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Fetal-maternal nitrite exchange in sheep: experimental data, a computational model and an estimate of placental nitrite permeability

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Abstract

Introduction—Nitrite conveys NO-bioactivity that may contribute to the high-flow, low-resistance character of the fetal circulation. Fetal blood nitrite concentrations depend partly on placental permeability which has not been determined experimentally. We aimed to extract the placental permeability-surface (PS) product for nitrite in sheep from a computational model.

Methods—An eight-compartment computational model of the fetal-maternal unit was constructed (Matlab® (R2013b (8.2.0.701), MathWorks Inc., Natick, MA). Taking into account fetal and maternal body weights, four variables (PS, the rate of nitrite metabolism within red cells, and two nitrite distribution volumes, one with and one without nitrite metabolism), were varied to obtain optimal fits to the experimental plasma nitrite profiles observed following the infusion of nitrite into either the fetus (n=7) or the ewe (n=8).

Results—The model was able to replicate the average and individual nitrite-time profiles ($r^2 > 0.93$) following both fetal and maternal nitrite infusions with reasonable variation of the four fitting parameters. Simulated transplacental nitrite fluxes were able to predict umbilical arterial-venous nitrite concentration differences that agreed with experimental values. The predicted PS values for a 3 kg sheep fetus were $0.024 \pm 0.005 \text{ l} \cdot \text{min}^{-1}$ in the fetal-maternal direction and $0.025 \pm 0.003 \text{ l} \cdot \text{min}^{-1}$ in the maternal-fetal direction (mean $\pm$ SEM). These values are many-fold higher than the reported PS product for chloride anions across the sheep placenta.

Conclusion—The result suggests a transfer of nitrite across the sheep placenta that is not exclusively by simple diffusion through water-filled channels.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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Keywords

sheep; nitrite; placental transfer; simulation

INTRODUCTION

Nitric oxide (NO) is a potent vasodilating agent that is generated *in vivo* by NO synthases (NOS) in endothelial [1] and other cells. NO itself is short lived, but related compounds such as nitrite (NO_2^-) and nitrosothiols carry NO-like bioactivity throughout the body [2].

Among the many pathways that interconvert these species are those mediated by nitrite reductase proteins such as members of the heme-containing globin superfamily. These reactions are favored when O_2 concentrations are low [3], constituting an hypoxia-dependent mode of NO production. Thus, for the mammalian fetus *in utero*, where average oxyhemoglobin saturation is far less than in the adult, nitrite as a source of NO bioactivity becomes of particular interest and may account in part for the vasodilated state of the fetal circulation [4, 5].

Fetal plasma nitrite levels are in the range of 200–500 nM [5, 6] and likely to depend on placental transfer of nitrite from mother to fetus, as well as in the reverse direction. Evidence for placental transfer is shown by fetal levels closely following maternal levels [5], and then falling about 50% immediately after birth [7]. Placental permeability of nitrite has not yet been determined experimentally, and such measurements would be difficult to interpret because nitrite is metabolized quickly in blood [8], moves rapidly between body fluid compartments [9], is produced endogenously from NO [10], and may be metabolized in placental tissues. [8], moves rapidly between body fluid compartments [9], is produced endogenously from NO [10], and may be metabolized in placental tissues.

We therefore decided to build a computational model of fetal-maternal nitrite exchange based on experiments in chronically instrumented fetal sheep. In these experiments, nitrite was infused intravenously to either the fetus or the ewe, and the time courses of plasma nitrite concentrations in both circulations were measured, as were uterine and umbilical flow rates and other physiological variables. The nitrite concentration profile is then partly dependent on placental nitrite permeability, and we sought to separate this parameter from other factors using the model, and to gain insight into the distribution of nitrite in various body compartments.

MATERIALS AND METHODS

Animal experiments

The animal protocol was pre-approved by the Loma Linda University Institutional Animal Care and Use Committee. Ewes and their fetuses (gestational age 127 to 131, term 145 days) were surgically instrumented with femoral arterial and venous catheters, with catheters in a uterine vein and an umbilical vein, and with Transonic® flow probes around one uterine artery and the intra-abdominal common umbilical vein. The instrumentation allowed for intravenous infusion of sodium nitrite into either a maternal or fetal femoral vein, the

withdrawal of blood samples after selected time intervals (5 minutes) for the measurement of plasma nitrite concentrations in arteries as well as in the umbilical and uterine veins, for the determination of arterial blood parameters (pH, PO₂, PCO₂, percentage of hemoglobin oxygen saturation, percentage methemoglobin, hemoglobin concentration) and the continuous measurement of uterine and intra-abdominal umbilical vein blood flow rates, blood pressures, and heart rates. In the fetus, intra-abdominal umbilical venous flow is identical with fetal placental flow whereas in the ewe maternal placental flow is mirrored only partially by the uterine flow measured at *one* side, and where the latter also supplies not only placental tissues (cotyledons) but also the myo- and endometrium. Thus, our experimental data are based on the assumption of equal division of flow between the two uterine horns and negligible net nitrite exchange with the myo- and endometrium, which comprise ~10% of uterine blood flow [11]. The duration of an experiment was 200 min. During this time, flow rates and hemoglobin oxygen saturations remained relatively stable, and thus their time-averaged values were used in this study.

