Developmental toxicity in rats of a hemoglobin-based oxygen carrier results from impeded function of the inverted visceral yolk sac

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ABSTRACT

HBOC-201 is a bovine-derived, cross-linked, and stabilized hemoglobin (250 kDa) in physiological saline. Daily intravenous infusions of HBOC (1.95, 3.90, or 5.85 g/kg/day) during gestational days (GDs) 6–18 in Sprague-Dawley rats caused fetal mortality, reduced birth weight, and malformations. Subsequent single-day infusions (5.85 g/kg/day) showed that developmental toxicity was limited to GDs 7–9 when histiotrophic nutrition via the inverted visceral yolk sac (invVYS) is essential. Histiotrophic nutrition is receptor-mediated endocytosis of bulk maternal proteins and subsequent lysosomal degradation providing amino acids and other nutrients for embryonic growth. Controls for protein content, oncotic properties, and hemoglobin content indicated that toxicity was due to hemoglobin. Rat whole embryo cultures verified HBOC interference with invVYS transport capacity and resultant deficient embryonic nutrition. These mechanisms of action are not expected to impact human development based on differences in VYS morphology and function, although a complete understanding of early human embryonic nutrition is lacking.

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1. Introduction

Blood transfusions are frequently life-saving procedures that are received by nearly 5 million people each year [1]. Currently, the blood supply is solely dependent upon volunteer donations, and the population of potential donors has recently decreased due to stricter requirements necessary to keep the blood supply safe. For instance, because severe acute respiratory syndrome (SARS) and bovine spongiform encephalopathy have been detected abroad, individuals who frequently travel are deemed unfit to donate for prolonged periods of time. The unfortunate result is that the blood supply is dangerously low, thus there is an acute need for human blood substitutes.

Hemoglobin-based oxygen carriers (HBOCs) are a promising class of blood substitutes currently in development for intravenous administration to patients suffering from anemia and/or blood loss.

They are used for the dual purposes of expanding blood volume and acting as oxygen-bridging agents [2]. The substance used in these experiments, HBOC-201, is derived from bovine hemoglobin that is thoroughly purified to remove bacteria and viruses. In its native form, bovine hemoglobin is a noncovalently bound tetramer that has a total molecular weight of 64,000 Da (64 kDa). HBOC-201 is formed by covalent bonding of four covalently stabilized tetramers, resulting in a polymeric hemoglobin molecule that exhibits a mean molecular weight of 250,000 Da.

Observations made during pilot studies of HBOC-201 infusion, including resorptions that occurred only early after implantation but not later, raised questions about the appropriateness of the rodent model for human risk assessment [3]. The purpose of the set of studies reported here was to ascertain and characterize the maternal and, particularly, the developmental toxicity associated with intravenous infusion of HBOC-201 in pregnant rats. An understanding of the toxicological properties of HBOC-201 during these sensitive periods provides insight into particular gestational mechanisms likely to underlie the observed effects.

The intrinsic physicochemical characteristics and intended use of HBOC-201 posed significant challenges for designing rational

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dosing protocols for toxicology studies because of the high concentration of protein, oncotic properties, and large injection volumes. Initial experiments examined developmental toxicity resulting from a dose of HBOC-201 (1.95 up to ∼6 g/kg/day) chosen to represent multiples of the highest proposed human dose to be given in one day. HBOC-201 was infused during gestational days (GDs) 6–18, a treatment period that encompasses major organogenesis in the rat. This treatment was associated with embryolethality and a high malformation rate in the initial experiments; further experiments in pregnant rats were performed in order to identify specific sensitive periods during organogenesis when HBOC-201 infusion produces the most pronounced developmental toxicity. Additional studies were performed in order to investigate whether changes in osmotic pressure, properties of the HBOC-201 component not related to hemoglobin, and/or an increase in oxidative stress were underlying factors that might explain or exacerbate the observed toxicity. Because developmental toxicity was only present when dams were intravenously infused with HBOC-201 during the GD 7–9 ranges, a period when the inverted visceral yolk sac (invVYS) provides the primary source of embryonic nutrition, supplementary experiments in whole embryo culture were conducted to determine if HBOC-201 interferes with the normal endocytotic functions of the invVYS in the rodent.

2. Materials and methods

2.1. Infusion studies

Continuous and single-day infusion studies were performed at ClinTrials BioResearch Ltd. Laboratories (CTBR) in Quebec, Canada, and hemodilution studies were performed at WIL Research Laboratories, Inc. (WIL) in Ashland, Ohio. Techniques used in the two laboratories were comparable; any variations are noted. Both facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and animals were maintained in accordance with the principles set forth by the Guide for the Care and Use of Laboratory Animals [4].

2.2. Animals

Female Sprague-Dawley Crl:CD® (SD)BR rats in apparent good health were received from Charles River Breeding Laboratories (Kingston, NY and Portage, MI). Rats were individually housed in stainless steel, wire-mesh cages in temperature and humidity controlled rooms maintained on a 12 h light/12 h dark cycle. Animals were given time to acclimate to the environment and provided with ad libitum access to food and water. At the start of treatment, animals were 10–15 wks old. To mate the animals, one female was placed with one proven male of the same strain and source. The females were examined daily for evidence of mating by detection of a copulatory plug in the vagina and/or examination of the vaginal lavage for spermatozoa. The day of positive identification of copulation was considered GD 0. Females were assigned to experimental groups using a computer-based randomization procedure which provided homogeneity of group means and variances for body weight. At the end of each experiment, animals were humanely euthanized by CO_2 asphyxiation.

2.3. Control and test articles

The infusate vehicle (saline) control consisted of a 0.9% sodium chloride solution (Baxter Healthcare Corporation; Deerfield, IL) stored at room temperature. Solutions of HBOC-201 (13 g/dL) in saline were prepared by Biopure® (Cambridge, MA). A 13% solution of human serum albumin (HSA) in saline (Pharmacia & Upjohn, Inc.; Kalamazoo, MI) was used as a control for protein content. To

control for colloidal composition, a 6% solution of hetastarch in saline (DuPont®; Wilmington, DE) with colloidal properties similar to HBOC-201 was used. An additional control for hemoglobin content was performed using purified bovine hemoglobin (PBH; 13 g/dL) in isotonic saline in Tris/Acetate buffer, pH 7.8 (Biopure®). Aseptic techniques were applied to fill the syringes for infusion.

