

EFFECT OF PREGNANCY AND TEMPERATURE ON RED CELL OXYGEN-AFFINITY IN THE VIVIPAROUS SNAKE *THAMNOPHIS ELEGANS*

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Summary

The oxygen affinity of red cell suspensions from fetal garter snakes was higher than that of cell suspensions from their mothers. This difference appeared to be due to different concentrations of nucleoside triphosphate (NTP, primarily adenosine triphosphate). NTP concentrations were significantly higher, and oxygen affinities were significantly lower, in red cell suspensions from pregnant females compared with those from nonpregnant females or males; there is no precedent for such a pronounced effect of pregnancy on the oxygen affinity of maternal blood. These data indicate that pregnancy may result in an enhanced ability of adult blood to deliver oxygen to the fetus. Since the binding of organic phosphates and oxygen to hemoglobin is sensitive to temperature, and since these animals experience diurnal changes in temperature, we examined the influence of relatively low (20°C) and high (34°C) temperatures on red cell oxygen-affinity. The temperature increase of 14°C resulted in a lowered oxygen-affinity of all red cell suspensions examined. However, this increase in temperature lowered the affinity of maternal red cells to a greater extent than it did the affinity of fetal red cells. This suggests that daytime temperatures may further enhance the ability of maternal blood to deliver oxygen to the fetus at times when fetal oxygen demand is probably greatest.

Introduction

In all viviparous animals examined, with the notable exception of the cat (Novy and Parer, 1969), fetal blood has a higher affinity for oxygen than does maternal blood. This difference in oxygen affinities is thought to facilitate maternal–fetal oxygen transfer (Meschia, 1978; Bauer *et al.* 1981; Itskovitz *et al.* 1984; Edelstone *et al.* 1989) and is due to the presence of unique hemoglobins and/or low

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concentrations of organic phosphates (allosteric modifiers of hemoglobin function) in the red cells of the fetus. Hypothetically, the difference in maternal and fetal blood oxygen-affinities could be due to a decrease in affinity of the maternal red cell relative to that of the nonpregnant adult; that is, pregnancy could cause a reduction in the oxygen affinity of the female's blood. There is, however, no previous evidence that pregnancy induces physiologically significant changes in the oxygen affinity of maternal blood (Battaglia and Meschia, 1986).

Our studies of the garter snake *Thamnophis elegans* have shown that the hemoglobin (Hb) of the fetus is indistinguishable from that of the adult, that the oxygen affinity of purified hemoglobin is reduced by the presence of adenosine triphosphate (ATP), and that fetal red cells contain significantly less nucleoside triphosphate (NTP) (primarily as ATP) than do those of the mother (Berner and Ingermann, 1988). Further, preliminary observations in our laboratory suggested that red cell NTP levels of nonpregnant adults are also lower than those of pregnant females in this species. Consequently, we have measured NTP concentrations and oxygen affinities of red cells from pregnant and nonpregnant females, males and fetuses.

Since the pregnant females, and consequently the fetuses, are exposed to dramatic diurnal fluctuations in temperature (and presumably oxygen demand), and since the binding of organic phosphate to hemoglobin is temperature-sensitive (Benesch *et al.* 1969; Weber *et al.* 1985), we also examined the oxygen affinities of red cell suspensions from fetal and pregnant and nonpregnant adult snakes under simulated night-time and daytime temperatures.

Materials and methods

Adult garter snakes were collected in early summer in Latah Co., Idaho, and identified as *Thamnophis elegans* (Baird and Girard) according to Nussbaum *et al.* (1983). Sex was determined by probing for the hemipenes and pregnancy was determined by abdominal palpation. The developmental stage of the fetus was determined according to embryonic/fetal morphology as described by Zehr (1962). Fetuses used in this study ranged from Zehr stage 35 to stage 37 (the last of the prenatal stages). Adults were kept at 17–20°C with access to a heat source, on approximately natural photoperiod, and fed mice, beef heart or fish on a biweekly basis. Water was available *ad libitum*.

