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Assessing the Effects of Low Boron Diets on Embryonic and Fetal Development in Rodents Using In Vitro and In Vivo Model Systems

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ABSTRACT

To date, boron (B) essentiality has not been conclusively shown in mammals. This article summarizes the results of a series of in vitro and in vivo experiments designed to investigate the role of B in mammalian reproduction. In the first study, rat dams were fed either a low (0.04 μg B/g) or an adequate (2.00 μg B/g) B diet for 6 wk before breeding and through pregnancy; reproductive outcome was monitored on gestation day 20. Although low dietary B significantly lowered maternal blood, liver, and bone B concentrations, it had no marked effects on fetal growth or development. The goal of the second study was to assess the effects of B on the in vitro development of rat postimplantation embryos. Day 10 embryos collected from dams fed either the low or adequate B diets for at least 12 wk were cultured in serum collected from male rats exposed to one of the two dietary B treatments. Dams fed the low B diet had a significantly reduced number of implantation sites compared to dams fed the B-adequate diet. However, embryonic growth in vitro was not affected by B treatment. The aim of study 3 was to define the limits of boric acid (BA) toxicity on mouse preimplantation development in vitro. Two-cell mouse embryos were cultured in media containing graded levels of BA (from

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6 to 10,000 μM). Impaired embryonic differentiation and proliferation were observed only when embryos were exposed to high levels of BA (>2000 μM), reflecting a very low level of toxicity of BA on early mouse embryonic development. Study 4 tested the effects of low (0.04 μg B/g) and adequate (2.00 μg B/g) dietary B on the in vitro development of mouse preimplantation embryos. Two-cell embryos obtained from the dams were cultured in vitro for 72 h. Maternal exposure to the low B diet for 10, 12, and 16 wk was associated with a reduction in blastocyst formation, a reduction in blastocyst cell number, and an increased number of degenerates. Collectively, these studies support the concept that B deficiency impairs early embryonic development in rodents.

Index Entries: Boron; preimplantation; postimplantation; embryonic development; reproduction; in vitro model systems.

INTRODUCTION

Boron (B) has been recognized as an essential nutrient for plants for over 60 years *(1)*. In numerous plant species, a deficit of this mineral element can result in severe growth-stunting, morphological and physiological alterations, and potentially death *(1,2)*. During the last 5 years, evidence has been accumulating that B may be essential for reproduction in two separate fish model systems *(3,4)*, and for the early embryonic development of frogs *(Xenopus laevis; 5)*. Given the known essentiality of B in plants, the recent strong evidence for a B requirement for amphibian and fish reproduction, and earlier studies suggesting that B may be needed by the developing chick *(6)*, we have initiated a series of studies aimed at characterizing the role of B in mammalian reproduction.

In vivo and in vitro model systems are available to study mammalian reproduction. The in vivo models allow us to investigate the effects of a deficit of a nutrient, such as B, during a single pregnancy or multiple generations. With respect to in vitro systems, a number of models can be used including pre-, peri-, and postimplantation embryos, allowing direct experimental manipulations on small and otherwise inaccessible mammalian embryos. The in vitro models in concert with the in vivo model allow the investigator to make serial observations on the effects of select nutrient deficiencies over defined developmental periods (Fig. 1). When detrimental developmental consequences are observed in response to a nutrient deficiency (or excess), the underlying biochemical, cellular, and molecular mechanisms can then be studied with a high level of precision.

In this article, we will first describe in some detail the different in vitro model systems and how they can be applied to the study of specific nutrients, using Zn as a paradigm. In the second part of this article, we will summarize our preliminary results on the effects of B on embryonic, fetal, and postnatal rodent development.