2.4. Surgical insertion of catheters

The surgical implantation of catheters was performed on non-pregnant or GD 0 animals. At CTBR, animals were anesthetized with ether, and at WIL, animals were anesthetized using a 4:1 mixture of Ketamine HCl and Xylazine (1 mL/kg). All animals remained under anesthesia for the entire surgical procedure. A small incision was made in the groin region, the femoral vein was isolated, and a medical grade silastic catheter was inserted. The catheter was secured in place with suture material and exteriorized via a trocar between the shoulder blades. A jacket was placed on the animal to hold the tether system. A constant infusion of 0.9% sodium chloride at approximately 0.4–0.5 mL/h until initiation of treatment kept the catheter patent. A topical antibiotic was administered daily to both the catheter exteriorization site and the femoral site until considered unnecessary.

2.5. Continuous and single-day infusion procedures

For the continuous infusion study, 8 animals were assigned to each of the 1.95, 3.90, and 5.85 g/kg/day HBOC-201 groups, as well as to saline and 5.85 g/kg/day hetastarch control groups following implantation of the catheters. The rats received a continuous 24-h intravenous infusion from the morning of GD 6 to the morning of GD 18. Rats were weighed on GD 6, 9, 12, 15, 18 and 20, and the infusion rate was based on the most recent body weight of each animal. Infusions were interrupted only for weighing.

Similarly, for the single-day infusion study, six animals were assigned to 1.95 or 5.85 g/kg/day HBOC-201, saline and 2.7 g/kg/day hetastarch[1] control groups. With the exception of saline control animals, rats received intravenous infusions over a single period of approximately 24 h starting in the morning from GD 6–7, 7–8, 8–9, 9–10, 10–11, 11–12, or 12–13. Saline control rats were continuously infused with saline from GD 6–13. For both the continuous and single-day studies, a dose volume of 45 mL/kg saline, HSA or hetastarch was administered each day. The HBOC-201 was administered at dose volumes of 15, 30 or 45 mL/kg each day for the 1.95, 3.9 and 5.85 (5.9) g/kg/day groups, respectively.

2.6. Hemodilution procedures

HBOC-201 is a colloid that was administered in large volumes to the pregnant rats in these experiments. To reduce the likelihood of fluid overload and attendant adverse consequences such as pulmonary edema or hypertension, a hemodilution procedure was developed in which some of the blood volume of animals was removed immediately prior to infusing the test material. The hemodilution procedure is illustrated in Fig. 1.

Following mating, groups of eight animals were assigned to HBOC-201 and saline control groups to be used in hemodilution studies performed to assess whether anemia or osmotic pressure

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[1] Hetastarch was used to control for oncotic effects. The osmolality of 6% hetastarch is 309 mOm [44]; the osmolality of HBOC-201 is calculated to be 290–310 mOsm [45] Due to molecular weight differences (HBOC is approximately 250 kD; hetastarch is approximately 800 kD), 45 mL/kg of HBOC-201 at the high dose results in 5.85 g HBOC/kg whereas 45 mL of hetastarch results in 2.7 g/kg.
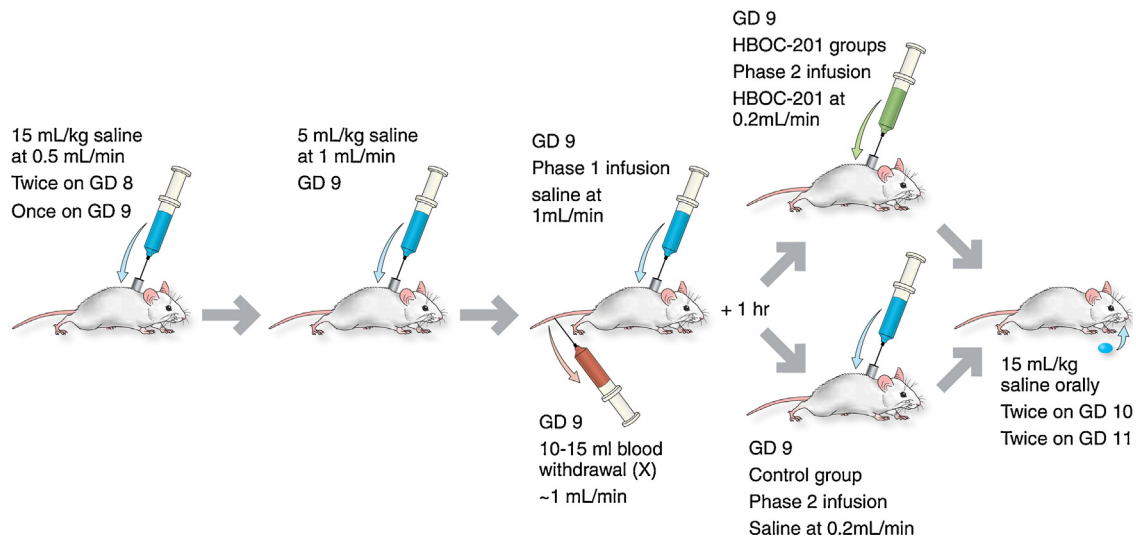
Fig. 1. Diagrammatic representation of the steps involved in the hemodilution procedure described in the text.

changes contribute to developmental toxicity. Test and control rats were given a dose of 15 mL/kg of saline, administered via the indwelling catheter twice on GD 8, and once in the morning on GD 9 at a rate of approximately 0.5 mL/min. These doses of saline were in addition to the maintenance saline administered for cannular patency. On GD 10 and 11, the same dose of saline was administered orally. The hemodilution procedure targeted a 50% decrease in hemoglobin.

The HBOC-201 infusion was conducted on the afternoon of GD 9, wherein each animal was administered 5 mL of saline via the indwelling catheter at a rate of 1 mL/min just prior to being placed in an incubator at approximately 40 °C for 10 min to allow the veins to dilate. The animals were then removed from the incubator, placed in a restrainer, and connected to an infusion pump administering saline at a rate of approximately 1 mL/min via the indwelling cannula. Simultaneously, blood was slowly withdrawn using a heparinized, temporary indwelling catheter (24 gauge), placed in the lateral tail vein. During blood withdrawal, the tail was stroked in a caudal direction to assist blood flow. The target blood withdrawal volume was 10–15 mL at a rate of approximately 1 mL/min. The total amount of infusate given to HBOC-201-treated and control animals through the indwelling catheter was three times the total volume of withdrawn blood. Infusion occurred in two phases. The first phase was 46 mL/kg HBOC-201 administered at a rate of approximately 0.2 mL/min over an approximate one-hour infusion period. The volume of the first phase of infusate was recorded. The second phase was made up of saline infused at a rate of 1 mL/min until the total volume of saline plus phase 1 infusate totaled three times the volume of blood that had been withdrawn from the tail vein. Control animals received saline only in both phases of infusion. After the rats had been removed from restraint they were returned to their cages, and reconnected to their tethering system.

