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INFLUENCE OF *GINKGO BILOBA* ON BIRTH WEIGHT AND HISTOMORPHOMETRIC CHARACTERISTICS OF NEONATAL KIDNEYS IN ALBINO RATS

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ABSTRACT

To elucidate the effects of *Ginkgo biloba* extract during pregnancy on the fetal growth in terms of weight and renal histogenesis, 28 pregnant albino rats (Wistar) were divided into four groups A, B, C and D (n=7). *Ginkgo biloba* was administered orally @ 3.5, 7 and 14 mg/kg/day to group A, B and C, respectively, from 8th to 20th day of gestation. Group D served as normal group. The animals were weekly weighed and observed for toxicity during pregnancy. After parturition, different morphological features of neonates were measured and euthanized to collect kidneys for length, width, weight, cortical and medullary thickness measurement. Renal sections were prepared by paraffin tissue technique followed by hematoxylin and eosin staining for histological examination. The weight gain was non-significant in dams although significant (P≤0.01) weight reduction was witnessed in neonate's weight. kidneys weight showed highly significant (P≤0.01) increase in treated groups than controls in dose-dependent manner. Interstitial edema, inflammation, tubular degenerations and hemorrhages were observed in renal sections. This experimental data suggested that extensive use of *Ginkgo biloba* during second and third trimester of pregnancy has a negative effect on renal genesis, thus, proving its safety to mothers during pregnancy but deleterious effects on renal histogenesis.

Keywords: Ginkgo biloba; birth weight; renal genesis; histogenesis; herbal medicine, reproductive toxicity; organogenesis.

INTRODUCTION

Ginkgo biloba (Family: Ginkgoaceae) has been used in China and its native countries as oriental cuisine, folkloric medicine and nutritional supplements (Brenner *et al*., 2005) for over 5000 years. *Ginkgo Biloba* was introduced in Europe and America only since 1980 as a part of the herbal repertoire. It is among the highest selling herbal tonics along with other medicinal plants (Qureshi *et al.*, 2017; Ali *et al*., 2017, Majeed *et al*., 2018, Dogan *et al*., 2018). The uncertainty it holds in the treatment of various diseases has created an unprecedented interest to determine its biological activities. Its embryo-toxic or fetotoxic effects are likely to remain unrecognized as it is only being tested in few randomized controlled trials.

Clinically, the extracts of *Ginkgo biloba* are used in the treatment of vascular and avascular (Weinmann *et al*., 2010; Tan *et al*., 2015) including Alzheimer's disease (Mancuso *et al*., 2012), cerebrovascular diseases like stroke (Zeng *et al*., 2005), peripheral vascular diseases like intermittent claudication (Nicolai *et al*., 2013), migraine (Esposito and Carotenuto, 2011; Allais *et al*., 2013), erectile dysfunction (Yeh *et al*., 2008; Wu *et al*., 2015), antidepressant induced sexual dysfunctions (Ashton and Gupta, 2000; Kang *et al*., 2002). It is very popular in the female for improving the symptoms associated with menopause or premenstrual syndrome (Ozgoli *et al*., 2009). It has strong antiaging property (Dong *et al*., 2004; Huang *et al*., 2012).

The standardized dried leaves extract contains – 24 percent flavonoids (Quercetin, Kaempferol andIsorhamnetin in large quantities) U.S Pharmacopeia and 6 percent terpenoids (ginkgolides and bilobalides). These are believed to account for *Ginkgo biloba's* beneficial health effects. Terpenes improve circulation while flavonoids are neuro-protective (Wollschlaeger *et al.,* 2003; Smith *et al*., 2004). The toxicity can be attributed to its constituents which includes ginkgolic acids, bilobalides, biflavones, cardols, cardanols and quercetin *Ginkgo biloba* is excreted either through kidneys (21%) or expired through lungs (16%)**.**It flavonoids can cross the placenta and enters the fetus where its concentration in tissues was found to be more than that of mother's (Schröder-van *et al*.,1998). Colchicine was also found in the placental blood of women using ginkgo biloba. Colchicine is linked to Down's syndrome and inhibits the cell division; therefore, it is strongly contraindicated in pregnancy (Petty *et al*., 2001).No specific literature regarding the teratogenic effect of ginkgo biloba was available despite of its wide medicinal usage. So, the purpose of this study is to assess the effects of *Ginkgo biloba* on the fetal growth in terms

of weight and histomorphometric changes in neonatal kidney after its maternal ingestion during gestation period from day 8 to day 21.