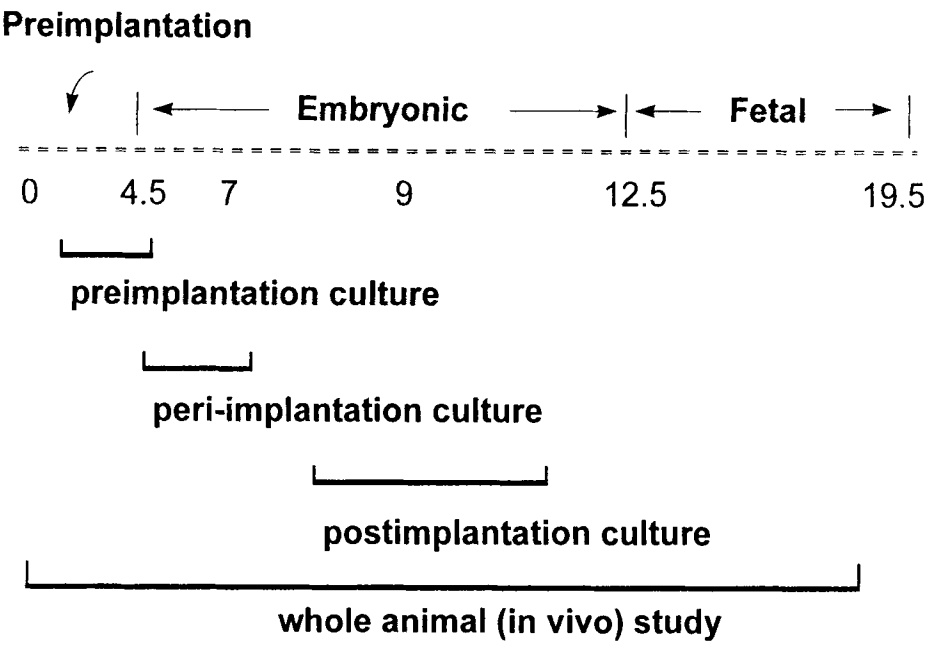

Fig. 1. Developmental periods covered by the different in vivo and in vitro model systems. The hatched line represent the different phases of mouse development in days: preimplantation (0–4.5 d); embryonic (4.5–12.5 d); fetal (12.5–19.5 d). The time periods covered by the different models are depicted: preimplantation (1.5–4.5 d); peri-implantation (4.5–8.5 d); postimplantation (8.5–10.5 d). In vivo models of investigation can be used to study any phases of development.

EMBRYO CULTURE MODEL SYSTEMS

Procedures for the recovery or isolation of embryos, their growth in defined media or sera, and the assessment and processing of the embryos at select end points are described below. The different culture media have been optimized to sustain the metabolic, energetic, and hormonal requirements of the developing embryos. For pre- and peri-implantation culture, a modified Hanks' balanced salt solution (L15) is used to collect the embryos; for postimplantation culture, embryos are dissected in Tyrodes' salt buffer.

Preimplantation

Preimplantation development is summarized in Fig. 2. Preimplantation embryos up to the eight-cell stage cannot utilize glucose efficiently, and the medium must contain lactate and pyruvate as energy sources. Glucose is added to sustain blastocyst formation. Preimplantation culture medium is composed of a modified bicarbonate buffered medium supplemented with bovine serum albumin (BSA) and antibiotics. Procedures

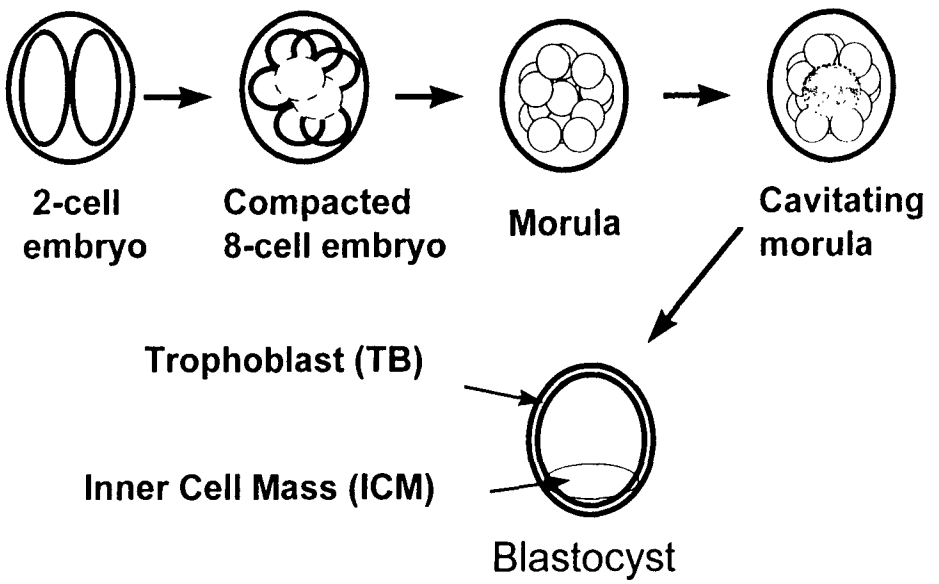

Fig. 2. Overview of preimplantation development. Two-cell mouse embryos are collected 1.5 d postfertilization or hCG injection. The embryos proceed through a phase of early cleavage as they replicate every 12 h to form 4- and 8-cell embryos. At the eight-cell stage, the embryos undergo compaction when cells increase their cell-to-cell contact and acquire polarity. By 3 d, the embryos have between 16 and 32 cells, and are called morulae. During cavitation, the morulae absorb fluid forming a cavity and become blastocysts by day 4. Blastocysts consist of two distinct cell populations: the trophoblast, a single-layered epithelium that will form the placenta, and the inner cell mass that will differentiate into the embryo proper.

for the culturing of preimplantation mouse embryos from the two-cell to the blastocyst stage were developed in the late 1950s from standard tissue-culture techniques *(7)*. The conditions used in our laboratory for culturing preimplantation embryos are described schematically in Fig. 3. Briefly, female mice are superovulated with an initial intraperitoneal injection of pregnant mare serum gonadotropin (5 UI PSMG) followed by a dose of human chorionic gonadotropin (5 UI hCG) 48 h later. Females are then mated overnight and killed 48 h after ovulation. The oviducts are isolated from the reproductive tract and flushed with L15 medium using a tuberculin syringe to collect two-cell embryos. As a consequence of the superovulation, typically 20–30 embryos can be recovered from an animal (this can be compared to an average in vivo litter size of 10–12). Using a mouth pipet, the embryos are washed in warm droplets of L15, transferred to 25 µL droplets of defined culture medium (T6) under mineral oil, and incubated at 37°C in 5% CO_2 humidified air. With this model, cultured two-cell embryos develop normally and reach the blastocyst stage over a 72-h period (Plate 1 A,B). Developmental and survival kinetics can be assessed at specific time-points throughout the culture