The molar NTP/Hb ratios and mean red cell NTP concentrations of pregnant and male snakes were determined as a function of time following their capture in early summer 1988. Four snakes that had given birth in 1987 were maintained in the laboratory in the absence of males and used as the nonpregnant snakes during the summer 1988 portion of this study. For the analysis of red cell oxygen-affinities, five of the pregnant snakes from summer 1988 served as the nonpregnant animals in summer 1989.

For analysis of red cell organic phosphate concentrations, adult snakes were bled by tail clip or heart puncture under ether anesthesia into ice-cold heparinized,

Ringer's saline (110 mmol l^{-1} NaCl, 1.9 mmol l^{-1} KCl, 1.1 mmol l^{-1} CaCl₂, 2.4 mmol l^{-1} NaHCO₃, pH 7.6). To obtain fetuses, pregnant females were chilled in ice then decapitated. Fetal red cells were collected by sectioning the fetus in ice-cold Ringer's buffer then filtering through glass wool to eliminate debris. (Examination of hematocrit tubes verified that final fetal and adult red cell preparations contained more than 93 % red cells.) Red cells were washed three times in this saline by centrifugation at 1000 g for 5 min at 4°C . Cells were resuspended in this buffer and samples were saved for measurement of NTP and hemoglobin concentrations and hematocrit.

To measure NTP concentrations, the red cell suspension was extracted with an equal volume of ice-cold 12 % trichloroacetic acid and centrifuged for 10 min at 10000 g . The acid extraction of the cells was completed within 30 min of blood collection. NTP concentrations of the supernatant fractions were determined using an enzymatic assay kit (no. 366-UV; Sigma Chemical Co., St Louis, MO). Red cell NTP concentration (in mmol l^{-1}) was calculated as suspension NTP concentration divided by suspension hematocrit. The hemoglobin concentration of the red cell suspension was determined spectrophotometrically as the cyanmet derivative using a millimolar heme extinction coefficient of 11.0 at 540 nm. The NTP/Hb tetramer ratio was calculated as the molar NTP concentration of the red cell suspension divided by the molar concentration of the hemoglobin tetramer.

For studies of oxygen affinity, red cells were washed three times and resuspended in the following buffer: 143 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 1.5 mmol l^{-1} MgCl₂, 1.5 mmol l^{-1} CaCl₂, 20 mmol l^{-1} Tris, adjusted with HCl to pH 7.4. The cell suspension was adjusted to a hematocrit of 10–18 %. Oxygen affinity measurements were conducted by the method of Tucker (1967) with a TC500 Tucker cell and model 781 oxygen meter from Strathkelvin Instruments (Glasgow, Scotland). Cells were incubated for 10–15 min in a water bath set at either 20 or 34°C with partial pressures of oxygen established by mixing nitrogen and compressed air (The Plant, Spokane, WA). P_{50} values were determined from Hill plots, each using 8–10 data points corresponding to cells that were 25–75 % saturated with oxygen. To test the precision of this system at high and low oxygen-affinities and in the absence of carbon dioxide, we analyzed the P_{50} values of fresh, washed human red cells from a single donor at 20°C and red cells from a single male northern Pacific rattlesnake (*Crotalus viridis oreganus*) at 34°C . The P_{50} for human cells was $1.11 \pm 0.16 \text{ kPa}$ and that for rattlesnake cells was $7.52 \pm 0.36 \text{ kPa}$ ($N=6$ for each). (All data are presented as mean \pm 1 s.d.) To test the accuracy of measurements made on washed red cells in the absence of carbon dioxide, we measured the P_{50} of red cells from the rainbow trout (*Oncorhynchus mykiss* = *Salmo gairdneri*) in the buffer of Nikinmaa (1983) at 20°C . Our value was $4.44 \pm 0.28 \text{ kPa}$ ($N=3$); literature values are 3.48 (Vorger, 1986), 4.21 (Vorger and Ristori, 1985) and $5.33 \pm 0.27 \text{ kPa}$ (Nikinmaa, 1983).