Hemoglobin values were measured using a Hemocue® (Derbyshire, UK) hemoglobin instrument prior to the hemodilution procedure, upon completion of blood removal/infusate administration, and approximately 1 h after completion of infusate administration.

2.7. Laparohysterectomy

Following euthanasia of the dams on GD 20, the reproductive tract was excised, the ovaries removed and the corpora lutea counted. The gravid uterus was weighed, the uterine contents, including the placentae, were examined, and the number and position of live fetuses, dead fetuses, and early and late resorptions were recorded. Early resorptions were defined as nonviable embryos having no discernible features such as the head or limbs. Late resorptions were defined as nonviable fetuses having discernible features with some indication of autolysis. The uterus of any animal that was not pregnant at the time of necropsy was stained with 10% aqueous (v/v) ammonium sulfide solution and examined for implantation sites as described in Salewski [5].

2.8. Offspring morphological examination

All viable fetuses were weighed, sexed, given a detailed external examination, and live fetuses were euthanized by subcutaneous injection of T-61 euthanasia solution or intrathoracic injection of sodium pentobarbital. The detailed examination included, but was not limited to, the eyes, palate and external orifices. External findings were recorded as developmental variations or malformations. Developmental variations include alterations in anatomic structure that are considered to have no significant biological effect on animal health or body conformity. Malformations include structural changes that alter general body conformity, disrupt or interfere with body function, or are generally thought to be incompatible with life. The percentages of malformed fetuses and of litters that had fetuses with malformations were determined.

2.9. Statistical analysis

Statistical analysis was performed on parametric data such as body weight data using a one way ANOVA [6] with a Dunnett's post hoc test [7] for statistically significant ($p \leq 0.05$) results. Statistical analysis of non-parametric data such as group mean live litter size was calculated using the Kruskal–Wallis test [8]. When statistically significant results were found ($p \leq 0.05$), the Mann–Whitney "U" test was used to analyze the differences between control and test groups [8]. Any animals that were deemed non-gravid or that had not been treated according to the study protocols were excluded from analysis.

3. Whole embryo culture studies

3.1. Animals

Sprague-Dawley rats used in the whole embryo culture studies were time-mated by the University of Michigan Reproductive

Sciences Program Small Animal Core. The morning after mating, vaginal smears were performed, and when sperm was detected, that animal was considered to be on GD0. Rats were received on GD 6–9.

3.2. Chemicals for embryo culture studies

Fluorescein isothiocyanate (FITC-albumin (11.2 mol FITC/mol albumin) was obtained from Sigma Chemical Company (St. Louis, MO). Embryo culture reagents were obtained from Gibco BRL (Gaithersburg, MD). Penicillin (10,000 U/mL)/streptomycin sulfate (10,000 U/mL) was obtained from Invitrogen, Co. (Grand Island, NY). HBOC-201 was supplied by Biopure®. All other reagents were of the highest quality commercially available.

3.3. Embryo culture

On GD 10, uteri were removed from pregnant dams, and the conceptuses were dissected from the uterus and prepared for culture as described previously [9,10]. Conceptuses of an appropriate stage (8–10 somites; with elevated, open neural tubes; and dorsal flexure) were cultured in medium consisting of 33% heat-inactivated rat serum in 67% Hank's balanced salt solution (HBSS) plus penicillin-streptomycin (4.3 μL/mL), allowing at least 1 mL of medium per conceptus. The culture medium and headspace were warmed to 37 °C and saturated for 30 min with 20% O_2/5% CO_2/75% N_2 before culturing. Conceptuses were allowed to equilibrate to culture conditions in a roller incubator (37 °C) for 1–2.5 h in untreated medium before being exposed to one of the following procedures.

3.4. Endocytosis assay

Simultaneous exposure of conceptuses to FITC-albumin and HBOC-201 allows for the determination of whether HBOC-201 affects endocytosis. Following the equilibrium period, conceptuses were cultured for an additional 3 h in fresh control medium or medium containing HBOC-201 at concentrations ranging from 0.5% to 12.5% (v/v) plus FITC-albumin (100 μg/mL). Medium samples of 20 μL and 250 μL were taken at the beginning and end of FITC-albumin exposure. After culture, conceptuses were removed and processed for FITC-albumin analysis as described below.

3.5. Protein and DNA assays

Protein content was measured by the method of Bradford [11] as modified by Harris et al. [14]. Bovine gamma globulin (BioRad) was used as the standard. Further slight modifications were needed for assays of invVYS (see below). DNA content was measured fluorometrically by the method of Labarca and Paigen [12], using a bovine DNA standard.

3.6. FITC-albumin measurement

After completing culture treatments, all conceptuses were removed from culture and processed as previously described by Ambroso et al. [13]. The following is an overview of the assay with slight modifications. Conceptuses were rinsed and transferred to a drop of sodium phosphate buffer (50 mM), two conceptuses per 250 μL drop. Conceptuses were dissected in the drop, releasing the extra-embryonic fluid (EEF) contained in the invVYS and amnion into the drop, which was transferred into a microcentrifuge tube. The EEF also contains a small amount of conceptal blood that leaks into the collection fluid. The invVYS were placed in cold 0.1% Triton X-100. Previous experimentation has confirmed that no significant amounts of intact FITC-albumin, FITC bound to

peptide fragments, or other forms of solubilized FITC reach the embryo proper during this procedure. The embryos were, therefore, discarded and not included in the subsequent analyses. The invVYS were sonicated and 20 μL taken from each sample for protein determination by the method described by Bradford [11] as modified by Harris et al. [14]. Proteins were precipitated from the EEF, invVYS, and 250 μL media samples by adding 750 μL of 6% trichloroacetic (TCA) to each. To determine the total FITC-albumin in the 20 μL media samples, protein was solubilized using 750 μL of 6% TCA containing 1% sodium dodecyl sulfate (SDS). Samples were refrigerated overnight for 15 h to fully precipitate or solubilize protein, respectively, and then centrifuged at 14,000 × g for 10 min, to remove the supernatant (which contains acid soluble albumin fragments). One milliliter of 500 mM Tris buffer and 150 μL of 1 N NaOH were added to the acid-soluble supernatant and the samples (approximate pH of 8.8) were quantified on a Perkin–Elmer Model LS 5 Fluorescence Spectrophotometer, 495 nm excitation, 520 nm emission, 10 nm slit widths and scale 8. One hundred-fifty mL of 1 N NaOH was added to the pellets which contain acid insoluble albumin. The pellets were allowed to dissolve at room temperature for about 1 h. One milliliter of 500 mM Tris, 250 μL of 0.1% Triton X-100, and 750 μL of 6% TCA were then added to these acid-insoluble pellets, and the solubilized material was analyzed for fluorescence.