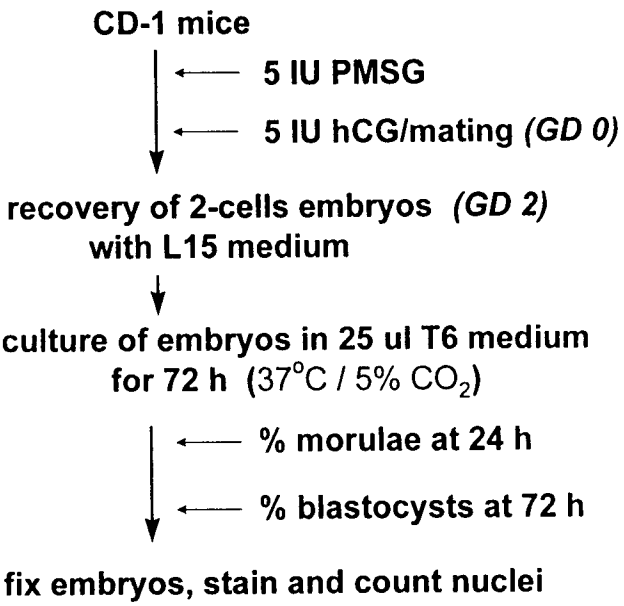

Fig. 3. Summary of the preimplantation culture system. *See text* for description.

period, and the blastocysts can be collected for morphological and biochemical analyses.

We have shown that the preimplantation culture system can be used to investigate the effects of Zn deficiency on early embryonic development. Using this model, we have shown that embryonic development in vitro is sensitive to changes in Zn status either directly in the culture medium *(8)* or through manipulations of dietary Zn *(9)*.

Peri-implantation

Peri-implantation embryo culture requires a more complex medium, reflecting the complexity of the developmental processes occurring at this period (Fig. 4). It is composed of Eagle's basal medium (BME) supplemented with amino acids and 10% fetal calf serum. Using a methodology developed by Wiley and Pedersen *(10)*, blastocyst growth can be sustained for up to 7 d through the egg cylinder stage and the embryos appear morphologically normal. More significantly, these embryos demonstrate implantation-like processes (*see* Plate 1 C,D). Ninety-six hours after mating, blastocysts are recovered from superovulated female mice by flushing the uterine horns with L15 medium using a 25-gauge needle inserted in the cervix opening. Blastocysts are cultured in 450 µL medium (Fig. 5).

A preliminary experiment by Hanna et al. *(11)* indicated that the peri-implantation development of mouse embryos in vitro showed the effects of Zn deficiency. When blastocysts were grown in Zn-deficient conditions, they failed to hatch and died. Zn repletion of the culture

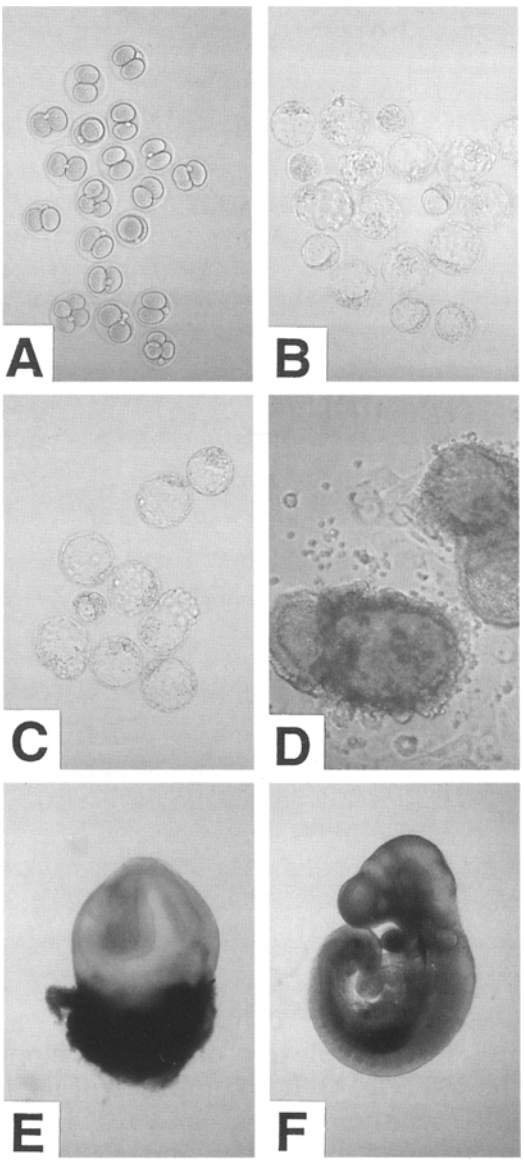

Plate 1. In vitro model systems: Representative photomicrographs of embryos taken at initial and end points of their respective culture system. Pre-implantation: two-cell embryos **(A)** grown 72 h in vitro will form blastocysts **(B)**; peri-implantation: blastocysts **(C)** grown for 7 d in vitro will differentiate to the egg cylinder stage **(D)**; and postimplantation: 3–5 somite pairs mouse embryo **(E)** will grow to 20–25 somite pairs **(F)** after 24 h of culture.

medium improved the survival rate, but not all of the differentiation processes observed in control blastocysts.