Three days after instrumentation, sodium nitrite was infused either into a fetal (7 animals) or a maternal (8 animals) femoral vein (cf. Figure S1 in Supplement). The mode of infusion was identical in both groups: at *time zero*, a saline bolus (6 ml) containing sodium nitrite (12 mmol l⁻¹ in fetuses) was injected within about 6 sec, followed immediately by a continuous infusion (1 ml·min⁻¹) for 60 min. Then the bolus injection was repeated, and the nitrite infusion was continued at twice the initial rate (by doubling the nitrite concentration) for another 60 min when the infusion was stopped. Data were collected until 200 min after the start of the nitrite infusion.

The two groups differed in the amount of nitrite infused. With fetal application, the amount of nitrite infused was the same for each animal, which was 72 µmoles for the boluses and 4.2 and 8.4 µmol·min⁻¹ (concentrations: 4.2 and 8.4 mmol l⁻¹, respectively) for the first and second infusion rates, respectively, a total of 900 µmoles. For maternal application, the amount of nitrite depended on maternal body weight (BW): each bolus transferred 3.6 µmol·kg⁻¹ BW into the ewe, and infusion rates were 0.121 and 0.242 µmol·min⁻¹·kg⁻¹ BW, respectively. On average, about 1500 µmoles were infused.

Blood sample assays

Average arterial blood gas values, corrected to maternal and fetal body temperatures, were measured at experimental time = 120 minutes using an ABL 800 blood gas analyzer (Radiometer, Copenhagen), with PO₂ and PCO₂ reported in mmHg and bicarbonate reported in mmol/l. During the fetal nitrite infusion protocol, maternal arterial levels were as follow: pH 7.44±0.01, PO₂ 109.9±2.0, PCO₂ 33.6±1.0, bicarbonate 22.6±0.9. Fetal arterial levels were as follow: pH 7.31±0.01, PO₂ 20.0±1.5, PCO₂ 47.2±0.7, bicarbonate 23.0±0.8. During the maternal nitrite infusion protocol, maternal arterial levels were as follow: pH 7.46±0.01, PO₂ 108.4±2.7, PCO₂ 34.1±0.49, bicarbonate 23.0±0.9. Fetal arterial levels were as follow: pH 7.33±0.01, PO₂ 22.4±1.7, PCO₂ 47.7±1.4, bicarbonate 23.6±0.9. These values are within the normal range for the chronic fetal sheep animal preparation.

Blood samples for plasma nitrite measurement were centrifuged for 30 sec at 12,000rpm immediately after collection. Plasma was stored at <80 C until assay. Nitrite was measured

using the triiodide chemiluminescence method as previously described [12, 13]. It should be noted that the method measures the combined concentrations of nitrite, *S*-nitrosothiols, *N*-nitrotyrosines and iron-nitrosyl species [14, 15] above 10 nM and has a precision of ± 5 nM. In our hands, removal of nitrite with acid sulfanilamide, as previously described [14, 15], eliminates >97% of the signal in samples collected from either maternal or fetal sheep (data not shown), and thus we designate these measurements here as nitrite. The assay does not detect nitrate.

The computational model

For model construction, the Simbiology® application of Matlab® (R2013b (8.2.0.701), MathWorks Inc., Natick, MA) was used. The program allows the build-up of a model structure and its parameters, and it then generates the necessary differential equations. The model structure is illustrated in Fig.1 in a simplified form. A detailed version representing experiments with fetal or maternal nitrite application is available for download from the *Placenta* website as MatLab® *.sbproj files and a supplemental document which lists all model equations, including the average and individual maternal and fetal plasma nitrite profiles.

Because nitrite was administered to either the fetus or the ewe, a fetal and a maternal infusion model were used, both of identical structure but with a different set of parameter values.

In the model, nitrite can either move between compartments, or it can be irreversibly “converted” and thus removed permanently from the system, as in red cells, for example. Compartments are the maternal and fetal blood volumes that are connected by placental nitrite exchange, and two different distribution volumes for nitrite, one of which is “passive” (A), and the other is “active” (B) in that it is able to convert nitrite.

Nitrite conversion in the red blood cell (RBC) compartment (see below) is controlled by the parameter Kex^* (* indicates either fetal or maternal parameters) which is $Kexf$ in fetal RBC when nitrite is applied to the fetus, and $Kexm$ when applied to the ewe. The fetal and maternal compartments (Figure 1, large boxes) have identical structures: a blood volume compartment divided into a plasma and a red blood cell volume, and two compartments accessible for nitrite outside the blood volume compartment. The flow of nitrite *between* compartments (squares), which includes “placental transfer” (ellipses), depends on their nitrite concentration difference and a multiplier factor which has the unit of a “permeability-surface-product” constant ($PS, l \cdot min^{-1}$); therefore, depending on the concentrations, nitrite movement may be bidirectional between fetal and maternal plasma. The parameters $Kplacf$ and $Kplacm$ ($l \cdot min^{-1}$) are considered to reflect placental nitrite PS in fetal-maternal and maternal- fetal direction, respectively. The rate constants for nitrite exchange between plasma and the red cell volume are chosen so that equilibrium would be quickly approached within 10 sec, and nitrite concentrations in RBC and plasma are assumed to be equal (though an option for higher RBC concentration is included, cf. below).