3.7. HPLC analysis of glutathione

GSH was resolved and quantified as its monobromobimane (mBBr) derivatives using the high performance liquid chromatographic (HPLC) method, as described and modified from Fahey and Newton [15]. Briefly, visceral yolk sacs were rinsed free of culture media in cold phosphate buffered saline (PBS) or HBSS and placed in microcentrifuge tubes containing 100 μL of methanesulfonic acid (MSA, 200 mM). Samples were frozen immediately in liquid nitrogen and stored at −75 °C until prepared for HPLC.

Thawed samples were homogenized by ultrasonic disruption; 100 μL of 4 M sodium methanesulfonate were added. Samples were centrifuged at 14,000 × g to pellet precipitated protein. The supernatant was transferred into a clean microcentrifuge tube and the following components were added in succession: 400 μL of 200 mM HEPPS-methanesulfonate (pH 8.0), 20 μL of 5 mM diethylenetriaminepentaacetic acid (DTPA), and 20 μL of 3 mM mBBr. Samples were mixed and allowed to react in the dark for 20 min (25 °C) followed by addition of 400 μL of 200 mM MSA to reacidify samples prior to storage at −75 °C. Samples remain stable under these conditions for up to 3 months. HPLC analyses were carried out using a Waters NovaPak C_{18} 4 μm Radial-Pak Cartridge preceded by a NovaPak Guard-Pak guard column. The columns were equilibrated and the samples eluted with an isocratic mobile phase consisting of 14.2% methanol (v/v) and 2.5% glacial acetic acid (adjusted to pH 3.4) at a flow rate of 1.0 mL/min. After each run, the column was washed and re-equilibrated for 15 min with a regeneration mobile phase consisting of 90% methanol and 2.5% glacial acetic acid. Detection and quantitation of bimane-GSH were accomplished using a Waters Model 470 Scanning Fluorescence Detector (excitation 360 nm; emission 455 nm) followed by peak recording and analysis using a Waters Model 746 Data Module.

3.8. Statistical analysis

Overall differences among treatment means was assessed by one-way analysis of variance (ANOVA). If significant differences were present, pairwise comparisons were analyzed by Fisher's protected least significant differences test. Differences were considered statistically significant when $p < 0.05$.

Table 1
Maternal and developmental toxicity in rats infused continuously with HBOC-201 throughout organogenesis.

Parameter	Saline N = 5	13% HSA (5.85 g/kg/day) N = 6	HBOC 201		
			1.95 g/kg/day N = 5	3.90 g/kg/day N = 5	5.85 g/kg/day N = 5
Total infusion volume (mL/kg/day)	45	45	15	30	45
Maternal toxicity: Clinical findings	–	–	Petechial hemorrhages on tails, darkened eyes and/or skin	Petechial hemorrhages on tails, darkened eyes and/or skin	Petechial hemorrhages on tails, darkened eyes and/or skin
Mean maternal body weight gain (GD 6–18; g ± SD)	101.5 ± 18.8	76.3 ± 26.3	48.0 ± 16.0[*]	27.4 ± 17.8[*]	19.2 ± 8.6[*]
Mean no. viable fetuses per litter (±SD)	12.8 ± 3.4	10.8 ± 7.78	10.2 ± 5.4	2.0 ± 1.58[*]	0
Mean fetal weight (g ± SD)	4.27 ± 0.30	4.25 ± 0.97	3.77 ± 0.90	1.82 ± 0.16[*]	N/A
% Litters with external malformations (N)	0	0	33.3 (3)	100[*] (6)	N/A
% Fetuses with external malformations (N)	0	0	25.9 (29)[*]	100[*] (17)	N/A
Predominant external fetal malformations	–	–	Anophthalmia/microphthalmia, kinked tail	Anury, hydrocephaly, exencephaly, micrognathia/brachygnathia, anophthalmia/microphthalmia, shortened trunk, anal atresia, protruding tongue	N/A

[*] Statistically significant, $p < 0.05$ compared to the saline control group.

4. Results

4.1. Continuous infusion study

Maternal and developmental toxicity data in pregnant female rats continuously infused with 1.75, 3.90 or 5.85 g/kg/day of HBOC-201, HSA, or saline treatments during GD 6–18 are summarized in Table 1. In comparison to saline controls, HBOC-201 infused dams in all dosage groups exhibited a reduction in maternal weight gain. One female in the 5.85 g/kg/day HBOC-201 treated group was euthanized on GD 8 due to overt discomfort, and another female in this group was found dead on GD 15. Gross pathologic changes were suggestive of cardiac insufficiency in both animals. Overt signs of maternal toxicity were observed in all HBOC-201 groups and included petechial hemorrhages on the tails and darkened eyes and/or skin. The onset of occurrence of these observations was dose-related. The mean number of viable fetuses per litter was significantly decreased in a dose-dependent manner following HBOC-201 infusion such that animals treated with 1.95, 3.90, and 5.85 g/kg/day bore an average of 10.2 ± 5.4, 2.0 ± 1.58 and 0 live fetuses per litter, respectively. Dose-related decreased fetal body weights following HBOC-201 administration were observed in both the 1.95 and 3.90 g/kg/day groups; the decreases were statistically significant in the 3.90 mg/kg/day group. The percentage of fetuses (litters) with external malformations in the 1.95 g/kg/day HBOC-201 group was 25.9% (33.3%). All fetuses (100%) of dams in the 3.90 HBOC-201 g/kg/day group exhibited external malformations. Predominant fetal malformations seen in the 1.95 g/kg/day group included anophthalmia/microphthalmia and kinked tail, and predominant fetal malformations seen in the 3.90 HBOC-201 group included anury, hydrocephaly, exencephaly, micrognathia/brachygnathia, anophthalmia/microphthalmia, shortened trunk, anal atresia and protruding tongue. No maternal or developmental toxicity was observed in the 13% HSA control, indicating that HBOC-201 toxicity was not directly attributable to protein content.