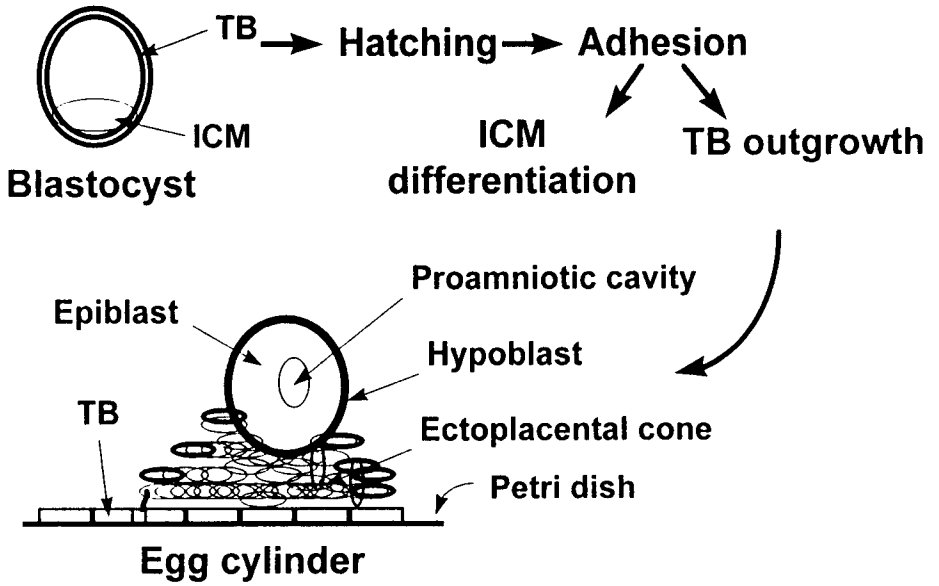

Fig. 4. Overview of peri-implantation development. Blastocysts are collected at day 4 of gestation. They are formed of the inner cell mass (ICM), a compact mass of cells on one side of the cavity (the embryonic pole), surrounded by an outer layer of cells, the trophoblast (TB). By day 5, blastocysts hatch from the zona pellucida (zp) by enzymatically "piercing" a hole in the zp membrane and "squeezing" out. The trophoblast cells at the embryonic pole of the "naked" blastocysts adhere to the bottom of the Petri dish as it would implant in the maternal endometrium. After implantation, the trophoblast cells enlarge, become polynucleated, and form a layer of giant cells or syncytium. The portion of syncytium at the site of attachment will eventually form the ectoplacental cone. Concomitantly, cells from the ICM differentiate into two layers of cells: the hypoblast or primitive endoderm and the epiblast or primary ectoderm surrounding the amniotic cavity.

Postimplantation

Culture of postimplantation embryos cannot be sustained in a chemically defined medium; the medium must contain >75% rat serum. In the postimplantation culture system, rat and mouse embryos are removed on embryonic days 9–11 in the rat and days 8–10 in the mouse, and can be successfully cultured for 48 h. This window represents a critical period of development, encompassing processes, such as gastrulation, neurulation, and organogenesis. The extent of the growth and morphogenesis occurring over 24 h can be appreciated by comparing photomicrographs of mouse embryos at embryonic day 8.5 (3–5 somites) with embryos after 24 h of culture (Plate 1 E,F). The development, morphology, and growth of embryos in vitro compares favorably with the embryonic development *in utero (12).* The basic culture conditions described by New et al. *(12)* have been adapted for rat *(13)* and for mouse *(14)* embryos. Briefly, embryos are explanted

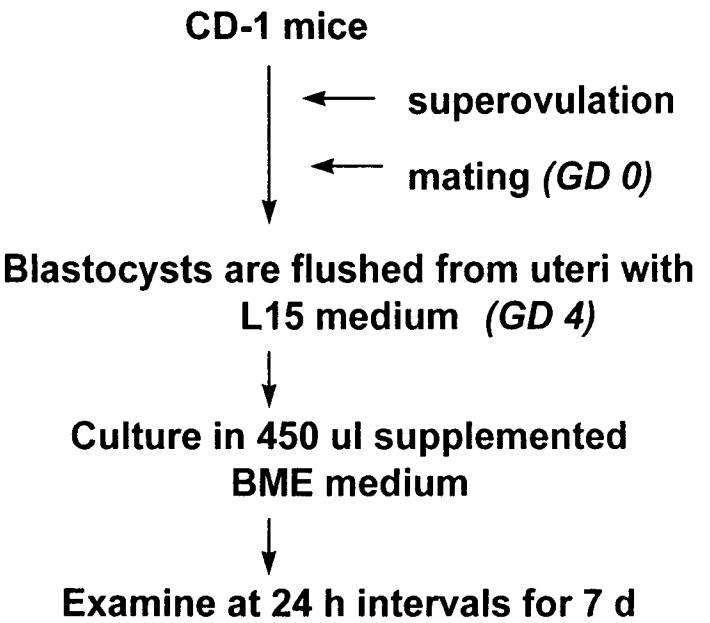

Fig. 5. Summary of the peri-implantation culture system. *See text* for description.

from the uterus and decidua, leaving the yolk sac and ectoplacental cone intact. The embryos are cultured in 30-mL stoppered vials containing 3 mL heat-inactivated rat serum. Vials are rotated at 30 rpm in a 37°C roller incubator and gassed every 12 h with the appropriate $O_2/CO_2/N_2$ mixture. At the end of culture, embryos are dissected free from their yolk sacs and examined by light microscopy for abnormalities. Embryos are scored for morphology based on the criteria developed by Brown and Fabro *(15)*, and growth is assessed by counting somite pairs and measuring crown-rump lengths. Embryos can be frozen, fixed, or embedded for further analyses.