In contrast to nitrite fluxes between compartments, nitrite “conversion” is irreversible and removes nitrite permanently from the system. Conversion is assumed to follow first order

kinetics and to be proportional to nitrite concentration. The various “real” metabolic nitrite conversion processes are lumped together and not specified in any detail. They include mechanisms such as oxidation to nitrate, reduction to NO, conversion to nitrosothiols, renal excretion and others. Nitrite conversion is allowed to occur in the RBC compartments (parameters Kexf and Kexm) and in one of the two virtual nitrite “distribution” compartments (distribution volumes B), with parameters KexDistf and KexDistm. Nitrite may move into or out of the other “passive” compartments (distribution volumes A) but it is not converted there. The physical counterpart of these distribution or storage compartments could be interstitial fluid, the *reversible* transformation of nitrite into other compounds, or the capacity of red blood cells [16] or tissues [17, 18] to store nitrite in concentrations higher than that of plasma (cf Discussion), or any process that might remove nitrite from plasma temporarily but return it later.

Nitrite concentrations in the compartments are derived from the respective nitrite quantities and the compartment volumes which, in turn, are determined in principle by body weights. For the sheep fetus, the assumed blood space volumes (including blood in the vessels of the placenta) are 111 ml·kg⁻¹ body weight (BV), 36 ml·kg⁻¹ RBC volume, and 75 ml·kg⁻¹ plasma volume (PV) [19]. For the ewe (gestational age 120 days), the values are: 75 ml·kg⁻¹ BV, 19 ml·kg⁻¹ RBC volume and 56 ml·kg⁻¹ PV [20]. These values were used also for individual animals using the respective body weights.

The volumes available for nitrite distribution or temporary “storage” outside the blood space are unknown but were found to be important for simulation of the experimental nitrite profiles. A glance at Fig. 2A exemplifies the problem. At time point *zero*, about 70 μmol of nitrite have been given to the fetus as a bolus and five minutes later, after infusion of an additional 20 μmol of nitrite, the average fetal plasma nitrite concentration is about 25 μmol·l⁻¹. In the blood volume of a 3 kg fetus, the *expected* nitrite concentration, provided that nitrite had not left the blood, would thus be 90 μmol/0.333 l or about 270 μmol·l⁻¹. It is obvious from the large difference between expected and measured concentration that nitrite has, in fact, quickly left the blood space. Three mechanisms for removal would seem available. Firstly, nitrite could be metabolized in the red blood cells. If a nitrite metabolism of this high capacity did exist, removing roughly 80 μmol nitrite within 5 minutes or 16 μmol·min⁻¹, then the increase in nitrite concentration at an infusion rate of only 4.2 μmol·min⁻¹ immediately after the bolus injection could not be explained. Secondly, nitrite could be transferred to the maternal circulation via the placenta. Again, a placental transfer rate of 16 μmol·min⁻¹ would not be possible even if all the nitrite carried in the umbilical arterial blood were transferred across the placenta. Model calculations demonstrate that even a combination of both of the above mechanisms could not account for the fetal and maternal nitrite profiles. Thus, thirdly, a more likely mechanism is the existence of a “distribution” compartment that is able to quickly accept and return nitrite to the blood space.

Values for these volumes are not based on experimental data but were derived from the model fitting procedures. They depend on body weight and were included in the model as two parameters: the maximal available total distribution volume MaxFactor* (either MaxFactorf or MaxFactorm), and the fraction DistPartition* (either DistPartitionf or DistPartitionm) available for the *passive* distribution volume (Fig. 1) that is quickly

accessible from the blood space but has no nitrite conversion capabilities (distribution volume A). An example may illustrate the calculation of storage volumes in the fetus: assuming a fetal weight of 2.5 kg, total BV would be 0.278 l ($2.5 \text{ kg} \times 0.111 \text{ l kg}^{-1}$). The remaining maximal possible distribution volume in the fetal body would be approximately $2.5 - 0.278 = 2.222 \text{ l}$, assuming body volume roughly equals body weight. With a MaxFactorf of 0.8, this would be reduced to 1.78 l of which (DistPartitionf=0.6) 1.07 l would be available for compartment A, and $1.78 - 1.07 = 0.7 \text{ l}$ for compartment B. Thus fetal body weight, blood volume and remaining possible maximal nitrite distribution volume are based on experimental data but what fraction of the maximal volume is used, and the partition between distribution volumes A and B is the result of modeling.