We were concerned that NTP concentrations might change during the course of the oxygen-binding analysis since the cells were stored on ice prior to analysis. A change in NTP concentration would probably lead to changes in oxygen affinities.

We therefore analyzed the concentrations of NTP in red cells from adult female garter snakes and found that they decreased by $0.9 \pm 1.5\%$ ($N=5$) over 3 h at 0°C . (Each oxygen-binding study took 2–2.5 h to complete.)

Data were analyzed for significance by paired *t*-test for fetuses and their mothers and by independent *t*-test for the remaining results.

Results

Oxygen-binding studies of red cells from pregnant females and their fetuses showed that fetal cells had a higher affinity for oxygen than did the cells from their mothers at 20°C . Furthermore, the P_{50} values of red cells from both nonpregnant females and males were significantly lower than values from pregnant females and significantly above those of the fetuses at 20°C (Table 1). P_{50} values rose in each group when the temperature was elevated to 34°C . The difference between maternal and fetal P_{50} values at 20°C was 3.62 ± 0.71 kPa ($N=5$), while at 34°C that difference was 5.17 ± 1.39 kPa ($N=5$). Over this 14°C rise, the P_{50} of red cells from nonpregnant females rose from 4.49 ± 0.65 kPa to 8.53 ± 0.63 kPa; this latter value at 34°C was significantly above that of the fetus but was not significantly different ($P=0.051$) from that of the pregnant female. At 34°C , the P_{50} value of red cells from the adult male was significantly less than those of the nonpregnant female and the pregnant female.

NTP levels, expressed either as mean corpuscular concentration or as a molar ratio to hemoglobin tetramer, were lower in the fetus than in any form of the adult examined (Fig. 1). Further, NTP values rose and then appeared to plateau near term in pregnant snakes during the summer (Fig. 1). In contrast, the levels in nonpregnant females and males showed insignificant changes during the same period. The high NTP levels in pregnant snakes had dropped to the levels in nonpregnant adults when they were assayed 6–7 months after giving birth.

Table 2 presents additional hematological variables for pregnant and nonpregnant females. These data show that pregnancy had no influence on blood hemoglobin levels, mean red cell hemoglobin concentrations or hematocrit values.

Table 1. Mean P_{50} values (in kPa) for fetal and adult red cells at 20 and 34°C

	20°C	34°C
Fetus	2.48 ± 0.63^a	4.72 ± 0.81^c
Pregnant female	6.11 ± 0.75^b	9.89 ± 1.17^f
Nonpregnant female	4.49 ± 0.65^c	8.53 ± 0.63^g
Male	4.29 ± 0.91^d	7.32 ± 0.83^h

Values are mean \pm s.d. ($N=5$).

Significant differences in P_{50} data: $P \leq 0.001$: a:b, a:c, a:e, c:g, d:h, e:f, e:g, e:h; $P \leq 0.01$: a:d, b:c, b:d, b:f, f:h; $P \leq 0.05$: g:h; NS c:d, f:g ($P=0.051$).