MATERIALS AND METHODS

Collection of Animals: A total of twenty-eight healthy female Albino rats (Wistar) weighing 200-250 g were used in this study. These animals were acclimatized for 15 days under optimally maintained room temperature, humidity and a 12-hour light/dark cycle.

Experimental Design: After mating, at the ratio of 3:1, the females with male albino rats, the pregnant females were separated and divided into four groups (A, B, C and D) containing seven biological replicates each. The first three groups A, B and C were experimental while the D served as control. Standardized extract of *Ginkgo biloba*, 120mg/5ml liquid preparation was purchased from Trimax Pharmaceuticals, Pakistan. The highest dose for human is 240mg/day, which is equivalent to 3.5mg/kg/day in an adult human being. In this study, an aqueous solution of 3.5, 7 and 14 mg/kg/day was administered in the total 1ml of water volume to Group A, B, and C, respectively while only water was given to Group D. The medicine was given once daily via oral gavage from 8th to the21st day of pregnancy. The mothers were weighed every seventh day to look for weight gain. Daily water and food intake, piloerection, any locomotor alterations, vaginal bleeding and maternal death were also noted. After delivery, neonates were weighed by using electrical balance while Crown-rump length (cm), head circumference (cm), abdominal circumference (cm) were measured by measuring tape. They were examined for any gross congenital abnormalities.

Collection of Samples: Each neonate was euthanized and midline abdominal incision was made to expose the abdominal viscera. The viscera were inspected for any visible deformity; color and consistency of the organs were checked and recorded. Neonatal kidneys were collected and cleared from fat and fascia. Kidneys were weighed on electric balance in grams. Renal length (mm), width (mm), cortical and medullary thickness (mm) were measured with the help of Vernier's caliper. The fetal tissues were fixed in 10% buffered formalin for 72 hours, later they were processed in automatic tissue processor and embedded in paraffin (Suvarna *et al*., 2013). Labeled blocks were kept for fifteen minutes in the freezer before cutting. Five micron (µm) thin sections were obtained using a rotary microtome (Leica RM 2125), floated in warm water at 45°C and transferred to pre-cleaned albumenized glass slides. Haematoxylin and Eosin (H & E) were used for general Staining.

Histomorphometric Parameters: The sections were studied under a light microscope (Leica DM 1000) at 200X. Diameter (µm) of glomeruli, and Bowman's capsule, proximal and distal convoluted tubules (µm), were measured using automated image analysis system Image J® version 1.49v. All measurements were made with a standardized ocular micrometer.

Statistical analysis: Descriptive statistics were applied for each parameter under study with the help of computer software Microsoft Excel. The means of parameters were compared with one-way analysis of variance (ANOVA) and least significance difference (LSD) test helped to compare the group means at 1% and 5% level of significance.

RESULTS

Gross Anatomical Parameters: The gain in maternal weight during pregnancy was compared with that of control group (Table I). Non-significant ($P>0.05$) changes were observed in body weight of the gravid rats.

Mean values of morphological parameters of neonates from experimental groups compared with control group (D) (Table II). Mean birth weight and crown-rump length in neonates of albino rats fed at different doses of *Ginkgo biloba* extract reduced significantly ($P\leq0.01$) from the control group. Average weight, length and width of kidneys in albino neonates revealed significant ($P\leq0.01$) increase in a dose-dependent manner (Table III).

Histological Parameters: The histological picture of the neonatal kidney from Group D showed normal interstitial space with no inflammation and hemorrhages (Fig 2). Glomeruli appeared normal and well defined. Tubular epithelium (T) was well defined with normal cell height both in proximal and distal convoluted tubules. Tubular epithelial cells showed no changes with the loss of nuclei and decrease in the height of the epithelial cells. Lumen (L) appeared to be dilated. Early fibrotic changes were also seen in proximal tubules.