Of similar importance are the nitrite conversion rates. The conversion parameters in the respective RBC compartments Kex* (either Kexf or Kexm) were derived, in combination with the permeability parameters Kplac* (either Kplacf or Kplacm), by the fitting mechanism of Simbiology® as a simultaneous four-parameter (Kplac*, Kex*, Maxfactor*, DistPartition*) fit, and no experimental values were entered into the model. Only conversion within the red cells needed to be considered because maternal or fetal plasma do not convert nitrite [21].

The conversion rate of nitrite in the proposed active compartments B is unknown. In simulations of the *maternal* infusion of nitrite the model results clearly indicated that nitrite conversion in maternal RBC and “placental transfer” were not rapid enough to account for the decay of nitrite following discontinuation of nitrite infusion. By trial and error, a decay parameter of $0.67 \text{ l} \cdot \text{min}^{-1}$ was assumed for nitrite conversion from the maternal compartment B. The respective value in the fetal compartment B was assumed to be $0.008 \text{ l} \cdot \text{min}^{-1}$.

The model variables DistPartition*, MaxFactor*, Kplac* and Kex* (the “fit parameters”) were determined using the fetal and maternal plasma concentrations *simultaneously* for fitting. The four variables were adjusted first using the *average* plasma nitrite profiles for fetal ($n=7$) or maternal ($n=8$) nitrite application. Then, starting with these average values, the fitting procedures were repeated for each *individual* pair of fetus and ewe (using their individual nitrite profiles and body weights) while the parameters of the respective recipient side were kept constant at their average values. Figs. 3 and 4 show the individual nitrite profiles of experiments with fetal (Fig. 3) and maternal (Fig. 4) nitrite application as experimental and simulated data. It is reassuring that the simulated profiles come to agree with the experimental profiles for the separate experiments quite well ($r^2 > 0.93$ on the infusion side). The larger variability of fetal profiles (Fig. 3) is possibly due in part to the constant nitrite dose given, independent of body weights. Tables 2 and 3 summarize the fit parameters and the weights for each experiment. These data then could be correlated with physiological variables such as blood flow rates and oxyhemoglobin saturations.

Of special interest is a comparison of the placental PS parameters in the two directions. Kplacf, which characterizes nitrite transfer in fetal-maternal direction, is of the same size as Kplacm that describes nitrite flux in the opposite direction (Tables 2 and 3).

There are clear-cut differences between individual animals but the values are in a plausible range, perhaps with the exception of the MaxFactorf of Experiment #32 where the value of 1.6 implies a total distribution volume considerably larger than the fetal body weight, even when an estimated placental mass was included.

The Matlab® solver used for simulation and fitting procedures was ode23t (Model stiff./trapezoidal). The numerical results did not differ when other solver types available in Simbiology® were used.

Statistics

Average values are given as mean±SEM. Linear regression was used to examine possible correlation between experimental variables and fitted model parameters. $P<0.05$ (two-tailed tests) was accepted for significance.

The fit between experimental and simulated curves was evaluated by calculating r^2 as $1 - \text{SSE}/\text{SST}$ with SSE =sum of the squared differences between experimental and simulated nitrite plasma concentrations, and SST =sum of the squared differences between experimental nitrite plasma concentrations and their experimental mean value.

RESULTS

Average experimental and simulated nitrite profiles, as shown in Fig 2, agreed well with r^2 values for the nitrite profiles on the application side mostly >0.93 (Tables 1, 2, 3). The model variables used for fitting and the average weights of the fetal sheep and ewes are shown in Table 1.

Following fetal infusion, the model predicts a fetal distribution volume that includes the whole fetal body (MaxFactorf about 1). After infusion into the ewe, the total distribution volume is about 45% of body weight (MaxFactorf=0.45). In the ewe, a greater portion of the infused nitrite was found in the active compared to the passive distribution volume than in the fetus, as shown by the parameter 'DistPartition' in Table 1, Table 2 and Table 3. Thus on the maternal side nitrite conversion in this compartment is required in order to account for the time course of changes in plasma nitrite.

The mean values (Tables 2 and 3) for the permeability-surface-product in the fetal-maternal direction are $0.024 \pm 0.005 \text{ l} \cdot \text{min}^{-1}$ in the fetal-maternal direction and $0.025 \pm 0.003 \text{ l} \cdot \text{min}^{-1}$ in the maternal-fetal direction, values that are not different. Because the electrical potential difference across the sheep placenta is less than one millivolt [22], identical permeability values for an ion in both directions of transfer are possible.

With fetal nitrite infusion the conversion rate of nitrite in red cells did not correlate with oxyhemoglobin saturation. Nor were correlations found between K_{placf} and umbilical blood flow or between K_{placm} and uterine blood flow. These results are consistent with diffusion limitation rather than flow limitation for transplacental nitrite flux.

These various results provide confidence that the proposed model reflects in an appropriate manner the physiology of fetal-maternal nitrite exchange in pregnant sheep. We would like

to use the model now to simulate experiments that would be difficult or impossible to perform in real life.