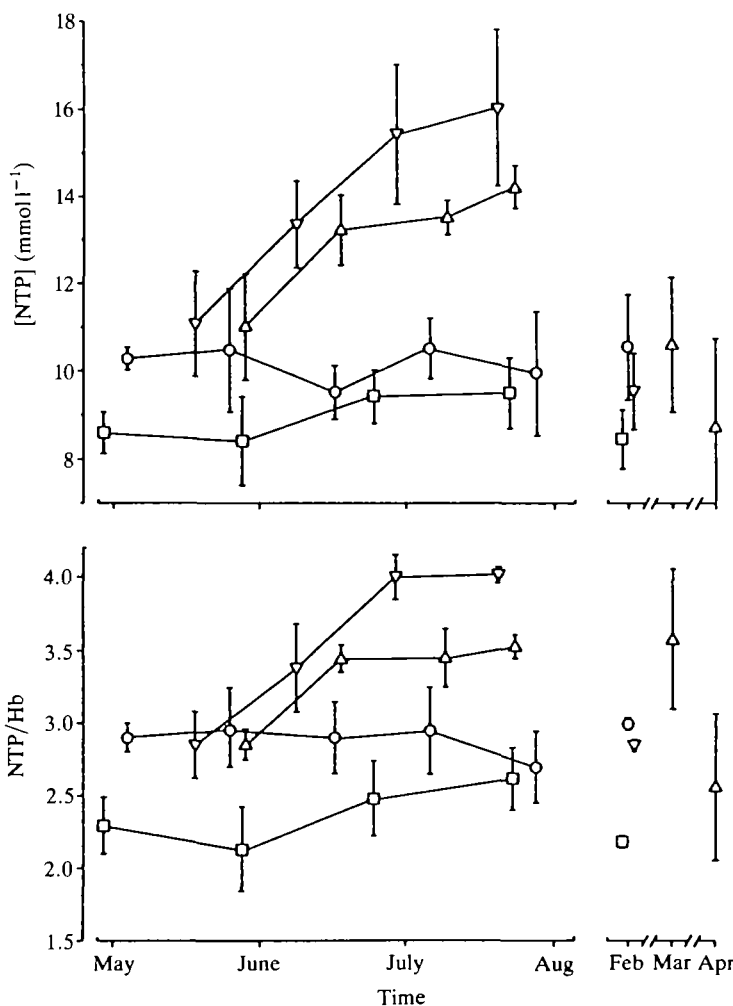


Fig. 1. Red cell NTP, expressed as a concentration (A) and as a molar ratio to hemoglobin tetramer (B) in cells from pregnant (∇ , $N=6$, \triangle , $N=5$) and nonpregnant females (\circ , $N=4$) and males (\square , $N=8$) analyzed between May 1988 and April 1989. Fetal values were: $NTP/Hb=1.69\pm 0.32$ and red cell $[NTP]=5.58\pm 0.77\text{ mmol l}^{-1}$, $N=11$. The two groups of pregnant females (triangles) were kept in different, but adjacent, terraria and were treated and maintained in the same way; we cannot explain why their data appear to be quantitatively different.

Discussion

In almost all viviparous vertebrates that have been examined, fetal red cells have a higher affinity for binding oxygen than do the red cells of the adult. Consistent with these findings, and with those of Manwell (1960) on *T. elegans* in particular, red cells of fetal *T. elegans* had a higher affinity for oxygen than did the red cells of the adult. This difference does not appear to be due to differences in

Table 2. *Blood hemoglobin concentrations, mean corpuscular hemoglobin concentrations (MCHC) and hematocrit values for pregnant versus nonpregnant females*

	[Hemoglobin] (mmol l ⁻¹)	MCHC (mmol l ⁻¹)	Hematocrit (%)
Pregnant female (N=7)	1.18±0.21	3.95±0.36	30.1±5.8
Nonpregnant female (N=8)	1.21±0.28	3.88±0.16	31.3±7.1

Values are mean±s.d.

There are no significant differences between the values of pregnant *versus* nonpregnant females.

the hemoglobins; it appears, instead, to be due to the relatively low concentrations of NTP in fetal red cells (Berner and Ingermann, 1988).

There is no precedent for pregnancy causing an appreciable reduction of the oxygen affinity of adult red cells (Battaglia and Meschia, 1986). Our preliminary findings on the garter snake suggested, and our current results confirm, that pregnancy is associated with a decreased oxygen-affinity of the maternal red cell at 20°C. This pregnancy-associated difference in oxygen affinity suggests that the blood of the pregnant female is better able to unload oxygen to the fetus (and to the tissues) than would be the blood of the nonpregnant adult. The basis of this affinity difference appears to be changes in red cell NTP (probably ATP) levels. Near the end of gestation, the fetal red cell contains appreciably less NTP than does the red cell of its mother, which, in turn, contains more NTP than does the red cell of the nonpregnant adult.