Each of the in vitro models can be used to monitor the effects of select nutrient deficiencies on conceptus development. These models can be applied to study the biological role of B in mammalian development.

THE EFFECTS OF LOW B DIET ON FETAL DEVELOPMENT: AN IN VIVO STUDY (STUDY 1)

Materials and Methods

Animals and Diets

Sixty-four male and 88 female Sprague-Dawley rats (50–60 g body wt; Simonsen, Gilroy, CA) were used in this study. The animals were housed in stainless-steel cages enclosed in a laminar flow unit (BioClean, LabProduct Inc., Maywood, NJ) in a temperature- and light- (12-h cycle)

controlled environment. Following a brief period of adaptation, the animals were randomly assigned to either a low (0.04 μg/g) or an adequate (2.00 μg/g) B diet. The detailed composition and method of preparation of the experimental diets have been described previously *(16)*. Ultrapure water and diets were provided *ad libitum* in plastic containers to minimize B contamination. Maternal food intake and body weight were recorded every other day.

Study Design

Rats were fed either the B-adequate or the low B diet for 6 wk. Four male and 4 female rats were killed at 0, 4, and 6 wk of dietary treatment to evaluate the effects of the diets on blood B concentrations. Animals were killed by CO_2 exposure, and blood was collected in plastic tubes by cardiac puncture, and frozen until analyzed. After 6 wk of dietary treatment, the remaining females were mated with males of the same dietary group, and mating performance was recorded. These females were fed their respective adequate and low B diets through mating and gestation. On gestation day 20, females were killed and whole blood, plasma, tibia, liver, and thymus were collected, weighed, and stored at –70°C for mineral analyses. Reproductive performance was assessed by counting the number of implantations, resorptions, and live fetuses per litter. Fetal weight, crown-rump length, gross morphology, and skeletal development were end points used to evaluate the effects of maternal dietary B intake on fetal growth and development. Fetuses were examined for malformations using the *Mid-Atlantic Reproductive Toxicology Association/Midwestern Teratology Associate Glossary and Thesaurus of Fetal/Neonatal Alterations (17)*. Additionally, half of the fetuses of each litter were processed for skeletal staining by the method of McLeod *(18)* and examined for structural defects using standard illustrations *(17,19)*.

B Analysis

The B concentration of ultrapure water, diets, and animal blood and tissues was determined by inductively coupled plasma mass spectrophotometry (ICPMS). The accuracy of the method was verified by titrating adult female blood, plasma, and serum samples with known concentrations of a boric acid (BA) stock solution made from the National Institute of Standards and Technology approved BA (Standard Reference Materials 951, Gaithersburg, MD).

Results

Food intake of male and female rats fed the low and adequate B diets was recorded over a 28-d period and was not statistically different from one group to the other. However, both males and females displayed a cyclic pattern of intake resulting in significant differences between the low and adequate groups at particular time-points (Fig. 6 A,B). There was no significant difference in mean body weight in males and females of the two dietary groups.