Model simulations

Long term steady state—Following fetal infusions, plasma nitrite concentrations do not reach a steady state within 120 min, whereas they do following maternal infusions (Fig. 2). Thus, in steady state, when an average of $12.6 \mu\text{mol}\cdot\text{min}^{-1}$ ($=51.9 \text{ kg} \times 0.2416 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$) is infused into the ewe in the interval from 60 to 120 min, the total conversion rate of nitrite in maternal and fetal compartments equals this infusion rate. With fetal nitrite application, the model predicts that stable nitrite concentrations would be reached after about 250 min with a nitrite infusion of $8.4 \mu\text{mol}\cdot\text{min}^{-1}$, some two hours after infusion was stopped in the experiments. Fetal plasma nitrite concentrations then would have reached nearly $100 \mu\text{mol}\cdot\text{l}^{-1}$. Maternal concentrations still would only be about $3 \mu\text{mol}\cdot\text{l}^{-1}$ because overall nitrite conversion in the maternal compartments would have prevented any further rise.

Once plasma concentrations reach a steady state during nitrite infusion, the nitrite infusion rate and the rate of nitrite disappearance from the fetal or maternal compartments must be identical. Nitrite may leave the fetal or maternal system via the placenta or via conversion in the RBC or in compartment B. These three nitrite fluxes are detailed in Figures 5A and 5B, which also show the sum of these fluxes and the infusion rates.

With fetal infusions of $8.4 \mu\text{mol}\cdot\text{min}^{-1}$ more than 50% of the infused nitrite is predicted to leave the compartments via conversion in the RBC, about 30% via the placenta (about $2.2 \mu\text{mol}\cdot\text{min}^{-1}$), and about 15% through conversion in the secondary distribution compartment. With maternal infusions of $12.6 \mu\text{mol}\cdot\text{min}^{-1}$ the predicted pathways are quite different. About 2/3 of nitrite is converted in compartment B, and nearly 1/3 in the maternal RBCs while placental transfer is almost negligible ($0.24 \mu\text{mol}\cdot\text{min}^{-1}$). Placental flux is of less importance after maternal infusions because once nitrite is diluted in the ewes' body fluids only small gradients are available to drive flux across the placenta.

Prediction of arterio-venous nitrite concentration in placental exchange

vessels—Results of the model may be used to predict the arterial-venous difference for nitrite as it passes through placental vessels and the result may be compared with experimental values. In the interval from 100 to 120 min, for example, with a fetal arterial nitrite concentration of $90 \mu\text{mol}\cdot\text{l}^{-1}$ (Fig. 2), and a predicted placental nitrite flux of $2.2 \mu\text{mol}\cdot\text{min}^{-1}$ with an average umbilical flow rate of $0.62 \text{ l}\cdot\text{min}^{-1}$, the expected nitrite concentration difference between umbilical artery and umbilical vein would be $3.5 \mu\text{mol}\cdot\text{l}^{-1}$. This result may be compared to experimentally measured arterial-venous differences that averaged $4.6\pm 1.7 \mu\text{mol}\cdot\text{l}^{-1}$ in this interval. The acceptable agreement with experimental data supports the simulated nitrite fluxes across the placenta and thus the model.

DISCUSSION

This model was designed to predict the permeability-surface area product for nitrite movement across the sheep placenta *in vivo*. Based on experimentally measured nitrite

profiles, the predicted PS values were similar for movement in both the fetal-maternal and maternal-fetal directions, but remarkably high when compared to a similar anion such as chloride. The model was *not* designed to simulate the details and complexities of nitrite metabolism, as has been done elsewhere [23–25], but rather sought to reproduce the fetal and maternal nitrite plasma profiles. It is thus comparable to the holistic approach of Hon et al [26].

The credibility of a model depends on the correspondence of real and simulated data but its significance is based on the capability to predict results that have not, or perhaps cannot, be obtained in experiments. As noted, this model was intended to gain estimates of the placental PS for nitrite in sheep. Because transplacental nitrite fluxes will depend on the plasma nitrite concentrations in the maternal and fetal blood compartment, our primary goal was to simulate these concentrations as accurately as possible. To obtain an optimal simulation, it was necessary (cf. Methods) to implement two types of extravascular compartments accessible for nitrite. Whether these compartments are “real” in an anatomic sense does not affect the determination of the placental PS for nitrite (cf. Discussion below).

We used the chronically instrumented fetal sheep with two reasons in mind. The first was to assess metabolic handling of nitrite under conditions of lower O₂ tensions that prevail in the fetus. There is growing recognition that the conversion of nitrite to NO generally occurs in inverse proportion to O₂ tensions [3], and thus nitrite may serve as a more significant source of NO in the fetus than in the adult. The chronically instrumented fetal sheep enables the controlled infusion of nitrite, the simultaneous withdrawal of blood and measurement of blood flow rates, blood gases, blood pressure and other physiological variables in animals without anesthesia, providing the time course of nitrite concentrations for use in the model simulations. A second reason for interest is the possibility of using nitrite for therapy. In experimental animals as well as in humans, nitrite reduces vascular resistance to blood flow [27–30] and lessens damage caused by ischemia/reperfusion in a variety of tissues (see review by Rassaf *et al.* [31]). Work has already begun to explore the potential benefits of nitrite in adult humans [31, 32] and there is the possibility of administering nitrite to the mother for the treatment of fetal ischemic diseases. This will require knowledge of nitrite transfer across the placenta.