4.2. Single day infusion study

Developmental toxicity data in rats infused with HBOC-201 over single day periods are presented in Table 2. Control animals received a single day infusion of hetastarch at 1.95 g/kg. In dams treated with hetastarch neither the mean fetal weight nor the percentage of fetuses with external malformations significantly differed from controls during any 1-day interval (data shown for GD 8–9; other days were similar). This indicates that the toxicity induced by HBOC-201 is treatment-related and not attributable to its colloidal properties. However, rats infused with 5.85 g/kg/day HBOC-201 between GD 7–8 and 8–9 bore malformed pups (litters) at a statistically significant increased rate of 10.1% (16.7%) and 76.3% (83.3%), respectively. No significant increases in the percentage malformed pups born to dams infused with 5.85 mg/kg/day HBOC-201 were observed over any other 1-day interval of exposure.

The early screening level infusion studies were conducted on single days of gestation using only 5 or 6 litters per group. The absence of statistical significance with regard to pairwise comparisons between HBOC-201 versus saline is both a function of the small number of litters and also the reduced intensity of the fetal examinations. In screening studies, complete evaluations of fetuses (which would include visceral and skeletal examinations in a definitive study) are typically not performed. However, because developmental toxicity includes alterations in fetal weights in addition to malformations, it is possible to obviate the statistical issues by combining the fetal weight data from the four-day span of GD 6–7 to GD 10–11 (the time period that precedes the full functioning of the chorioallantoic placenta). The comparison for potential developmental toxicity of the combined GD 6–10 hetastarch-treated and HBOC-201-treated groups includes a total of 23 litters in each treatment group, with a total of 332 fetuses from hetastarch-treated dams and 318 fetuses from HBOC-201-treated dams. The hetastarch fetuses (3.69 ± 0.42 g) were significantly heavier than those in the HBOC-201 group (3.27 ± 0.32 g) when analyzed by Dunnett's test ($p \leq 0.05$). This analysis demonstrates a developmentally toxic effect of treatment that was not related to the osmolality of the HBOC. Because fetal weight is considered the most sensitive endpoint of developmental toxicity, this shows that during the pre-chorioallantoic placenta period, HBOC-201 fetuses experienced developmental toxicity even though external malformations were observed only during treatment on GDs 6–9.

The absence of external malformations in fetuses from dams treated on GD 10–11 is likely due to the normal redistribution of maternal (HBOC-containing) blood within the maternal-fetal

Table 2
Summary of multiple single-day infusion exposures in rats.

Treatment	No. examined fetuses (Litters)	Mean fetal Wt. (g ± SD)	External malformations (%)	
			Fetuses	Litters
Saline (45 mL/kg) [GD 6–13]	63 (5)	3.96 ± 0.28	0	0
Hetastarch (2.7 g/kg)				
GD 6–7	91 (6)	3.71 ± 0.26	1.1	16.7
GD 7–8	82 (6)	3.65 ± 0.28	0	0
GD 8–9	66 (5)	3.69 ± 0.23	0	0
GD 9–10	90 (6)	3.71 ± 0.29	0	0
GD 10–11	94 (6)	3.72 ± 0.75	0	0
GD 11–12	67 (5)	4.02 ± 0.23	0	0
GD 12–13	67 (5)	3.92 ± 0.24	0	0
HBOC-201 (5.85 g/kg)				
GD 6–7	77 (6)	3.88 ± 0.28	1.2	16.7
GD 7–8	89 (6)	3.27 ± 0.12*	10.1*	16.7
GD 8–9	76 (6)	3.10 ± 0.37*	76.3*	83.3*
GD 9–10	67 (5)	3.34 ± 0.32	0	0
GD 10–11	86 (6)	3.39 ± 0.41	0	0
GD 11–12	68 (5)	3.30 ± 0.33*	0	0
GD 12–13	92 (6)	3.53 ± 0.65	0	0

* Statistically significant, $p \leq 0.05$ compared to the saline control group.

interface that occurs during this time in rats [16]. The chorioallantoic placenta of the rat develops from the ectoplacental cone which begins to become vascularized on the fetal side by allantoic arteries during GD 11 [17]. Prior to that time, however, the tissue of the ectoplacental cone develops sinusoids that become filled with maternal blood. This diverts some of the volume of maternal blood away from the invYSP, as shown by the detailed microsphere data of Buelke-Sam et al. [18]. This reduction in flow to the invYSP is likely sufficient to have reduced the impact of HBOC on that membrane to the extent that only fetal body weight was affected.

4.3. Hemodilution studies

To determine whether the maternal and developmental toxicity observed following HBOC-201 infusion could be attributed to a change in oncotic pressure, rats were hemodiluted and infused with 46 mL/kg (6 g/kg) HBOC-201 or 46 mL/kg saline on GD 9 (Table 3). The data indicate that maternal weight gain from the day of infusion until euthanasia (GD 9–20) was markedly lower in hemodiluted dams infused with HBOC-201 (42 ± 31.7 g) compared to hemodiluted dams infused with an equivalent volume of saline (80 ± 20.8 g). Because net body weight gain (weight gain minus the weight of the gravid uterus) in the HBOC-201 group was similar to the saline control group, the lower maternal weight gain was attributed to the high rate of embryolethality and lower fetal body weights observed in the HBOC-201 group. Other overt signs of maternal toxicity, observed only in hemodiluted dams infused with HBOC-201, included reddened body areas and red urine.

Table 3
Maternal and developmental toxicity observed as a result of hemodilution/infusion with saline or HBOC-201.

Parameter	Saline infused on GD9 (46 mL/kg)	6 g/kg HBOC 201 infused on GD9 (46 mL/kg)
Number	7	8
Maternal clinical findings	–	Reddened body areas, red urine
Mean maternal body weight gain GD 9–20 (g ± S. D.)	80 ± 20.8	42 ± 31.7*
Mean no. viable fetuses	11.30 ± 5.35	5.60 ± 5.48
Mean fetal weight (g ± S. D.)	3.50 ± 0.40	2.70 ± 0.84
External malformations (% per litter ± S.D.)	0.0	61.8 ± 48.7*

* Statistically significant, $p < 0.05$ compared to the saline control group.