The histological picture of neonatal kidney Group A, whose mothers were exposed to Ginkgo biloba @ 3.5 mg/kg/day, showed mild to moderate interstitial edema with few foci of inflammation (In)and hemorrhage. Glomeruli (G)were well defined with normal Bowman's space (A, Fig 2). Tubular epithelium(T)was intact. Mild congestion could also be visualized in blood vessels of the medulla. No fibrosis or tubular damage was observed. Cortex appeared to be unremarkable. The histological picture of the neonatal kidney from Group B, whose mother was exposed to Ginkgo biloba @ 7 mg/kg/day (B, Fig. 2) showed moderate interstitial edema with multiple foci of inflammation (In) and hemorrhages (H). Glomeruli (G) were well defined with normal Bowman's space. Tubular epithelium (T) was foamy in appearance more prominent in distal convoluted tubules. Tubular epithelium (T)

showed degenerative changes with foci of mild fibrosis. Cortex appears to be unremarkable but slight thinning was observed.

The histological section of the neonatal kidney from Group C, whose mothers were exposed to Ginkgo biloba @ 14 mg/kg/day, (C, Fig. 2) showed severe interstitial edema with multiple foci of inflammation (In) and hemorrhage(H). Glomeruli (G) appeared to be poorly defined with a tuft of capillaries irregularly arranged. Bowman's space seemed to be increased due to flattening of lining epithelium and edema around capillaries. The tubular epithelium (T) was foamy, more prominent in the distal convoluted tubules. Tubular epithelial cells showed marked atrophic changes with the loss of nuclei and decreased in the height of the epithelial cells. Lumen appeared to be dilated. Early fibrotic changes were also seen in proximal tubules. Cortex was, however, preserved withaslight reduction in its thickness.

Table 1. Mean ± SEM values of live weight of pregnant females of group A, B, C treated with *Ginkgo biloba* @ 3.5,7 and 14 mg/kg/day respectively, as compared to group D on Day 1, 8, 15, 21 of pregnancy.

Groups	Days after pregnancy			
	Day 1	Day 8	Day 15	Day 21
Group A	208.00^{A}	217.86^{B}	227.86^{C}	243.43^{D}
Group B	205.63^{A}	216.63^{B}	226.50^{C}	241.50^{D}
Group C	211.43^{A}	220.29^{B}	230.71^{C}	241.71^{D}
Group D	211.43^{A}	220.71^{B}	231.29^{C}	242.71^{D}

Different superscripts in each column indicate significant difference at P≤0.01.

Table 2. Mean ±SEM values of morphological parameters of neonates of albino rats delivered by mothers treated with the *Ginkgo biloba* extract@ (A) 3.5,(B) 7, and (C) 14mg/kg/day from the 8th to 20th day of pregnancy as compared to Group (D).

Parameter	Group A	Group B	Group C	Group D
Birth weight(gm)	4.57 ± 0.05^{A}	4.39 ± 0.043^{B}	4.20 ± 0.05^{C}	4.69 ± 0.04^{A}
Crown rump Length (cm)	6.50 ± 0.03^{B}	6.48 ± 0.06^{B}	6.07 ± 0.03^{C}	6.78 ± 0.01^{C}
Head circum-ference (cm)	3.23 ± 0.01^{A}	3.23 ± 0.01^{B}	2.90 ± 0.03^{C}	3.23 ± 0.01^{A}

Different superscripts in each row indicate significant difference atat P≤0.01.

Table 3. Mean±SEM values of renal morphological parameters of neonates delivered vy mothers treated with the *Ginkgo biloba* extract @ (A) 3.5,(B) 7and (C) 14mg/kg/day from the 8th to 20th day of pregnancy as compared to Group (D).

Parameter	Group A	Group B	Group C	Group D
Weight (mg)	20.84 ± 0.02^{D}	22.62 ± 0.05^{B}	23.30 ± 0.05^{A}	21.75 ± 0.04^{C}
Length (mm)	3.68 ± 0.06^{D}	4.98 ± 0.05^{C}	6.00 ± 0.03^{A}	5.27 ± 0.10^{B}
Width (mm)	2.95 ± 0.04^{C}	3.68 ± 0.060^{B}	3.70 ± 0.06^{B}	2.89 ± 0.10^{A}

Different superscripts in each row indicate significant difference atP≤0.01.

Table 4. Mean ± SEM values of morphological and histomorphometric parameters of neonatal kidneys delivered by mothers treated with the *Ginkgo biloba* extract, A@ 3.5, B@7and C@14mg/kg/day) from the 8th to 20th day of pregnancy as compared to Group (D).

