Periconceptional nutrition programs development of the cardiovascular system in the fetal sheep

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Edwards, L. J., and I. C. McMillen. Periconceptional nutrition programs development of the cardiovascular system in the fetal sheep. Am J Physiol Regul Integr Comp Physiol 283: R669–R679, 2002.—It has been proposed that fetal adaptations to intrauterine nutrient deprivation permanently reprogram the cardiovascular system. We investigated the impact of restricted periconceptional nutrition and/or restricted gestational nutrition on fetal arterial blood pressure (BP), heart rate, rate pressure product, and the fetal BP responses to ANG II and the angiotensin-converting enzyme inhibitor captopril during late gestation. Restricted periconceptional nutrition resulted in an increase in fetal mean arterial BP between 115 and 125 days gestation (restricted 41.5 ± 2.8 mmHg, n = 12; control 38.5 ± 1.5 mmHg, n = 13) and between 135 and 147 days gestation (restricted 50.5 ± 2.2 mmHg, n = 8; control 42.5 ± 1.9 mmHg, n = 10) as well as an increase in the rate pressure product in twin, but not singleton, fetuses between 115 and 147 days gestation. Mean BP and fetal plasma ACTH were also positively correlated in twin, but not singleton, fetuses. This is the first demonstration that maternal undernutrition during the periconceptional period results in an increase in fetal arterial BP. This increase occurs concomitantly with an increase in fetal ACTH but is not dependent on activation of the fetal renin-angiotensin system.

A world-wide series of epidemiological studies has demonstrated associations between low birth weight and poor adult health outcomes, including raised arterial blood pressure (BP) and coronary heart disease (1–3). The reproducibility of these associations across many populations has generated the “fetal origins of adult disease hypothesis.” This hypothesis states that fetal adaptations to a period of intrauterine nutrient deprivation result in a permanent reprogramming of the developmental pattern of key organ systems and in subsequent pathological consequences in adult life (3, 22). Recent data from the Dutch “Winter Hunger” Famine study provided evidence that the timing of fetal nutrient restriction in pregnancy is important in determining specific pathophysiological outcomes (30, 32, 33). Individuals exposed to famine in the first trimester only, when the nutrient demands of the conceptus are minimal, had an increased prevalence of coronary heart disease compared with individuals not exposed to the famine during this period (30, 32, 33). Although adult hypertension was not specifically associated with exposure to maternal malnutrition during any one trimester, it was associated with any level of maternal undernutrition that resulted in reduced fetal growth (33). In experimental studies, rats born to mothers fed a low-protein diet either during the preimplantation period or during mid or late gestation also developed high BP in later life (17, 20). It has been proposed that maternal undernutrition may result in exposure of the embryo or fetus to excess glucocorticoids and that this exposure permanently changes the program of structural or functional development of the cardiovascular system (6, 18, 19, 21, 35). We previously showed in the sheep that maternal undernutrition imposed throughout the last month of pregnancy results in an increase in fetal glucocorticoid exposure, an increase in fetal BP, and in the fetal BP response to ANG II (9, 11). Thus an interaction between exposure to excess glucocorticoids and the renin-angiotensin system (RAS) may play a role in the programming of fetal and adult BP.

In the present study we tested the hypotheses that maternal undernutrition during the preimplantation period or during the subsequent gestational period would result in an increase in fetal arterial BP and that the increase in BP would be related to the basal level of activity of the fetal hypothalamo-pituitary-adrenal (HPA) axis and to an increased activation of the fetal RAS.

We, therefore, investigated the effects of exposure of the embryo and fetus to maternal undernutrition during either the periconceptional period (from 0 to 7 days gestation) or during the gestational period (from 8 to 140 days gestation) on fetal arterial BP and the relationship between fetal arterial BP and plasma ACTH and cortisol concentrations in singleton and twin fetal sheep in late gestation. We also investigated the effects of maternal undernutrition on the fetal BP responses to ANG II and the angiotensin-converting enzyme (ACE) inhibitor captopril during late gestation as well as the rate pressure product (rpp), which is a marker of...
myocardial oxygen consumption and fetal cardiac work (14).

**MATERIALS AND METHODS**

All procedures in sheep were approved by The University of Adelaide Standing Committee on Animal Ethics and Experimentation.