Additionally, the mean number of viable fetuses (5.6 ± 5.48 g compared to 11.3 ± 5.35 g) and the mean fetal weight (2.7 ± 0.84 g compared to 3.5 ± 0.40 g) in the HBOC-201 group were lower than in the saline-infused controls. The mean litter percentage of external malformations in the HBOC-201 group was ∼62% while no external malformations were observed in fetuses born to hemodiluted animals infused with saline. Malformations consisted primarily of microphthalmia and cephalocele.

In a second hemodilution study, hemodiluted dams were infused with saline, hetastarch, or 6 g/kg PBH on GD 9. This is the same dosage of HBOC-201 used in the first hemodilution study. Maternal and developmental toxicity data associated with these groups are presented in Table 4. Neither maternal nor developmental toxicity was observed in the hemodiluted animals infused with saline or hetastarch. However, hemodiluted dams infused with PBH gained significantly less weight (52 g ± 32.7 g) from the day of infusion until euthanasia (GD 9–20) than hemodiluted dams infused with saline and hetastarch; 126 ± 38.2 g and 110 ± 17.9 g, respectively. Because net body weight gain in the PBH group was similar to the saline control group, the lower maternal weight gain was attributed to the high rate of embryolethality and lower fetal body weights observed in the PBH group. Overt signs of maternal toxicity in the hemodiluted group infused with 6 g/kg PBH were similar to those observed in the HBOC-201 treatment group. PBH infusion of hemodiluted dams led to a reduction in the number of viable fetuses, as well as a reduction in fetal weights. In litters from hemodiluted animals the mean number of viable fetuses per litter and mean fetal weights were reduced after infusion with PBH, but not after infusion with saline or hetastarch. The mean litter percentage of external malformations was increased (75.1 ± 35.46%) in hemodiluted dams infused with 6 g/kg PBH, whereas the incidence of external malformation was very low in fetuses of hemodiluted dams infused with saline and hetastarch. The malformations observed in the PBH group fetuses consisted primarily of microphthalmia and hydrocephaly. Thus, HBOC-201 and PBH resulted in similar teratogenic profiles, indicating that the hemoglobin component of HBOC-201 is essential to the observed toxicity.

4.4. Whole embryo culture studies

Protein and DNA levels measured in embryos and invVYS exposed to various concentrations of HBOC-201 are presented in Table 5. Both protein and DNA levels were significantly decreased in a dose–response fashion indicating that HBOC-201 interferes

Table 4
Maternal and developmental toxicity observed as a result of hemodilution followed by infusion with saline or purified bovine hemoglobin.

Parameter	Saline (46 mL/kg)	Hetastarch (2.76 g/kg)	PBH (6 g/kg)
Number	5	7	8
Clinical findings	–	–	Reddened body areas, red urine
Mean maternal body weight gain GD 9–20 (g ± S.D.)	126 ± 38.2	110 ± 17.9	52 ± 32.7[*]
Mean no. viable fetuses	14.0 ± 1.41	13.6 ± 2.30	5.1 ± 4.16[*]
Mean fetal weight (g ± SD)	3.9 ± 0.27	3.8 ± 0.15	2.4 ± 0.38[*]
External malformations (% per litter ± SD)	1.5 ± 3.44	1.3 ± 3.44	75.1 ± 35.46[*]

[*] Statistically significant, $p < 0.05$ compared to the saline control group.

Table 5
Growth parameters in cultured embryos treated with HBOC-201.

| Treatment	% Viable (Viable/Total)	Protein (µg)[a]		DNA (µg)	
HBOC-201 (%)		Embryo	Visceral yolk sac	Embryo	Visceral yolk sac
0.0	100 (20/20)	337.8 ± 100.8	195.3 ± 56.5	38.3 ± 23.7	17.2 ± 6.1
0.5	100 (16/16)	194.0 ± 65.9[*]	130.5 ± 42.9	19.3 ± 11.8[*]	10.7 ± 5.7
1.0	79 (19/24)	171.4 ± 68.1[*]	133.2 ± 49.8	23.7 ± 18.2	12.8 ± 6.1
2.5	76 (19/25)	141.4 ± 54.0[*]	141.3 ± 4.4	15.1 ± 9.5[*]	10.1 ± 5.4
5.0	70 (16/23)	109.2 ± 3.0[*]	118.6 ± 58.5[*]	14.2 ± 13.0[*]	N/A
7.5	38 (9/24)	122.2 ± 13.5[*]	120.3 ± 87.4	12.4 ± 0.6[*]	7.7 ± 4.7
10.0	61 (11/18)	129.2 ± 7.7[*]	140.0 ± 49.3	15.5 ± 8.2[*]	9.0 ± 4.7
12.5	67 (6/9)	140.4 ± 46.7[*]	160.2 ± 39.6	16.0 ± 4.9[*]	8.4 ± 2.0

[a] Data are expressed as means ± SE.
[*] Values are statically significantly different from control ($p < 0.05$).

Table 6
Uptake and degradation of FITC-labeled albumin.

Treatment HBOC-201	Visceral yolk sac $\frac{Soluble}{Insoluble}$	Extraembryonic fluid $\frac{Soluble}{Insoluble}$	Media Soluble	Totals Soluble + insoluble
FITC Clearance[a] (µl/mg/h)				
Control (0)	$\frac{20.2 \pm 3.2}{17.3 \pm 2.4}$	$\frac{10.1 \pm 0.8}{0.9 \pm 0.0}$	6.8	55.2
0.5%	$\frac{18.8 \pm 2.2}{14.8 \pm 2.5^*}$	$\frac{4.0 \pm 0.4^*}{0.9 \pm 0.0}$	3.7	42.1
1.0%	$\frac{11.5 \pm 2.0^*}{10.7 \pm 0.9^*}$	$\frac{2.9 \pm 0.2^*}{1.0 \pm 0.0}$	3.1	29.2
2.5%	$\frac{8.1 \pm 3.0^*}{8.0 \pm 1.1^*}$	$\frac{2.0 \pm 0.2^*}{0.8 \pm 0.0}$	3.4	22.3
5.0%	$\frac{6.1 \pm 1.2^*}{6.4 \pm 1.8^*}$	$\frac{1.8 \pm 0.4^*}{0.9 \pm 0.0}$	3.2	18.4