Parameter	Group A	Group B	Group C	Group D
		Morphometric Parameters		
Cortical thickness (mm)	0.88 ± 0.02^{C}	1.21 ± 0.01^{A}	1.06 ± 0.01^{B}	0.80 ± 0.01^{D}
Medullary thickness (mm)	1.80 ± 0.01^{D}	2.50 ± 0.01^{B}	2.81 ± 0.01^{A}	1.89 ± 0.012^{C}
		Histometric Parameters		
Glomerular diameter (µm)	120 ± 0.01^{A}	121 ± 0.02^{B}	122.7 ± 0.01^{C}	119 ± 0.01^{D}
Bowman capsular area(µm)	10.80 ± 0.01^{D}	12.50 ± 0.04^{B}	14.81 ± 0.02^{A}	10.89 ± 0.06^{C}
Dia. of proximal tubules(µm)	59.93 ± 0.10^{B}	59.71 ± 0.18^{B}	55.18 ± 0.10^{C}	62.22 ± 0.29^{A}
Dia.of distal tubules (µm)	30.11 ± 0.074^{A}	30.09 ± 0.07^{A}	27.47 ± 0.18^{B}	30.27 ± 0.12^{A}

Dia = Diameter; Different superscripts in each row indicate significant difference at P≤0.01.

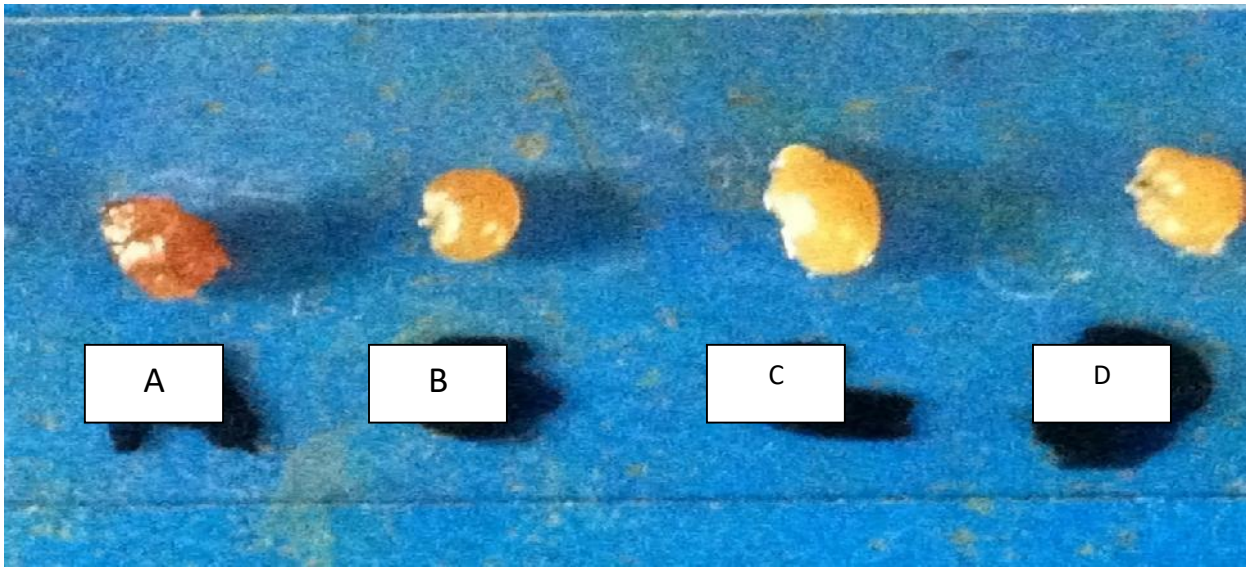

Figure 1. Morphological pictures of neonatal kidneys from group A, B and C after mother's treatment with the Ginkgo biloba extract (A@3.5, B@7and C@14mg/kg/day) respectively from the 8th to 20th day of pregnancy compared with control Group (D).