**Nutritional management.** Fifty-two Border-Leicester cross Merino ewes were used in this study. From 60 days before mating, ewes were randomly assigned to one of two feeding regimens, control (C, \( n = 23 \), starting weight: 55.7 ± 0.9 kg), which received 100% of nutritional requirements, or restricted (R, \( n = 29 \), starting weight: 56.6 ± 0.8 kg), which received 70% of the control allowance. The nutritional requirements for the control animals were calculated to provide sufficient energy for the maintenance of a nonpregnant ewe (7.8 MJ/day for a 60-kg ewe) (27). All animals were housed in individual pens and had free access to water. The diet consisted of lucerne chaff and pellets (Johnsons and Sons, Kandunda, SA, Australia) containing straw, cereal, hay, clover, barley, lupins, almond shells, oat husks, and limestone. Eighty percent of the total energy requirements were obtained from the lucerne chaff and twenty percent of the energy requirements from the pellet mixture. The lucerne chaff provided 8.3 MJ/kg metabolizable energy, 193 g/kg of crude protein, and contained 85% dry matter and the pellets provided 8.0 MJ/kg metabolizable energy, 110 g/kg of crude protein, and contained 90% dry matter. All of the dietary components were reduced by an equal amount in the restricted diet such that a 60-kg ewe would receive a total energy intake of 5.5 MJ/day.

After a minimum period of 60 days, ewes in the R nutrition group had lost significantly more weight (-2.2 ± 0.4 kg, \( n = 29 \)) than those in the C group (-0.5 ± 0.5 kg, \( n = 23 \)). A ram was introduced, and 7 days after mating, ewes from each feeding regimen were randomly assigned to the C or R plan of nutrition for the remainder of pregnancy. Four treatment groups were therefore generated: C-C (\( n = 12 \), 6 ewes carrying singleton pregnancies and 6 ewes carrying twin pregnancies); C-R (\( n = 11 \), 4 ewes carrying singleton pregnancies and 7 ewes carrying twin pregnancies); R-R (\( n = 16 \), 11 ewes carrying singleton pregnancies and 5 ewes carrying twin pregnancies); and R-C (\( n = 13 \), 6 ewes carrying singleton pregnancies and 7 ewes carrying twin pregnancies). The period from 60 days before mating until 7 days after mating is termed the “periconceptional period,” and from day 8 until the ewes were killed at post-mortem between 140 and 147 days gestation is termed the “gestational period” (term = 147 ± 3 days gestation). Pregnancy and fetal number were confirmed by ultrasound at 60 days gestation. The nutritional intake for animals on the restricted diet was maintained at 70% of control energy requirements, and both nutritional regimens were adjusted for gestational age and fetal number, as outlined by the Ministry of Agriculture, Fisheries and Food (27).

**Animals and surgery.** Pregnant ewes were transported into the Animal House between 90 and 100 days gestation. Surgery was performed under aseptic conditions between 105 and 110 days gestation (term = 147 ± 3 days gestation) with general anesthesia initially induced by an intravenous injection of sodium thiopentone (1.25 g; Pentothal, Rhone Merieux, Pinkenba, Queensland, Australia) and maintained with 2.5–4% inhalational halothane (Fluothane, ICI, Melbourne, Victoria, Australia) in oxygen. In all ewes, vascular catheters were implanted in a fetal carotid artery and jugular vein and a maternal jugular vein and the amniotic cavity, as previously described (10). Vascular catheters were inserted into one fetus in twin pregnancies. All catheters were filled with heparinized saline, and the fetal catheters were exteriorized through an incision made in the ewes’ flank. All ewes and fetal sheep received a 2 ml injection of antibiotics (procaine penicillin 250 mg/ml; dihydrostreptomycin 250 mg/ml; procaine hydrochloride 20 mg/ml, Penstrept Illium, Troy Laboratories, Smithfield, NSW, Australia) at the time of surgery. The ewes were housed in individual pens in animal holding rooms with a 12:12-h light-dark cycle and fed once daily at 1100 with water provided ad libitum. Animals were allowed to recover from surgery for at least 4 days before experimentation.

**Blood sample collection.** Fetal arterial blood (0.5 ml) samples were collected every day for 4 days after surgery and then three times per week thereafter for the measurement of arterial P\textsubscript{O}\textsubscript{2}, P\textsubscript{CO}\textsubscript{2}, pH, oxygen saturation, and hemoglobin (ABL 520 blood gas analyzer, Radiometer, Copenhagen, Denmark).

Fetal arterial blood samples (3.5 ml) were collected in chilled tubes three times per week between 0800 and 1100 for the measurement of ACTH, cortisol, and glucose concentrations throughout late gestation. All blood samples were centrifuged at 1,500 \( \times \) g for 10 min, and plasma was separated into aliquots and stored at -20°C for subsequent hormone and metabolite assays.

**ACTH.** Immunoreactive ACTH concentrations in fetal sheep plasma were measured by radioimmunoassay (ICN Biomedicals, Seven Hills, NSW, Australia), previously validated for fetal sheep plasma (26). The interassay coefficient of variation was 10.3%, and the intra-assay coefficient of variation was <10%.

**Cortisol.** Cortisol was extracted from fetal plasma using dichloromethane as previously described (4). The efficiency of recovery of \( ^{125}\text{I}-\text{cortisol} \) from fetal plasma using this extraction procedure was always >90%. Fetal cortisol concentrations were then measured using an Orion Diagnostica radioimmunoassay kit (Orion Diagnostica, Turku, Finland), previously validated for fetal sheep plasma (11). The interassay coefficient of variation was 20%, and the intra-assay coefficient of variation was <10%.