[a] Data expressed as clearance rates: µL of assay medium cleared of fluorescence per mg of VYS protein per hour ± SE.
[*] Statistically different from control at $p \leq 0.05$.
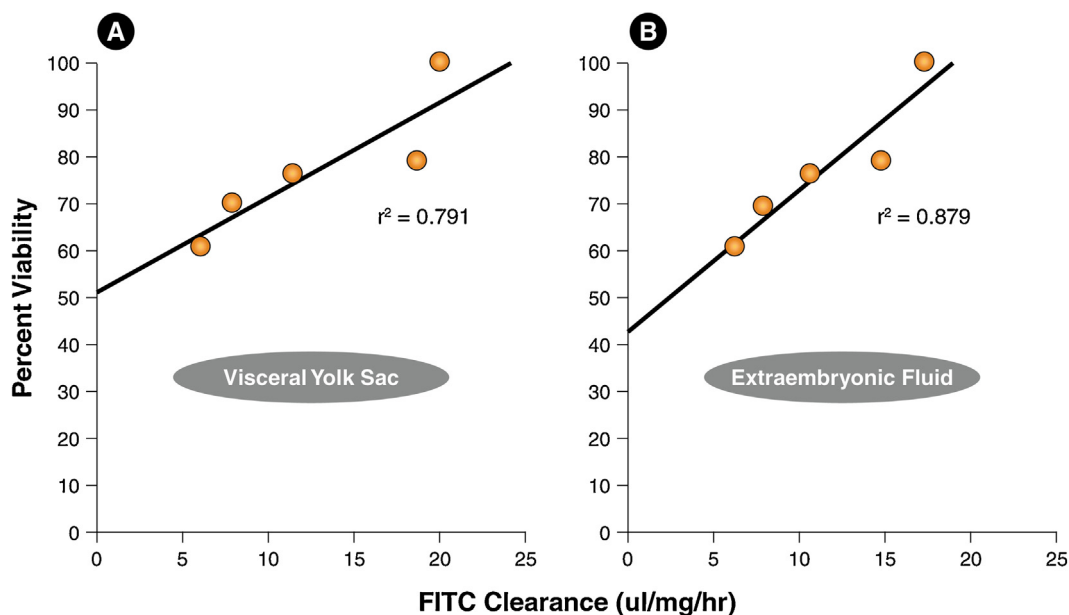
Fig. 2. Correlation between percent of viable embryos and FITC-labeled albumin clearance in visceral yolk sac membranes (A) and extraembryonic fluid (B). Embryonic viability is strongly associated with increasing rate of clearance. Data points (from left to right) are for values determined in 10%, 5%, 2.5%, 1%, and 0% (control) media concentrations of HBOC.
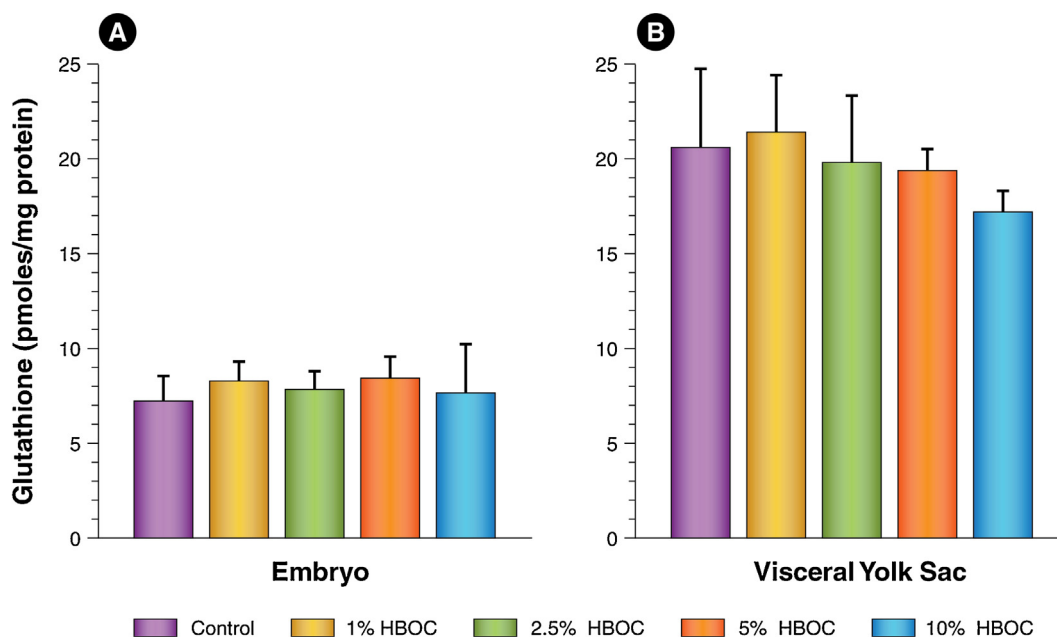
Fig. 3. Glutathione concentrations in embryos (A) and visceral yolk sac membranes (B) after a 24 h exposure to varying concentrations of HBOC on GD 10. No significant difference is observed among any of the treatment groups.

with normal nutritive mechanisms supporting protein and DNA biosynthesis. Studies assessing endocytotic capacity through measurements of the uptake and degradation of FITC-labeled albumin in embryos, invVYS, EEF and medium (Table 6) demonstrated that HBOC-201 decreases the clearance of protein in the invVYS. Positive correlations were observed between the percentage of viable embryos and FITC clearance in both the visceral yolk sac and the extraembryonic fluid (Fig. 2); $r = 0.791$ and 0.879, respectively.

Furthermore, concentrations of glutathione were measured in order to determine if the developmental toxicity of HBOC-201 can be attributed, in part, to an increase in oxidative stress. Levels of glutathione in the embryo were unchanged with all doses of HBOC-201 examined (Fig. 3a). The level of glutathione in the invVYS did decrease slightly (Fig. 3b), indicating the HBOC-201 seems to affect the invVYS more than the embryo proper but the data are insufficient to discern oxidative stress.