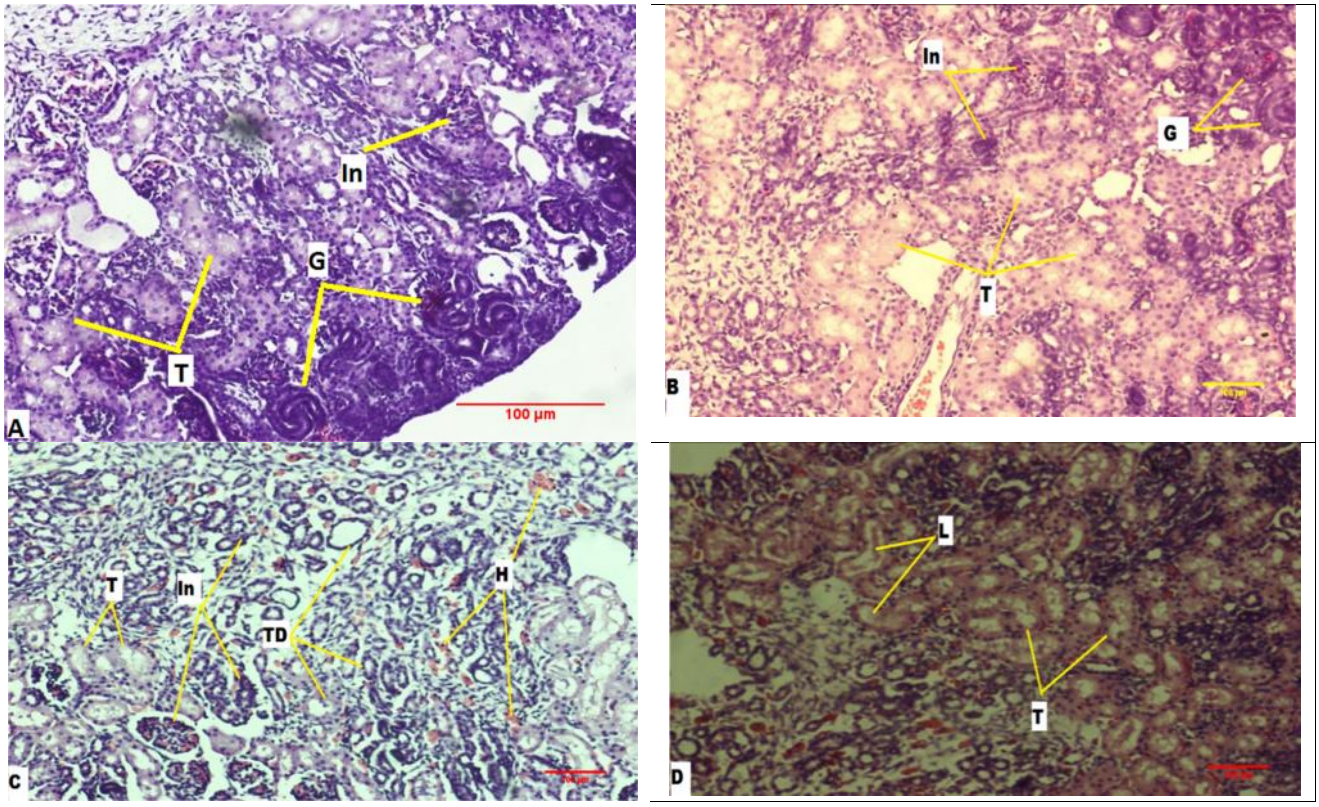

Fig. 2. Histomicrograph of neonatal kidneys of Group A, B, C and D after mother's treatment with the *Ginkgo biloba* extract@ 3.5, 7, 14mg/kg/day respectively. A: kidneys showed mild interstitial odema, well defined Glomeruli (G) with normal Bowman's space, few foci of inflammation (In) and intact cellular structure of Tubules (T). B: Renal histograph demonstrated moderate interstitial edema, multiple foci of inflammation (In), degenerative and foamy tubular epithelium (T) with normal glomerular (G) structure. C: Renal micrograph represented severe interstitial edema, hemorrhages (H), degenerative and atrophied tubular epithelium (T), morefoamy tubular appearance (T) and fibrotic changes. D: renal histology exhibited no inflammation, hemorrhages and tubular (T) degenerations to some extent dilated lumen (L).