**Glucose.** Plasma concentrations of glucose were determined by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (COBAS MIRA automated analysis system, Roche Diagnostica, Basel, Switzerland). The intra- and interassay coefficients of variation were both <5%.

**Arterial BP measurements.** Fetal arterial BP and intra-amyotatic pressure were measured directly from the fetal carotid arterial catheter and amniotic catheter, respectively, which were filled with saline and connected to MacLab 1050 displacement transducers (ADInstruments, NSW, Australia) that had been calibrated using a water-filled manometer. The transducers were placed at the level of the ewe’s abdomen corresponding to the position of the fetal body. The transducers were then connected to a MacLab data-acquisition system via a quad-bridge amplifier (ADInstruments). Arterial BP, corrected for amniotic pressure, was measured continuously using the MacLab Chart software on a Power Macintosh computer. Fetal BP was measured continuously, and fetal BP values were taken every 5 min during a 60- to 90-min baseline period between 115 and 125 days and then between 135 and 147 days for the calculation of basal diastolic and systolic arterial BP at each of these gestational age ranges.

**ANG II dose response.** Bolus intravenous doses of ANG II (0.75, 1.5, 3.0, 5.0, and 10.0 \( \mu \text{g} \), Peninsula Laboratories) were administered via the fetal jugular vein catheter in a random...
order with 20 min between each dose. These experiments were performed between 115 and 125 days gestation (C-C, n = 12, 6 singletons and 6 twins; C-R, n = 11, 4 singletons and 7 twins; R-R, n = 15, 10 singletons and 5 twins; R-C, n = 13, 6 singletons and 7 twins). Similarly, random doses of ANG II were also administered between 135 and 147 days gestation (C-C, n = 9, 3 singletons and 6 twins; C-R, n = 5, 2 singletons and 3 twins; R-R, n = 9, 6 singletons and 3 twins; R-C, n = 7, 3 singletons and 4 twins). Fetal arterial BP and intra-amiotic pressure were measured continuously throughout the ANG II dose-response experiments. Fetal BP values from the continuous recordings were analyzed every 20 s from 2 min before until 4 min after each dose of ANG II. A minimum of 12 h was allowed after ANG II experiments before captopril experiments were performed.

Captopril infusion experiments. Captopril ([25]-[3-mercapto-2-methylpropionyl]-L-proline, Sigma) was infused intravenously in fetal sheep for 4 h (Graseby Medical syringe driver M5–10A, Selby Scientific & Medical) between 135 and 147 days gestation (900 μg/h captopril). These experiments were performed in eight fetuses in the C-C group (3 singletons and 5 twins), five fetuses in the C-R group (3 singletons and 2 twins), ten fetuses in the R-R group (7 singletons and 3 twins), and seven fetuses in the R-C group (3 singletons and 4 twins). Intravenous saline (3 ml/h) was also infused in eight age-matched control experiments. Fetal arterial BP and intra-amiotic pressure measurements were recorded continuously from 60 min before until 240 min after the end of the infusion period. Fetal BP values from the continuous recording were analyzed every 10 min for 1 h before the infusion, then every 15 min for 1 h and every 30 min subsequently for 6 h.

Post mortem. Ewes were killed with an overdose of pentobarbital sodium (Virbac, Peakhurst, NSW, Australia) between 140 and 147 days gestation, and the fetuses were delivered by hysterotomy, weighed, and killed by decapitation.

Statistical analysis. Data are shown as the mean ± SE. When a significant interaction between major factors was identified by ANOVA, the data were split on the basis of the interacting factor and reanalyzed. The Duncan's new multiple range test was used post-ANOVA to identify significant differences between mean values, and a probability level of 5% (P < 0.05) was taken as significant.

Mean values for the arterial blood gas variables (PO2, PCO2, pH, O2 saturation, and hemoglobin) were calculated as the average of the values available between 112 and 147 days gestation for each fetus. The effects of periconceptional and gestational nutrition on PO2, PCO2, pH, O2 saturation, and hemoglobin were compared between fetal sheep using ANOVA and the Statistical Package for Social Sciences (Windows version 10.0.5, SPSS Science, Chicago, IL). Specified factors for the ANOVA included fetal number (singleton or twin) and the level of periconceptional nutrition (C or R) and gestational nutrition (C or R). Similarly, the effects of periconceptional and gestational nutrition on fetal weight were compared separately in singleton and twin fetuses using a multifactorial ANOVA. Specified factors for the ANOVA included periconceptional nutrition (C or R) and gestational nutrition (C or R).

Mean arterial BP diastolic BP + 0.4 (systolic – diastolic BP) was calculated for fetuses between 115 and 125 days and between 135 and 147 days gestation as the average of the BP values during the baseline period.