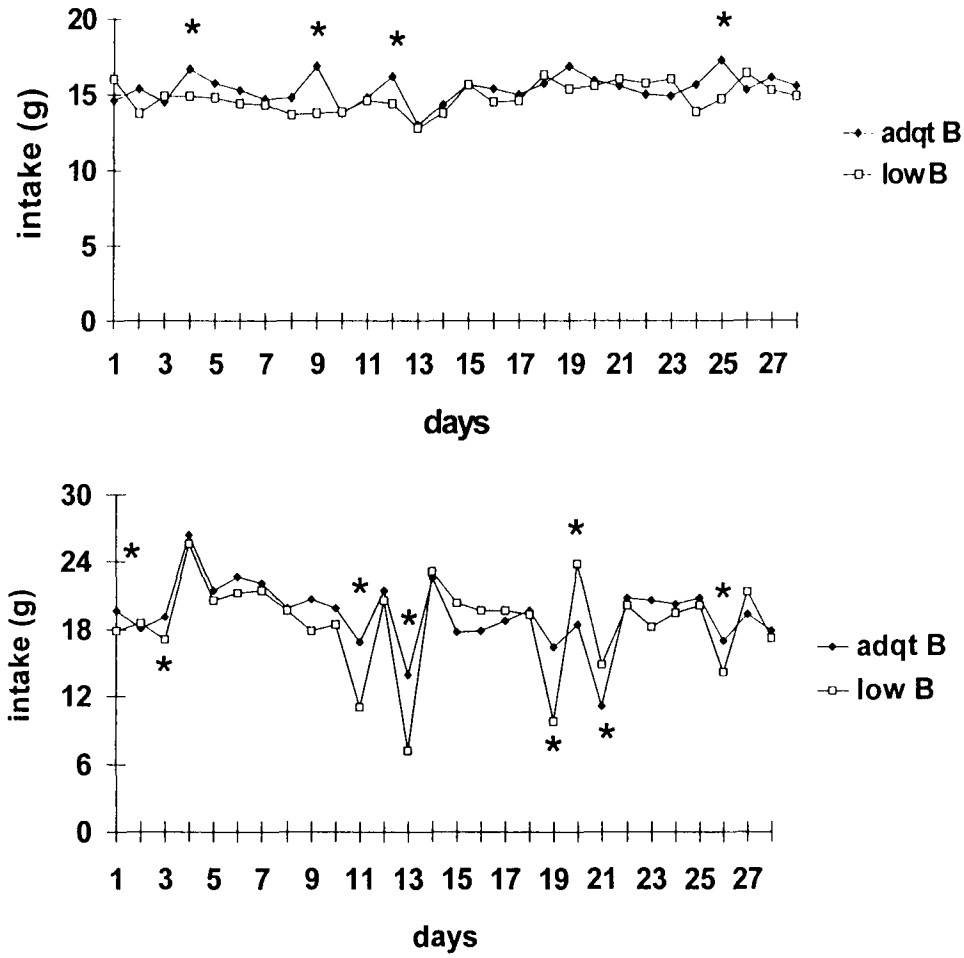

Fig. 6. Twenty-eight-day food intake patterns of female **(top)** and male **(bottom)** rats fed the low and adequate (adqt) B diets. *N* = 15 females and 8 males/diets. *Indicates statistical differences between the two diets.

Both B diets resulted in significant changes in blood B concentrations in male and female rats. Blood B concentrations dropped dramatically after 4 wk of dietary treatment and they further decreased after 6 wk (Table 1). At week 6, a 10-fold reduction in blood B was observed in rats fed the adequate B diet, and a 20-fold reduction was observed in rats fed the low B diet; this difference between the two dietary groups was statistically significant.

Dietary B also affected maternal B status. Gestational day 20 pregnant rats fed the low B diet had significantly lower tibia B content and lower blood and liver B concentrations, compared to dams fed the B-adequate diet (Table 2).

Despite the marked differences in tissue B concentrations, reproductive performance was not affected by the dietary B deficiency in

Table 1
Blood B Concentrations (nmol/mL) in Male and Female Sprague-Dawley Rats Fed a Commercial Stock Diet, and After 4 and 6 Wk Exposure to Low and Adequate B Diets[1]

Stock diet-fed rats (n = 8)		Boron diet-fed rats (n = 4)	
Week 0		Week 4	Week 6
		females	
	Dietary boron (μg/g)		
13.0 ± 0.5	2.00	1.6 ± 0.2	1.2 ± 0.1
	0.04	0.2 ± 0.04*	0.2 ± 0.0*
		males	
13.8 ± 0.6	2.00	2.0 ± 0.1	1.8 ± 0.07
	0.04	0.7 ± 0.04*	0.2 ± 0.01*

[1]Values are means ± SE. *Significantly different from the boron adequate group ($P < 0.05$, ANOVA).

Table 2
Blood, Liver, and Tibia B Concentrations (nmol/g) of Gestational Day 20 Rat Dams Fed Low or Adequate B Diets for 12 Wk[1]

Dietary Boron (mg/g)	Maternal tissues		
	Blood[3]	Liver	Tibia
2.00	4.1 ± 0.3	6.1 ± 0.9	24.7 ± 2.9
0.04	3.4 ± 0.3	4.0 ± 0.9	<b.d.l.*[2]

[1]Values are means ± SE. N = 20 dams per dietary groups.
[2]Tibia boron concentrations in dams fed the low boron diet was below the detection limit (b.d.l.) of the instrument (0.5 μg/g).
[3]Average of blood B values above the detection limit of the instrument. N = 16/19 (79%) for adequate B diet; 4/19 (15%) for low B diet.

that fetal resorption, fetal mortality, and fetal growth, as assessed by weight and length measurements, were similar between the two groups (Table 3). Furthermore, neither morphological nor skeletal defects were observed in fetuses of the low B group (Table 4). However, the average number of implantation sites and fetuses was slightly higher in the B-adequate group.

Discussion

It is important to point out that the terms used throughout these studies to describe the diets, "low" and "adequate," do not imply deficiency or adequacy. The concentration of B in the low diet was 0.04 μg/g of diet, an amount that certainly represented a very low level of intake

Table 3
The Effects of Dietary B on Reproductive Outcome[1]

Reproductive parameters	Dietary Boron	
	adequate (2.00 μg/g)	low (0.04 μg/g)
no. of dams	18	20
GD[2] 0 weight (g)	217.7 ± 4.6[a]	202.0 ± 3.2[b]
GD 13 weight (g)	275.9 ± 6.1[a]	254.5 ± 3.4[b]
GD 20 weight (g)	340.3 ± 3.7[a]	318.2 ± 4.3[b]
no. of implantation sites	13.2 ± 0.5[a]	12.4 ± 0.4[a]
no. of resorptions	0.6 ± 0.2[a]	0.7 ± 0.2[a]
no. of fetuses	12.6 ± 0.5[a]	11.7 ± 0.4[a]
placental weight (g)	0.7 ± 0.0[a]	0.7 ± 0.0[a]
fetal weight (g)	3.5 ± 0.1[a]	3.5 ± 0.1[a]
fetal length (cm)	3.8 ± 0.0[a]	3.9 ± 0.0[a]

