However, since glucose demand is directly propotional to the number of fetuses *in utero*, and glucose is being secreted into the amniotic fluid from the fetus. Thus the glucose concentration in the amniotic fluid of TP goats was significantly higher compared with the SP does (Hay 2006).

There was no variation in fetal fluid concentrations of urea, creatinine, AST, ALT and total protein. This could be attributed to the fact that these kidney and liver markers are not usually expressed in significant quantities in the fetuses. They are markers that depict ageing and injury. Previous studies stated that AST, ALT and total protein levels are not affected by pregnancy and the number of developing fetuses (Carter 1990; Pasciu et al. 2019). The values of total protein in this study were comparable to those recorded in amniotic fluids of cattle in mid to late periods of gestation (Essawi et al. 2020).

The significantly higher amniotic fluid cortisol concentration in TP Red Sokoto goats compared with their SP counterparts could be attributed to increased stress imposed by the twin fetuses. With maternal stress, corticotrophin-releasing hormone (CRH) is produced by the placenta, leading to activation of fetal Hypothalamus-Pituitary-Adrenal (HPA) axis and fetal cortisol production, which is secreted into the amniotic fluid (Challis et al. 2000). The cortisol increase in the TP group, therefore, could represent cummulative response to stress experienced by the two developing fetuses (Herman et al. 2016). This increase in amniotic fluid cortisol could also be due to the fact that cortisol is required for normal development of the fetal respiratory organs, liver and kidneys (Wood and Keller-Wood 2016).

The placentomes from mid-gestation goats had more diffuse villi formation and inter-digitations between the maternal and fetal tissues in TP goats compared with SP goats. This could be a reflection of the increased functional demands, in terms of blood supply and other feto-maternal relationships in the TP goats compared with the SP goats (Erdoğan et al. 2016). The diffuse nature of the villi permits adequate supply of comparatively higher nutrients/waste exchange occasioned by the presence of the twin fetuses. This may also be responsible for the previous observations that TP goats had heavier fetal cotyledons compared with SP goats (Ocak et al. 2015). On the other hand, the histological sections of the interplacentomal areas from the TP mid-gestation goats showed thicker endothelium of the maternal blood vessels compared with the SP goats. The lumen of the maternal blood vessels in the TP goats was also wider compared with the SP goats. These could be part of the functional adjustments towards exchanging nutrients/waste products in the face of the increased number of fetuses (Erdoğan et al. 2016).

Conclusion

In the present study, twin pregnancy in Red Sokoto goats caused an increase in the gravid uterine weight, placental fluid volume, placentome diameter and glucose level; but decreased the amniotic fluid concentration of calcium ions. The increased functional demands associated with twin pregnancy in Red Sokoto goats were structurally demonstrated histologically.

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