The effect of fetal number on mean arterial BP was compared in the C-C animals using a multifactorial ANOVA with repeated measures using the Statistical Package for Social Sciences (SPSS, SPSS Science) on a Vax mainframe computer (Adelaide University, Adelaide, SA, Australia). Specified factors for the ANOVA included fetal number (singleton or twin), gestational age, and animal.

The effects of periconceptional and gestational nutrition on mean arterial BP, systolic and diastolic BP, heart rate, and rpp (rpp = systolic BP × heart rate) were compared separately in singleton and twin fetuses using a multifactorial ANOVA with repeated measures. Specified factors for the ANOVA included periconceptional nutrition (C or R), gestational nutrition (C or R), gestational age (<125 days or >135 days), and animal.

The effects of periconceptional and gestational nutrition on the fetal mean arterial BP responses to ANG II and captopril were also compared separately in singleton and twin fetal sheep. The change from baseline in fetal mean arterial BP in response to either captopril or saline infusion was compared using a multifactorial ANOVA with periconceptional nutrition (C or R), gestational nutrition (C or R), time (in relation to captopril administration), and animal as the specified factors. The change from baseline in fetal mean arterial BP responses to increasing doses of ANG II (calculated as the difference between the basal mean arterial BP and the maximal mean arterial BP response to any given dose of ANG II) was also compared using a multifactorial ANOVA with repeated measures. Specified factors for the ANOVA included periconceptional nutrition (C or R), gestational nutrition (C or R), ANG II dose (0.75, 1.5, 3.0, 5.0, and 10 μg), and animal.

Linear regression analysis was used to determine the relationship between the mean arterial BP and plasma ACTH or cortisol concentrations (calculated as the average hormone value measured during the age range of the BP measurement, i.e., between 115 and 125 days gestation or between 135 and 147 days gestation). Similarly, linear regression analysis was used to determine the relationship between the arterial BP responses to captopril mean fetal plasma glucose concentrations.

RESULTS

Fetal well being and outcome. Arterial PO2 and oxygen saturation were significantly reduced (P < 0.05) in twin fetuses compared with singleton fetuses in all nutritional groups (Table 1). Restricted periconceptional nutrition resulted in a small but significant reduction (P < 0.05) in arterial PCO2 in singleton fetuses (Table 1). There was, however, no effect of restricted periconceptional or gestational nutrition on the arterial blood gas status in the twin fetuses (Table 1).

Restricted periconceptional or gestational nutrition did not affect fetal survival to post mortem between 140 and 147 days (number of fetal sheep to survive to post mortem: C-C, n = 9/12; C-R, n = 6/11; R-R, n = 9/16; R-C, n = 5/13).

There was no significant effect of either restricted periconceptional or gestational nutrition on fetal weight in singleton fetal sheep (C-C: 4.9 kg; C-R: 5.2 ± 0.2 kg; R-R: 4.9 ± 0.2 kg; R-C: 4.3 ± 0.6 kg). In twin fetal sheep, however, there was an interaction between the effects of periconceptional and gestational nutrition on fetal weight. Twin fetuses in the C-R group (2.8 ± 1.8 kg) were significantly smaller (P < 0.05) compared with the C-C group (4.5 ± 2.7 kg), whereas
there was no difference in the fetal weights between twins in the R-C (4.3 ± 2.3 kg) and R-R (3.9 ± 2.1 kg) groups.

**Fetal arterial BP.** In the group maintained on a C maintenance diet throughout the periconceptional and gestational periods (the C-C group), there was no significant difference in the mean arterial BP between singleton and twin fetal sheep at either 115–125 days gestation or at 135–147 days gestation. In the C-C group, the mean arterial BP was significantly higher in both singleton and twin fetuses at 135–147 days gestation (singleton: 44 ± 2 mmHg; twins: 44 ± 2 mmHg) compared with 115–125 days gestation (singleton: 41 ± 2 mmHg; twins: 39 ± 2 mmHg).

When all of the nutritional groups were considered, there was no effect of either restricted periconceptional or gestational nutrition or any interaction between the effects of these nutritional treatments on the mean, systolic, or diastolic BP in singleton fetuses, at either 115–125 days or at 135–147 days gestation (Fig. 1, A–C). In twin fetuses, however, restricted periconceptional nutrition resulted in a significant increase in systolic, diastolic, and mean arterial BP compared with the control periconceptional nutrition groups at either 115–125 days or at 135–147 days gestation (Fig. 1, D–F). There was no effect of restricted gestational nutrition or any interaction between the effects of periconceptional and gestational undernutrition on fetal BP in the twin fetuses. Thus the effect of periconceptional undernutrition on fetal BP is independent of whether the fetuses subsequently experienced restricted nutrition during the gestational period or not, i.e., fetal arterial BP was higher in twin fetuses in the R-C and R-R groups when compared with those in the C-C and C-R groups. There was, however, a significant effect of gestational age, such that the mean, systolic, and diastolic BP were each higher at 135–147 days gestation compared with 115–125 days gestation in twin fetuses in all nutritional groups (Fig. 1, D–F).