[1]Values are means ± SE. Values within a row with different superscripts are significantly different ($P < 0.05$, Student's *t* test).
[2]GD = gestational day

Table 4
The Effects of Dietary B on Markers of Fetal Skeletal Development[1]

Dietary boron (μg/g)	no. fetuses	Vertebrae	Centra	Caudal Vertebrae	Metacarpals	Metatarsals	Sternum	Rib Pairs
2.00	116	31.9 ± 0.1[a]	25.4 ± 0.1[a]	3.9 ± 0.1[a]	3.8 ± 0.1[a]	4.0 ± 0.0[a]	5.6 ± 0.1[a]	13.0 ± 0.0[a]
0.04	119	32.3 ± 0.1[b]	25.6 ± 0.1[a]	4.1 ± 0.1[a]	3.7 ± 0.1[a]	4.0 ± 0.0[a]	5.5 ± 0.1[a]	13.0 ± 0.0[a]

[1]Values are means ± SE. Values within a column with different superscripts are significantly different ($P < 0.05$, Student's *t* test). Skeletal parameters were examined following staining as described in material and methods section.

as indicated by the marked decrease in blood B concentration after 6 wk of treatment. On the other hand, the 2.00 μg of B/g of diet also resulted in a significant reduction in blood B concentration in weanling rats previously fed a commercial rodent diet; these diets typically contain 12.0–14.0 μg B/g *(16)*. Thus, whether the 2 μg/g diet represents an adequate B intake is an issue that needs further research. Differences in food

intake at specific time-points were observed between the two dietary groups. The mechanism underlying these differences in food intake needs to be determined. Although these patterns were not associated with differences in body weight in the nongravid females, the low B diet affected the weight of the pregnant rats.

During pregnancy, the low B diet markedly affected maternal B status as shown by the lower liver, tibia, and blood B concentrations in these dams. However, despite a significant effect on maternal B status, neither reproductive outcome nor fetal development was markedly compromised, since there were no differences in fetal body weight and length, or in the external morphology and skeletal structures of the fetuses. Although one interpretation of this study could be that B is not essential for mammalian reproduction, an alternative explanation is that a longer period of deficiency is needed before having consequences on fetal development. In addition, it should be emphasized that postnatal survival and development were not evaluated in this study.

THE EFFECTS OF LOW B ON POSTIMPLANTATION RAT EMBRYOS; AN IN VITRO STUDY (STUDY 2)

Materials and Methods

Animals and Diets

The rat strain and housing conditions were identical to those described for study 1. Thirty female and 16 male rats were divided into two experimental groups (low B diet or adequate B diet). Dietary treatment was maintained for 12 wk prior to and during gestation. The male breeders from study 1 were kept on their respective dietary treatments for a period of 8–15 wk before being killed to collect serum for the whole-embryo culture experiment.

Study Design

A crossover design was used in this study. Rat embryos were collected from dams fed either the low or adequate B diets, and cultured in serum collected from male rats fed either the low or adequate B diets. In addition, embryos from low or adequate B-fed mothers were cultured in low B serum repleted with BA to control serum concentrations. Thus, the following six treatment groups were examined:

1. Embryos from B-adequate mothers cultured in B-adequate serum (adqt B / adqt B).
2. Embryos from B-adequate mothers cultured in low B serum (adqt B / low B).

3. Embryos from low B mothers cultured in B-adequate serum (low B / adqt B).
4. Embryos from low B mothers cultured in low B serum (low B / low B).
5. Embryos from B-adequate mothers cultured in low B serum, supplemented with boric acid (adqt B/ low B + BA).
6. Embryos from low B mothers cultured in low B serum supplemented with BA (low B/low B + BA).

Additionally, embryos from B-adequate mothers cultured in glass vials served as a baseline control group.

Embryo Culture

The embryos were cultured as described above. Dams were killed on gestational day 10, and the number of implantation sites was recorded. Embryos were isolated from the decidua and cultured for 48 h in sterile polystyrene flasks containing 3 mL of rat serum. One to 4 embryos were cultured/flask with 3–6 flasks/treatment group. Culture flasks were gassed with the appropriate gas mixture at 0800 and 1600 h daily. At the end of the culture period, embryos were scored for development, and the number of somite pairs counted. Postculture serum samples were collected for B analysis. Serum was prepared from blood collected from male rats fed low or adequate B diets for a minimum of 8 wk. Males were anesthetized with ether and the blood collected from the abdominal aorta into trace mineral-free polypropylene tubes. The recovered serum was heat-inactivated at 56°C for 30 min. Streptomycin sulfate (50 mg/mL) was added, and the serum was frozen in aliquots.

Results

There were no differences in food intake or weight gain between the low and adequate B dams. However, the number of implantation sites in the low B diet group was significantly lower than that of the B-adequate diet group (9.75 ± 0.8, low B vs 12.25 ± 0.6, adequate B ($P < 0.05$, Student's *t*-test).