5. Discussion

In this study, malformed fetuses, reduced fetal viability, and maternal toxicity were observed after maternal infusion with HBOC-201. Investigation to define the timing of embryotoxicity revealed that malformed fetuses were observed after maternal infusion with HBOC-201 primarily on GDs 8 and 9. This time period coincides with the appearance and early functioning of the rat invVYS during GD 7 [17]. Additionally, the rather abrupt disappearance of malformations in exposed litters after GDs 10–11 was concurrent with the development of the chorioallantoic placenta of the rat, which becomes well established during GD 11. Because the histiotrophic mechanism for transport of nutrients and other cargoes by the invVYS differs from that of the chorioallantoic placenta, which works by countercurrent exchange of substances between closely apposed maternal and embryonal circulations [19–21], and because the adverse embryonic effects were not produced after the chorioallantoic placenta was fully functioning, the embryotoxic effects appear to be related to impaired invVYS function. The FITC-albumin uptake and DNA content studies also indicate that early exposure of embryos to HBOC-201 impaired the endocytotic capacity of the invVYS.

Thus, the single-day infusion data and in vitro embryo exposure data are consistent with the hypothesis that the early developing invVYS was the target organ for HBOC-201 toxicity. Reduced nutrition/starvation of pregnant rat [22] and mouse embryos [23,24] during the early period of organogenesis has previously been linked to the production of malformations in these species, which is also consistent with the findings in HBOC-treated litters in this report. Additionally, interference with invVYS function during the remainder of gestation would be expected to diminish transfer of some nutrients into the embryo, which could account for the fetal weight decrements seen in HBOC-treated groups.

It must be pointed out that cell-free hemoglobin (Hb) is toxic in adult animals. Much of the Hb toxicity is due to perturbed redox balance as the ferrous ion (Fe^{II}) in the heme moiety is oxidized to ferric ion (Fe^{III}) in the presence of oxygen, generating a variety of reactive oxygen species (ROS) and peroxide (reviewed in [2,25,26]). Young embryos, however, exist in a hypoxic state until the establishment of the uteroplacental circulation and subsequent oxygen enrichment by forming the chorioallantoic placenta. These two temporally distinct gestational milestones increase physiologic exchange of nutrients and gases between mother and the embryo. As the amount of oxygen reaching the embryo increases, the morphology of embryonic mitochondria changes to handle the increased oxygen exposure efficiently and without toxicity [27,28]. If the effects of HBOC were due to the creation of ROS, it would be expected that the effects on the embryos would increase concomitantly with elevated oxygen levels reaching the embryo subsequent to establishment of the chorioallantoic placenta. In this case, however, the adverse effects on embryos decreased after the chorioallantoic placenta was formed, suggesting that the Hb-associated toxicity is not due to ROS.

The use of the various control groups further supports the proposed mode of developmental toxicity. First, the lack of developmental toxicity in the hetastarch control group demonstrates that the developmental toxicity is not merely the result of an imbalance in oncotic pressure, but must be due to a separate property of the polymer or the hemoglobin component of HBOC-201. Second, the lack of developmental toxicity after infusion of the human serum albumin indicates that the developmental impact is not due to high

protein concentrations added to the maternal blood volume. Third, the hemodilution experiments investigated the possibility that the developmental toxicity could have been secondary to anemia or an increase in the circulating blood volume of the pregnant rats. Fetal outcomes in hemodiluted rats that were infused with HBOC-201, however, did not differ substantively from fetal outcomes of the non-hemodiluted rats. Together, these controls indicate that the developmental toxicity is not the result of increased oncotic pressure caused by increased blood volume or the colloidal properties of HBOC-201. Fourth, the developmental toxicity resulting from infusion of PBH shows that the effects caused by HBOC-201 *are* associated with the large molecular weight protein, hemoglobin.

Given these observations, it is possible that HBOC-201, like other molecules, that poison the invVYS (e.g. trypan blue and concanavalin A), leads to fetal malformations [29–32]. If this is indeed the mechanism of action of HBOC-201 in the rodent, differences in the anatomy and function of the vestigial human VYS suggest that these mechanisms may not be relevant in human development. Like all eutherian mammals, humans possess a well-defined yolk sac during early postimplantation development and organogenesis [33]. The yolk sac endodermal epithelium is polarized and, similar to other epithelia, will endocytose nutrient materials from the VYS lumen, especially during the first trimester prior to full activation of placental function [34–37]. Unlike the rodent, human yolk sac membranes never become directly apposed to the chorion (the outer most embryonic membrane within which the conceptus resides) or the maternal uterine wall or uterine lumen [16,20,33,38]. This means that the human yolk sac membrane does not function as a placenta in the sense of being able to transfer nutrients between maternal and conceptual circulations [39]. Furthermore, the human yolk sac does not invert to encompass the embryo which means that materials originating from human blood (such as HBOC) first must diffuse or be actively transported into the spherical yolk sac lumen prior to being endocytosed by the polarized yolk sac endothelium. The yolk sac remains small relative to the size of the developing, major organogenesis stage embryo [33,38], suggesting that that the surface area available for transport in human embryos is small compared to that of rodents. Based on these considerations, we believe that it is unlikely that interference with human VYS function will contribute to hemoglobin embryotoxicity.

To elucidate any potential risk for developmental toxicity of HBOC-201 in humans, safety testing should be conducted using an animal model that does not rely upon an inverted yolk sac for embryonic nutrition. Because rodents and lagomorphs rely on inverted yolk sac during early gestation, they are inappropriate models for assessing the developmental toxicity of molecular substances such as HBOC-201 that may poison the invVYS. Among commonly used experimental models used in safety testing, non-human primates and dogs do not have inverted yolk sacs [20,40]. Studies with non-human primates are lengthy, expensive, and pose ethical issues for use in general safety tests. However, there is a substantial amount of experience in using dogs in safety assessment studies for other disciplines and a significant body of general toxicological and physiological information has been developed [41,42]. Thus, developmental toxicity studies performed in a canine model ([43] submitted companion paper) could verify that the developmental toxicity of HBOC-201 in the rat is indeed due solely to poisoning of the invVYS.

Conflict of interest

Funding for the experiments was supplied by BioPure. Dr. Pearce had been an employee of BioPure; Drs. Stump and Holson were employees of WIL Research; Dr. Harris was an employee of the University of Michigan and worked as a subcontractor; Drs. Watson and DeSesso were consultants to BioPure.

Transparency document

The Transparency document associated with this article can be found in the online version.

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