**Arterial BP, ACTH, and cortisol.** In singleton fetuses at 115–125 days gestation, there was a negative relationship between mean arterial BP and fetal plasma ACTH concentrations ($r = -0.52, P < 0.01$) (Fig. 2A). In contrast, in twins, there was a positive relationship between the mean arterial BP and fetal plasma ACTH between 115 and 125 days gestation ($r = 0.57, P < 0.005$) (Fig. 2B). There was no relationship, however, between mean arterial BP and plasma cortisol concentrations in either singletons or twins at 115–125 days gestation.

At 135–147 days gestation, there was a positive relationship between mean arterial BP and fetal plasma ACTH ($r = 0.60, P < 0.05$) in twin but not singleton fetuses (Fig. 2, C–D). Furthermore, in this age range, there was also a positive relationship between mean arterial BP and fetal plasma cortisol ($r = 0.63, P < 0.05$) in twin but not singleton fetuses (Fig. 3).

**Fetal heart rate.** There was no effect of either restricted periconceptional or gestational nutrition or any interaction between the effects of periconceptional and gestational nutrition on fetal heart rate in singleton or twin fetuses at 115–125 days or at 135–147 days gestation (Table 2). Fetal heart rate was significantly lower at 135–147 days gestation compared with 115–125 days gestation in singleton ($F = 58.5, P < 0.001$) and twin ($F = 61.3, P < 0.001$) fetuses in all nutritional groups.

**rpp.** There was no effect of either periconceptional or gestational undernutrition or any interaction between the effects of periconceptional and gestational undernutrition on the rpp in singleton fetal sheep between 115 and 125 days gestation (C-C and C-R; $9.6 ± 0.5$ mmHg × beats/min × 10$^{-3}$, $n = 10$; R-R and R-C; $9.7 ± 0.3$ mmHg × beats/min × 10$^{-3}$, $n = 17$) or between 135 and 147 days gestation (C-C and C-R; $8.3 ± 0.5$ mmHg × beats/min × 10$^{-3}$, $n = 6$; R-R and R-C; $8.6 ± 0.4$ mmHg × beats/min × 10$^{-3}$, $n = 10$). In singletons, the rpp was significantly lower, however ($F = 7.8, P < 0.05$), at 135–147 days gestation compared with 115–125 days gestation.

In twin fetal sheep, the rpp was significantly higher in the restricted periconceptional nutrition group compared with the control periconceptional nutrition group at 115–125 days gestation (R-R and R-C; $9.4 ± 0.4$ mmHg × beats/min × 10$^{-3}$, $n = 12$; C-C and C-R; $8.6 ± 0.4$ mmHg × beats/min × 10$^{-3}$, $n = 13$) and at 135–147 days gestation (R-R and R-C; $9.0 ± 0.4$ mmHg × beats/min × 10$^{-3}$, $n = 8$; C-C and C-R; $7.4 ± 0.4$ mmHg × beats/min × 10$^{-3}$, $n = 10$). There was no effect of restricted gestational nutrition or any interaction between the effect of periconceptional and gestational nutrition on the rpp in twins.

### Table 1. Effect of periconceptional and gestational nutrition on fetal arterial blood gas characteristics between 112 and 147 days gestation in singleton and twin fetal sheep

<table>
<thead>
<tr>
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<th>Twins</th>
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<tr>
<td></td>
<td>C-C (n = 6)</td>
<td>C-R (n = 4)</td>
<td>R-R (n = 11)</td>
<td>R-C (n = 6)</td>
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<td></td>
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<tr>
<td>$P_{O_2}$</td>
<td>22.5 ± 1.0</td>
<td>22.6 ± 0.4</td>
<td>23.1 ± 0.6</td>
<td>24.3 ± 1.7</td>
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<tr>
<td>$P_{CO_2}$</td>
<td>45.4 ± 0.9†</td>
<td>44.7 ± 0.5†</td>
<td>42.9 ± 0.9‡</td>
<td>42.6 ± 0.8‡</td>
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<tr>
<td>$pH$</td>
<td>7.38 ± 0.01</td>
<td>7.38 ± 0.01</td>
<td>7.39 ± 0.01</td>
<td>7.39 ± 0.02</td>
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<tr>
<td>$SO_2$*</td>
<td>67.3 ± 3.9</td>
<td>69.5 ± 3.1</td>
<td>68.3 ± 1.6</td>
<td>69.0 ± 1.3</td>
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<tr>
<td>$Hb$</td>
<td>10.0 ± 0.3</td>
<td>9.3 ± 0.3</td>
<td>9.3 ± 0.3</td>
<td>10.0 ± 0.4</td>
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<td>$P_{O_2}$</td>
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<td>62.3 ± 5.1</td>
<td>62.4 ± 3.4</td>
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<td>$Hb$</td>
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<td>9.7 ± 0.3</td>
<td>9.4 ± 0.2</td>
<td>9.1 ± 0.3</td>
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Values are mean ± SE. *Significant difference of blood gas characteristics between singleton and twin fetuses. †‡Significant effect of restricted periconceptional nutrition on blood gas characteristics ($P < 0.05$). C, control; R, restricted.
**ANG II.** In singleton and twin fetuses, mean arterial BP increased in response to increasing doses of ANG II at both 115–125 days gestation ($F = 84.5, P < 0.001$; $F = 133.6, P < 0.001$, respectively) (Fig. 4, A and B) and 135–147 days gestation ($F = 38.0, \hat{P} < 0.001$; $F = 89.7, P < 0.001$, respectively) (Fig. 5, A and B).