The model structure as displayed in Fig.1 simplifies the real situation to a considerable extent. Detailed pathways in the circulatory system are not included because samples were taken at intervals of at least 5 minutes which is large in relation to circulation times, in the range of ~15 sec [11], and thus the blood space may be assumed to be well mixed. Values for arterial and venous blood are not distinguished because arterial-venous differences are small. And the complexities of placental exchange and biochemical pathways of nitrite metabolism within the red cells are not considered in detail.

It was found necessary, however, to introduce two types of “distribution” or “storage” compartments for nitrite in the model. The reasons for including them are based mainly on the discrepancy between the amount of nitrite infused and the resulting plasma nitrite concentrations (cf. Methods). In principle, both distribution volumes are hypothetical. However, both compartments may have “real” counterparts which may include, for

example, a “virtual” compartment due to *reversible* binding or metabolism of nitrite to proteins and other cell constituents outside of the blood. It may also include physical aqueous spaces, such as interstitial cell fluid, lymph, rapidly permeable cell water, urine, and fluids outside the fetus. The values presented for the maximum volume (MaxFactorf) and the partitioning (DistPartitionf) between the passive volume A and the active volume B (cf. Figure 1) in Tables 1, 2 and 3 are possible if the mass of cotyledons (about 0.4 kg, [33, 34]) and the volumes of the allantoic and amniotic fluids (combined about 1 to 3 liters) are allowed to be available for nitrite distribution on the fetal side.

On the fetal side it was not necessary to postulate that nitrite was metabolized within the distribution volume to a large extent. On the maternal side, however, it was necessary to assume rapid conversion of nitrite in the active distribution volume (Figure 1, distribution volume B). This could reflect a greater tendency for irreversible oxidation of nitrite to nitrate in the maternal tissues where oxygen tension is higher.

The red cell provides an easily accessible storage volume for nitrite (cf. [16]). However, when distribution volumes A and B are removed from the model (which leaves plasma and red blood cell volumes as the only volumes available for nitrite distribution), and the ratio of nitrite concentration in RBC to the concentration in plasma was allowed to change freely while fitting the nitrite profiles, nitrite concentration was required to be 29 times higher in RBC than in plasma for a reasonable fit; this seems highly unlikely. When the ratio was included in the fitting procedure together with the four standard parameters a ratio of 1.2 (RBC) to 1 (plasma) was predicted. Values of this order of magnitude have been reported for normal physiological blood nitrite concentrations ([16], ratio 2.4:1), but nothing is known when plasma nitrite concentrations reach values of $50 \mu\text{mole}\cdot\text{l}^{-1}$ and more, as in our experiments. We elected to assume equal concentrations in RBC and plasma in the model (cf. Hon et al. [26]).

At present, we have no concrete knowledge about the reality of the assumed distribution volumes, and on one hand this point may be regarded as a weakness of the model. This, however, does not invalidate the model’s estimate of the placental permeability for nitrite because the nitrite concentration vs. time profiles are simulated correctly as shown in Figs. 2, 3 and 4, and the rates of transplacental nitrite flux that also appear to be valid as shown in Fig. 5. On the other hand, the role of extra-vascular nitrite compartments in humans or animals does not seem to have been investigated experimentally, and this model may draw attention to this problem.

We base our confidence in the model on three observations:

1. Optimization within the fitting program of four parameters allowed the successful simulation of *average* experimental nitrite profiles in the plasma of fetuses and ewes.
2. The same optimization of the four model parameters permitted the successful simulation of *individual* nitrite profiles in the plasma of fifteen ewes and their fetuses. The values of the four parameters varied within reasonable ranges.

3. Using the predicted rate of nitrite flux across the placenta, an umbilical arterial-venous nitrite concentration difference is deduced that is close to the values observed in experiments.

We think it is reasonable to accept, therefore, the estimated placental PS products in fetal-maternal direction of $0.024 \pm 0.005 \text{ l} \cdot \text{min}^{-1}$ and in maternal-fetal direction of $0.025 \pm 0.003 \text{ l} \cdot \text{min}^{-1}$.

To our knowledge, the placental permeability, or PS of nitrite has not been determined for any placenta, either in the intact animal or using an isolated perfused placental preparation. The nitrite ion is identical in charge and very similar in its diffusion properties to the chloride anion. The diffusion coefficients for chloride in water of $20.3 \times 10^{-6} \text{ cm}^2 \cdot \text{sec}^{-1}$ at 25°C may be compared to a value of $19.1 \times 10^{-6} \text{ cm}^2 \cdot \text{sec}^{-1}$ for nitrite [35]. The chloride permeability-surface product has been determined for the sheep placenta by Thornburg *et al* [35] by observing the blood profiles of radioactively labeled chloride given to fetal sheep in chronically instrumented animals. They arrived at a value of $0.0018 \text{ l} \cdot \text{min}^{-1}$ for a 3 kg fetus. This value is strikingly less than the value estimated for nitrite in our model. This difference is unlikely to indicate an insufficient design of our model because when the data of Thornburg and coworkers [35] were used, our model predicted the observed (Fig. 6) chloride profiles reasonably well when a PS value of $0.0018 \text{ l} \cdot \text{min}^{-1}$ was assumed. Thus the 13-fold greater PS product for nitrite than for chloride suggests it crosses the placental membranes not only through water filled channels as chloride does, but also uses additional diffusive and other more complicated mechanisms for membrane transfer.