DISCUSSION

Ginkgo biloba is top-ranked prescribed medicine in western countries. It has been used traditionally to treat various reproductive and contraceptive diseases both in human and animals. To our knowledge, this is the first report to evaluate the effects of *Ginkgo biloba* on the histomorphometrical development of the neonatal kidneys.

In the present study, non-significant (P>0.05) changes in body weight of the rats were observed. Moreover, non-significant (P>0.05) differences in the estimated food and water intake of rats were observed during this experiment. There were no clinical signs and symptoms for maternal toxicity (tremors, locomotive changes, piloerection, head flicking, convulsions or death) in the study animals. Even after delivery, no gross congenital malformations were seen in the neonates during macroscopic analysis**.**

Mean birth weight and crown-rump length in neonates of albino rats fed at different doses of *Ginkgo biloba* extract reduced significantly (P≤0.01) from the control group. The observations are in line with various who (Pinto *et al*., 2007; Faria *et al*., 2008; Fernandes *et al*., 2010): claimed no effect on maternal body weight but intra-uterine retardation of the fetus. Macroscopic malformations were absent in neonates in the present study, in contrast to the observations recorded by Zehra *et al*. (2010) where the tendency for gross malformation in fetuses increased when pregnant mice were fed with *Ginkgo biloba* @100mg/kg/day throughout their pregnancy. Average weight, length and width of kidneys in albino neonates revealed significant (P≤0.01) increase in a dose-dependent manner in the present study (Table III).

Moreover, significant variations in the histomorphometric parameters of the kidneys i.e. thickness of cortex and medulla, the diameter of glomeruli, Bowman's capsule, proximal tubules and distal convoluted tubules were observed in the study animals (Table IV). Probably the inhibition of iNOS expression by Ginkgo biloba extract in a “dose dependent manner” explains the renal congestion in the fetuses. Nitric oxide (NO), synthesized by the endothelial NO synthase plays a vital role in stabilizing the renal microcirculation and protects the kidney from oxidative injury (Nishida *et al*., 1994; Wang and Abdel Rahman, 2005). There isevidence that ginkgolide A, B, and bilobalide may have a selective inhibitory effect on iNOS expression by inhibiting the transcriptional activity of nitric oxide synthase (DeFeudis *et al*., 2003). A similar trial revealed that after treatment with *Ginkgo biloba* extract (50 mg/ml), NO metabolites released by the endothelial cells were reduced (Cheung *et al*., 1999); This inhibition of NOS metabolites resulted in decreased blood flow to kidneys thus affecting renal agenesis. Significant (P≤0.01) renal damage observed, in the present study, may be have been caused by a similar mechanism. Consumption of plants containing terpenoids can cause fatal proximal tubular necrosis (Obatomi *et al*., 1998). Since terpenoids are one of the major components of Ginkgo extract, this strengthens our results. *Ginkgo biloba* extract also contains 24% phytoestrogens, which are responsible for its hormone replacement potential (Oh & Chung, 2006). There is evidence suggesting harmful effects of phytoestrogens on fetal outcomes (Padilla *etal*.,2012). Teratogenic effects of the Ginkgo extract have also been previously reported like Chan *et al*. (2006) stated that Ginkgo glides compounds in *Ginkgo biloba* halt early post-implantation embryonic development. Early degenerative changes in renal parenchyma, in the present study, may either be due to the vasodilator and anticoagulant properties of Ginkgo biloba or its metabolites. But the adulteration done with herbal medicine like the addition of colchicine can also be responsible for these atrophic and degenerative changes. (Dugoua *et al*., 2006) As renal cells are immature and their exposure to toxic substances can lead to destruction. It is causing more deleterious effects on interstitium, tubules (proximal and distal) and blood vessels. However, Bowman's capsules are preserved as they are somewhat resistant to toxicity. Clinicians and patients should also be concerned about the interactions that may occur between ginkgo and numerous other medications, particularly anticoagulant and antiplatelet drugs. This issue has greater significance when its exposure or toxicity can lead to malformations in fetuses and neonates. The traditional use of *Ginkgo biloba* has not indicated any substantive risks of taking this herb during pregnancy and lactation. Since animal studies are accepted as human risks so it can be generalized that products containing *Ginkgo biloba* pose a risk to the consumers. Nonetheless, rigorous and well-controlled research is needed to assess the toxic effects of *Gingko biloba* and other herbal medicines on maternal and fetal body systems.

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