There was no effect, however, of either restricted periconceptional or gestational nutrition or any interaction between these nutritional treatments on the BP responses to ANG II in singleton or twin fetal sheep at either gestational age range.

**Captopril infusion experiments.** There was no mean arterial BP response to a saline infusion. In singleton fetal sheep, mean arterial BP decreased in response to captopril infusion and remained low for the duration of the experiment in all nutritional treatment groups. In singletons, there was no effect of restricted periconceptional nutrition on the arterial BP responses to captopril. Restricted gestational nutrition, however, resulted in a smaller hypotensive response to the captopril infusion and recovery periods compared with hypotensive response in the control gestational nutrition group (Fig. 6A).

In twin fetal sheep, mean arterial BP decreased in response to captopril and remained low for the duration of the experiment in all nutrition groups (Fig. 6B). There was no effect, however, of either periconceptional or gestational nutrition or any interaction between the effects of these nutritional treatments on the BP responses to captopril.

Between 135 and 147 days gestation, there was a significant inverse correlation ($r = -0.53, P < 0.005$) between the maximal BP responses to captopril infusion and the mean plasma concentrations of glucose.
when data from singletons and twins were combined (Fig. 7).

DISCUSSION

We demonstrated that maternal undernutrition during the periconceptional period, at a time when the nutrient requirements of the embryo are minimal, resulted in an increase in arterial BP and in the rpp in twin fetal sheep in late gestation. This effect of periconceptional undernutrition was not reversed by the provision of a maintenance control diet for the remaining four and a half months of pregnancy. Maternal undernutrition during either the periconceptional or the gestational periods did not alter mean arterial BP or the rpp in singleton fetuses. There was no difference in mean arterial BP between twin and singleton fetuses in ewes fed a maintenance control diet during the periconceptional and gestational periods.

In the present study, we found no effect of a 30% decrease in the level of maternal nutrition from day 8 until the end of pregnancy (i.e., gestational undernutrition) or any interaction between the effects of periconceptional and gestational undernutrition on fetal arterial BP in either twin or singleton fetuses. It was previously demonstrated that a 15% decrease in maternal nutrition during the first 70 days of gestation resulted in a decrease in fetal arterial BP during late gestation (14). Differences between the effects of maternal undernutrition on the fetal cardiovascular system in these two studies may be explained by the impact of undernutrition on the relative development of the umbilical-placental and fetal vasculature at the two different stages of gestation.

Periconceptional undernutrition and fetal arterial BP. We found an important interaction between the effects of maternal undernutrition during the periconceptional period and embryo number on the programmed development of the cardiovascular system before birth. Interestingly, the offspring of pregnant rats fed a low-protein diet during the preimplantation period also develop increased BP at ~12 wk of age (17). Exposure to a low-protein diet during the preimplantation period also develop increased BP at ~12 wk of age (17). Exposure to a low-protein diet during the preimplantation period reduced the cell numbers in both the inner cell mass and the trophectoderm in the developing blastocyst. Thus maternal undernutrition may restrict early embryonic proliferation and the generation of appropriately sized stem-cell lineages (17). Changes in intrauterine oxygen and glucose concentrations also alter the expression of genes required for the formation of blood vessels in the mouse embryo, including hypoxia inducible factor 1 and VEGF (25). One possible explanation for the results of the present study, therefore, is that periconceptional undernutrition alters the intrauterine environment and changes the expression of those genes that control fetal and/or placental vasculogenesis, resulting in an increase in peripheral vasculature.
It is interesting that arterial BP is increased in twin but not singleton fetuses in response to periconceptional undernutrition. It appears that there is a different impact of restricted nutrition during the preimplantation period on the intrauterine environment of singleton and twin embryos that may be important in cardiovascular development.