One possibility for an additional diffusive pathway is non-ionic diffusion. Samouilov *et al* [36] speculate that nitrite may diffuse across cell membranes in its protonated form as HNO_2 , in spite of the large difference between its pK_a (3.1) and the pH in tissues. At a pH of 7.4, the non-dissociated and membrane-permeable $[\text{HNO}_2]$ would be less than 0.01% of the charged nitrite ion concentration. This proportion is higher than that of chloride (pK_a of HCl is -9), and thus non-ionic diffusion may contribute to the proposed difference between chloride and nitrite permeability in sheep.

Another possibility would be that during its placental passage nitrite is converted to NO , which can diffuse freely across cell membranes [37] and then be converted back to nitrite. Other options include specific transport proteins in cell membranes that would transport nitrite ions and indeed there is evidence for accelerated flux across red cell membranes but findings differ with regard to the effectiveness of various blockers [38]. Specific nitrite transport proteins have been identified in bacteria [39, 40] and plant cells [41, 42] but in animal cells the mechanism of trans-membrane nitrite movement remains poorly understood [36]. Particularly in the multilayered placenta nothing appears to be known about nitrite transfer across the endothelial, syncytio- and cytotrophoblastic layers of the placental membrane. The remarkably high value of the permeability-surface-product predicted by this model should be a challenge to the experimenter. One would expect the human placenta to be more permeable to nitrite than the sheep placenta [43], and therefore that fetal exposure to nitrite would be greater at given maternal doses. As a result, lower maternal nitrite concentrations would be required to reach concentrations needed for fetal therapy. However,

given that the NO-bioactivity of nitrite is enhanced under conditions of low PO₂ [2] such as those found *in utero*, future studies should also take into account that the high rate of maternal-to-fetal nitrite transfer may also place the fetus at increased risk of adverse effects of nitrite such as hypotension and methemoglobinemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

NO	nitric oxide
L-NAME	L-N ^G -Nitroarginine methyl ester
PS	permeability-surface product
RBC	red blood cell
BW	body weight
PV	plasma volume
BV	blood volume
HNO₂	nitrous acid
HCl	hydrochloric acid

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Highlights

- Nitrite can be reduced to NO by heme-containing proteins and other metalloenzymes under hypoxic conditions, and is thus a potential source of NO in the fetus.
- The placental permeability for nitrite has not been previously determined.
- Based on a computer model and experimental data, we determined the placental permeability to be many-fold higher than would be expected based on nitrite's size and charge.
- The results suggest the placental transfer of nitrite is not by simple diffusion through water-filled channels.

Fig. 1.

Structure of the model. Shown here is the arrangement for fetal nitrite infusion because nitrite infusion to the maternal plasma (K_{infm}) and “placental” transfer from maternal plasma to fetal plasma (K_{placm}) are inactivated. Circles (ovals) indicate nitrite fluxes into the fetus (K_{inf} , $\mu\text{mol}\cdot\text{min}^{-1}$) or mother (K_{infm} , $\mu\text{mol}\cdot\text{min}^{-1}$), out of the red cell volume (K_{exf} , K_{exm}) or distribution volumes B ($K_{exDistf}$, $K_{exDistm}$) and across the placenta (K_{placm} , K_{placf}). Squares indicate nitrite movements between compartments in the fetus or the ewe. FetPlasmaNitrite, FetRBCNitrite, MatPlasmaNitrite etc. refer to the *amount* of nitrite (μmol) within a compartment. The “passive” distribution volumes A (FetDistVolA, MatDistVolA) do not convert nitrite, in contrast to the “active” distribution volumes B (FetDistVolB, MatDistVolB) that do. Equal concentrations are reached inside the blood volumes within about 10 seconds. See text for more details.

Fig. 2.

Average nitrite profiles following nitrite infusion to the fetus (A) or the ewe (B). Symbols (closed circles: fetus; open circles: ewe) indicate experimental profiles and lines (dashed lines: fetus; solid lines: ewe) simulated nitrite profiles. Experimental data points are the mean of 7 (A) and 8 (B) animals, respectively. Note that the right y-axis in Panel A gives the appropriate nitrite concentration values for maternal data. Spikes in simulated profiles reflect the bolus infusion of nitrite that have no experimental equivalent. Arrows indicate injection/infusion of nitrite. Bars on symbols are $\pm$ SEM.

Fig. 3.