Since pregnancy appeared to be associated with changes in the NTP content and oxygen affinity of the adult red cell, we examined whether pregnancy was also associated with changes in other blood variables: blood hemoglobin, mean corpuscular hemoglobin concentration and hematocrit (Table 2). Consistent with the findings of Birchard *et al.* (1984) on the garter snake *T. sirtalis*, pregnancy was not associated with changes in these variables in *T. elegans*.

During the summer and throughout gestation, the garter snake experiences dramatic diurnal fluctuations in temperature (see review by Lillywhite, 1987). Based on a Q₁₀ effect, one would predict that the basal metabolism of the adult and fetus should be appreciably higher at daytime than at night-time temperatures. This suggests that there are diurnal changes in the metabolic oxygen demands of the adult and fetus. Since the oxygen affinity of reptilian hemoglobin and blood and the binding affinity of organic phosphates to hemoglobin are temperature-sensitive (Benesch *et al.* 1969; Pough, 1980; Weber *et al.* 1985), we examined the influence of temperature on red cell oxygen-affinities. A temperature increase from 20 to 34°C resulted in an increase in red cell P₅₀ values in each group of snakes examined (Table 1). However, the red cell P₅₀ of the pregnant female increased to a greater extent than did the P₅₀ of the fetal red cell; the maternal-fetal difference was about 43% greater at 34°C than at 20°C. Thi

suggests a greater ability of maternal blood to unload oxygen to the fetus at daytime temperatures, i.e. at those times when fetal oxygen demand is probably highest.

A concern with the experimental design of this study might be that pregnant and nonpregnant females had different histories. Pregnant females were collected from the field and were analyzed shortly after capture, whereas nonpregnant females had been maintained for an entire year in captivity after having given birth in our laboratory. Nonetheless, we believe that our findings of relationships between pregnancy and red cell NTP concentration and red cell oxygen-affinity are valid for the following reasons. Red cell NTP levels of just-obtained pregnant females (early in gestation) were comparable to those of the nonpregnant females and, at about 6 months after parturition, those levels had returned to the levels of the nonpregnant female (Fig. 1). Also, NTP levels of males analyzed just after capture did not rise as did those of the pregnant females (Fig. 1). The red cell P_{50} values for just-caught pregnant females and just-caught males were significantly different. Finally, Whittier *et al.* (1987) reported that the red-sided garter snake (*T. sirtalis parietalis*) does not show a classical stress response: captivity is not associated with sustained elevations of corticoids or with a change in the pattern and concentrations of steroid sex hormones. These considerations suggest there is a red cell NTP baseline in the *T. elegans* female and that captivity *per se* does not necessarily lead to a rise in this baseline. These considerations probably extend to red cell oxygen-affinity, since it is very likely that this is determined to a large extent by intracellular ATP concentrations (Berner and Ingermann, 1988).

The data from *T. elegans* thus suggest that the high oxygen-affinity of fetal red cells is due to lower organic phosphate concentrations in these cells compared to the values in the red cells of the adult. In this regard, the mechanism of maternal-fetal oxygen transfer is similar to that described for many other vertebrates. Nevertheless, it appears that pregnancy is associated with a transient increase in organic phosphate concentrations in the red cells of the female, and thus with a decrease in maternal red cell oxygen-affinity. There is no precedent for this finding and its implication that pregnancy may cause a markedly enhanced ability of the maternal blood to deliver oxygen to the fetus. Finally, a consideration of temperature indicates that daytime conditions lower the affinity of maternal blood to a greater extent than that of fetal blood. This suggests a further enhanced ability of maternal blood to deliver oxygen to the fetus at times of greatest fetal oxygen demand.

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