Table 2. Effect of periconceptional and gestational nutrition on fetal heart rate between 115 and 125 days and between 135 and 147 days gestation

<table>
<thead>
<tr>
<th>Gestational Age</th>
<th>Fetal Number</th>
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<th>Control-Restricted</th>
<th>Restricted-Restricted</th>
<th>Restricted-Control</th>
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<td>115–125 days</td>
<td>Singletons</td>
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<td>171 ± 4</td>
<td>168 ± 5</td>
<td>174 ± 2</td>
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<td></td>
<td>Twins</td>
<td>165 ± 9</td>
<td>172 ± 3</td>
<td>165 ± 3</td>
<td>179 ± 5</td>
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<td>135–147 days</td>
<td>Singletons</td>
<td>141 ± 4*</td>
<td>150 ± 7*</td>
<td>143 ± 5*</td>
<td>145 ± 3*</td>
</tr>
<tr>
<td></td>
<td>Twins</td>
<td>133 ± 5*</td>
<td>148 ± 9*</td>
<td>144 ± 10*</td>
<td>141 ± 6*</td>
</tr>
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Values are means ± SE of fetal heart rate (beats/min). *Significant effect of gestational age on fetal heart rate in singleton and twin fetal sheep ($P < 0.05$).
weights from multifetal human pregnancies has found that after embryo reduction in the first trimester, the birth weights of the remaining twin pregnancies were significantly reduced compared with the birth weights from nonreduced twin pregnancies (34). This provides evidence that in multifetal pregnancies, fetal development, at least in terms of growth trajectory, is set in early pregnancy. In the present study, therefore, the impact of restricted periconceptional nutrition on the intrauterine environment may differ between singleton and twin fetuses. In this regard, we found that there was no significant effect of either restricted periconceptional or gestational nutrition on fetal weight in singleton fetal sheep. In twin fetal sheep, however, there was an interaction between the effects of periconceptional and gestational nutrition on fetal weight, whereby fetuses in the C-R group were significantly smaller compared with the C-C group. There was no substantial effect, however, of restricted nutrition in the periconceptional period on the weight or blood gas status of twin fetuses. It is the case, however, that fetal weight at term may not be an

![Fig. 5.](image)

Fig. 5. There was no significant effect of restricted periconceptional or gestational nutrition on the mean arterial BP responses (means ± SE) to increasing doses of ANG II between 135 and 147 days gestation in singleton fetuses (A; C-C n = 3, C-R n = 2, R-R n = 6, R-C n = 3) or twin (B; C-C n = 6, C-R n = 3, R-R n = 3, R-C n = 4) fetal sheep. There was, however, a significant effect of increasing doses of ANG II on BP that was consistent across all nutritional groups, as denoted by the superscripts, *P < 0.05.

![Fig. 6.](image)

Fig. 6. A: mean arterial BP responses (means ± SE) to a 4-h captopril infusion were significantly smaller during the infusion and recovery periods in singleton fetuses in the restricted gestational nutrition groups (R-R & C-R, n = 5) compared with the control gestational nutrition groups (C-C & R-C, n = 5) between 135 and 147 days gestation, as denoted by *P < 0.05. B: there was no significant difference in the mean arterial BP responses (means ± SE) to a 4-h captopril infusion in twin fetuses in the control (data from C-C and R-C groups combined n = 9) and restricted (data from R-R and C-R groups combined n = 5) gestational nutrition groups between 135 and 147 days gestation. Systolic and diastolic BP were significantly lower between +30 and +240 min, relative to the start of the captopril infusion compared with preinfusion values in all nutritional groups, as denoted by #P < 0.05.
accurate measure of the impact of periconceptional undernutrition on the early growth trajectory of the twin fetus.

Differences in the arterial BP responses to maternal undernutrition during the periconceptional period, in twin and singleton fetuses, may also be attributed to the differential effects of periconceptional undernutrition on the development of the fetal HPA axis in twin and singleton fetuses. We previously reported that periconceptional undernutrition results in an increase in basal fetal ACTH concentrations and an increase in the cortisol response to corticotrophic releasing factor stimulation in twin but not singleton fetal sheep in late gestation (8). We investigated whether the differences in the responses of the fetal cardiovascular system to periconceptional undernutrition in twin and singleton fetuses may be related to the differences in the responses of the fetal HPA axis to restriction of maternal nutrition in the preimplantation period.