Experimental (A) and simulated (B) individual nitrite profiles following nitrite infusion to the fetus. Line patterns (B) and symbols (A) correspond to experiments in individual sheep. The corresponding four fitted model parameters are presented in Table 2. Maternal nitrite profiles are not shown but were taken into account in the fitting procedure.

Fig. 4.

Experimental (A) and simulated (B) individual nitrite profiles following nitrite infusion to the ewe. Line patterns (B) and symbols (A) correspond to experiments in individual sheep. The corresponding four fitted model parameters are presented in Table 3. Fetal nitrite profiles are not shown but were taken into account in the fitting procedure.

Fig. 5.

Simulated nitrite fluxes for fetal (A) and maternal (B) nitrite infusions. Dashed lines (--) represent fluxes from the red cell volume compartments into nitrite conversion, solid black lines are transplacental fluxes, and the interrupted lines (-) are fluxes from the secondary distribution volume into nitrite conversion. The sum of the three fluxes (-..) must equal the infusion rate (gray line) into the fetus or the ewe, respectively, at steady-state concentrations. Note that on the fetal side most of the nitrite is metabolized in the red cell volume, whereas on the maternal side nitrite is predicted to be removed mostly in the secondary distribution volume.

Fig. 6.

Experimental (symbols, taken from Figure 1 in [28]) and simulated “chloride” time courses (continuous lines) after a single injection of chloride (continuous infusion inactivated in the model) into a 3 kg sheep fetus [28]. The amount of “chloride” injected is adjusted to result in an initial fetal chloride concentration close to that observed by Thornburg, et al [28]. The model uses the values of all parameters and compartments of the average of our fetal infusion experiments (cf. Fig. 2A) but “nitrite conversion” is inactivated completely. The “chloride” curves (dashed and solid lines) are calculated with a PS values of $0.0018 \text{ l min}^{-1}$ [28] whereas the “nitrite curves” (dotted lines) are based on a PS value of 0.024 l min^{-1} , the proposed nitrite permeability of the sheep placenta. Regarding “nitrite permeability”, equilibrium is reached after about 10 hours, whereas it takes about 6 days to obtain equilibrium when simulation is based on “chloride permeability”. It is noteworthy that it is necessary for a reasonable fit to assume extravascular compartments for chloride also as is proposed for nitrite. Considering the unknown compartment volumes (esp. maternal body weight) of the experiment [28], the fit between the experimental data [28] and the simulated curves appears to be reasonably good.

Table 1

Variables in the model found to describe the time course of plasma nitrite, and average weights of the fetal sheep and ewes.

Nitrite infusion	Fetal	Maternal
DistPartition	0.84	0.0013
MaxFactor	1.016	0.447
Kplac (l·min ⁻¹)	0.0234	0.0263
Kex (l·min ⁻¹)	0.061	0.333
r ² fetal profiles	0.99	0.80
r ² maternal profiles	0.79	0.98
Fetal weight (kg)	2.9±0.2	3.1±0.3
Maternal weight (kg)	53.0±3.0	51.9±1.3

Weight values are means ± SE.

Table 2

Model-predicted values best describing fetal and maternal plasma nitrite concentrations after nitrite infusion in seven fetal sheep.

Exp.#	DistPartitionf	MaxFactorf	Kplacf (l·min ⁻¹)	Kexf (l·min ⁻¹)	Fetal Wt. (kg)	Mat. Wt. (kg)	r ² fetal profiles	r ² matern. profiles
4	0.942	0.853	0.0245	0.0568	2.8	49	0.95	0.95
5	0.748	1.12	0.01	0.073	2.6	50	0.94	0.49
9	0.051	1.16	0.0265	0.0471	2.3	50	0.95	0.92
12	0.048	0.83	0.006	0.046	2.8	49	0.99	na
21	0.773	1.06	0.0286	0.0768	3.2	46	0.99	0.82
25	0.011	0.965	0.0349	0.0557	3.0	65	0.98	0.93
32	1.00	1.56	0.0366	0.126	3.8	62	0.99	na

na: not available

Model-predicted values best describing maternal and fetal plasma nitrite concentrations after nitrite infusion in eight ewes.

Table 3

Exp.#	DistPartitionm	MaxFactorm	Kplacm (l·min ⁻¹)	Kexm (l·min ⁻¹)	Fetal Wt. (kg)	Mat. Wt. (kg)	r ² matern. profiles	r ² fetal profiles
6	0.0001	0.51	0.0227	0.222	2.8	49	0.73	na
7	0.638	0.449	0.013	0.31	2.6	50	0.90	0.82
10	0.0002	0.345	0.0415	0.248	2.8	49	0.97	0.81
11	0.005	0.388	0.0232	0.589	2.3	50	0.98	0.71
13	0.00006	0.411	0.0261	0.179	3.6	51	0.98	na
14	0.0	0.428	0.0293	0.577	4.6	52	0.96	0.61
17	0.00014	0.377	0.0235	0.190	2.6	55	0.97	0.87
22	0.0004	0.331	0.0194	0.623	3.2	59	0.94	0.16

na: not available