In the present study, we found a negative relationship between mean arterial BP and plasma ACTH concentrations in singleton fetuses at 115–125 days gestation across all nutritional groups. We also previously reported that a 50% decrease in the level of maternal nutrition imposed from 115 days gestation resulted in a similar negative relationship between fetal arterial BP and plasma ACTH concentrations at 115–125 days gestation (9). It is possible that in singleton fetuses, the association between an increase in fetal BP with low circulating ACTH concentrations is a result of cortisol acting to increase fetal arterial BP and concomitantly to suppress pituitary ACTH secretion by negative feedback. At 115–125 days gestation, however, there was no positive relationship between circulating cortisol and fetal arterial BP. In this context, it is important to note that at this stage in gestation when cortisol concentrations are relatively low, the cortisol radioimmunoassay that measures “total” circulating cortisol (i.e., free and bound cortisol) may not be able to distinguish small changes in the bioactive or “free” form of cortisol. In contrast to the singletons, there was a positive relationship between mean arterial BP and plasma ACTH concentrations in twin fetuses at 115–125 days and at 135–147 days gestation. This may reflect the impact of periconceptional undernutrition on the development of the fetal pituitary-adrenal axis in early gestation, which would result in fetal exposure to an increase in circulating cortisol that may program development of the fetal cardiovascular system, resulting in the emergence of an increase in fetal arterial BP in later gestation. It is known that fetal exposure to glucocorticoids at around 27 days gestation results in the delivery of lambs that develop hypertension in adult life (5, 7). It would not be expected that the increase in fetal cortisol would persist at 100–125 days gestation, because the fetal sheep adrenal is relatively quiescent in terms of growth and steroidogenesis during this period of gestation. Thus the association between an increase in fetal ACTH and an increase in fetal arterial BP at 115–147 days gestation may reflect the impact of an activation of the fetal HPA axis that occurred some 60–100 days earlier in gestation.

Fetal arterial BP responses to ANG II and captopril. We previously showed that maternal undernutrition (a 50% reduction in dietary intake) imposed during the last month of pregnancy results in an increase in maternal glucocorticoids and an increase in the fetal BP responses to ANG II (9). Studies in the rat also found that there is an interaction between fetal exposure to excess glucocorticoids and activation of the RAS, which is important in the programming of hypertension in offspring after exposure of the mother to a low-protein diet (18, 19).

In the present study, we found no effect of periconceptional or gestational undernutrition in singleton or twin fetuses on the fetal arterial BP responses to increasing doses of ANG II at either before or after 135 days gestation. Thus, in contrast to undernutrition imposed in late gestation, periconceptional undernutrition does not increase fetal BP through enhanced central or peripheral ANG II sensitivity. Thus any contribution of endogenous ANG II to a nutritionally related programmed increase in fetal BP may depend on the timing and the level of maternal undernutrition.

In the present study, there was a greater hypotensive response to captopril infusion in singleton fetuses in the control compared with the restricted gestational nutrition group. A number of studies have investigated the effects of captopril administration on fetal arterial BP, and different results have been reported. Infusion of captopril into the fetus (10, 28, 31) or the ewe (12, 23, 24) was found to decrease BP in normally grown fetuses by between 2 and 10 mmHg, suggesting a role for ANG II in the maintenance of vascular tone. In contrast, other studies found that captopril administration in normally grown, healthy fetal sheep had little (1–4 mmHg decrease) or no effect on fetal BP (10, 13). When data from all fetuses in the present study were com-
combined, we found an inverse correlation between the fetal hypotensive response to captopril and the fetal plasma concentrations of glucose. It is possible that feeding pregnant ewes a diet that meets the full energy demands of the growing singleton fetus has allowed the “unmasking” of a relationship that exists between fetal RAS activity and circulating plasma glucose concentrations. It has been shown previously that fetal sheep with higher plasma glucose concentrations have a higher level of expression of angiotensigen mRNA levels in the fetal liver (38), and high in vitro glucose concentrations also stimulate the production of ANG II by rat mesangial cells (36). It should be noted, however, that captopril also has the ability to inhibit the breakdown of bradykinin and thus the involvement of bradykinin in the fetal hypotensive responses to captopril cannot be excluded.

**Fetal heart rate, rpp, and maternal undernutrition.** There was no effect of either periconceptional or gestational undernutrition on fetal heart rate or on the decrease in fetal heart rate that occurs with increasing gestational age (16, 37). The rpp (rpp = systolic BP × heart rate) has been used as a marker of myocardial oxygen consumption and thus of cardiac work in the sheep fetus (14) and human adult (15, 29). Periconceptional undernutrition resulted in an increase in the rpp in twin but not singleton fetuses, which was primarily due to the increase in systolic BP associated with this nutritional regimen. Although this may suggest that periconceptional undernutrition results in an increase in fetal cardiac work, we found no effect of periconceptional undernutrition on the weight of either the fetal heart or ventricles.

In summary, this experimental study demonstrates for the first time that maternal undernutrition before and during the first week of twin pregnancies results in an increase in fetal arterial BP that cannot be reversed by the provision of a maintenance level diet from the end of the pregnancy. The increase may reflect an interaction between activation of the fetal pituitary-adrenal axis and the fetal cardiovascular system, but it does not depend on an activation of the fetal RAS. The study provides new insights into the potential mechanisms by which alterations of the nutrient environment of the early embryo may reprogram the development of the fetal cardiovascular system.

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