The results of the whole-embryo culture experiments are summarized in Table 5. Overall, embryonic growth in vitro was not affected by the different conditions as shown by the absence of differences in the number of somite pairs or crown-rump length measurements. However, compared to the control group, embryos from the treatment groups were smaller by 5–10 somite pairs; it is not clear whether this reflects a difference in diet composition (commercial stock diet vs experimental diets) or a difference in the culturing vials (glass vs plastic). Our preliminary results show that embryos collected from low B-fed dams had higher developmental scores than embryos collected from B-adequate-fed dams; this difference was not statistically significant (data not shown). The treatment effect on the developmental score appeared not to be corre-

Table 5
Media B Concentration and Postimplantation Performance Indices of Cultured Rat Embryos in Response to Various Treatment Conditions[1]

treatment groups dietary B/media B[2]	media boron nmol/ml (no. of flasks)	no. of embryos	no. of somite pairs	yolk sac diameter (mm)	crown-rump length (mm)
[1] adqt B / adqt B	2.5 ± 0.4[a] (4)	10	25.0 ± 1.3	3.7 ± 0.3[a]	3.5 ± 0.2
[2] adqt B / low B	< 0.46[abc] (1)	8	27.0 ± 0.4	4.5 ± 0.4[ac]	3.2 ± 0.1
[3] low B / low B	2.4 ± 0.5[ab] (7)	15	20.0 ± 2.3	4.2 ± 0.3[ad]	3.2 ± 0.2
[4] low B / adqt B	3.9 ± 0.7[ac] (10)	18	23.3 ± 1.4	4.9 ± 0.2[bc]	3.7 ± 0 2
[5] adqt B /low B + BA[2]	5.4 ± 1.5[abc] (5)	16	24.3 ± 1.7	4.7 ± 0.1[bcd]	3 7 ± 0.2
[6] low B / low B + BA	3.4 ± 1.2[abc] (6)	10	25 2 ± 6.9	4 6 ± 0.3[bcd]	3 8 ± 0.3
[7] control[2]	13 2 ± 0.7[bc] (3)	8	31.0 ± 1.6	4.2 ± 0 2[ab]	3 6 ± 0 2
p value[3]	< 0.05		NS	< 0 05	NS

[1]Values are means ± SE.

[2]Dietary B: embryos were collected from dams fed either low or adequate (adqt) boron diets for a least 12 weeks. Media B embryos were cultured in rat serum of males fed either low or adqt boron diets for at least 8 weeks. Groups [5] and [6]. media were repleted with 5.5 nmol/ml boron with addition of boric acid to the culture media. Control embryos were cultured usign our standards culturing conditions

[3]Results of ANOVA Values within a column with different superscripts are statistically different, NS. no statistical differences

lated with the number of somite pairs. We also observed differences in the B concentration of the different culture media, the positive control group having significantly higher B.

Discussion

In this second study, the chronic feeding of a low B diet was associated with a significant reduction of number of implantation sites. A similar trend was observed in study 1, but the difference between the two group was not statistically significant. In study 1, both males and females were kept on the experimental diets for a total of approx 12 wk with breeding starting at week 6 and gestational day 20 occurring as early as week 8 and through week 12. In the second study, the animals were exposed to the dietary treatments for approx 15 wk. However, breeding was not commenced until after week 12 of feeding and continued until week 14. We speculate that the longer exposure to the low B diet prior to breeding further compromised maternal B status and affected reproductive performance.

It is interesting to note that for the different groups tested, embryos cultured in serum supplemented with BA had the best morphological scores, regardless of the dietary groups from which they were derived. Repletion of the culture medium with BA may have contributed to the higher scores by providing more B for developmental processes yet to be

identified. Alternatively, BA may have acted as an antibacterial agent, making the media more suitable for supporting growth. Arguing against this second possibility is the fact that the media already contained an antibiotic. Overall, we observed no, or little, significant differences between the treatment groups with respect to embryonic growth. Although the morphological score varied somewhat broadly between groups, there were no overt malformations that could be clearly attributed to low B conditions, either dietary or in the culture medium.

B TOXICITY IN PREIMPLANTATION EMBRYOS (STUDY 3)

Maternal and fetal toxicity have been observed in rats and mice treated with BA throughout gestation (0.2% BA in the diet for rats, and 0.4% BA for mice; *20,21*). Signs of toxicity include decreased fetal body weight and increased number of malformations, namely skeletal defects, such as supernumerary lumbar ribs, and agenesis or shortening of the 13th rib *(22,23)*. Of note is the observation that BA treatment during gestation increased the number of resorptions in the presence of a normal number of implantation sites *(23)*. It has also been reported that a single dose of BA (3000 mg/kg p.o.) given to mice on the first day of pregnancy resulted in early embryonic losses, apparently owing to a failure of blastulation *(24)*. Thus, it seems that preimplantation development is affected following maternal dosing with boric acid. However, it is not clear whether this results from a specific adverse effect of BA on early embryonic development, or if it is secondary to maternal toxicity. Moreover, the threshold levels of BA toxicity on preimplantation embryos, if any, have not been clearly defined. For these reasons, we investigated the effects of BA exposure on the in vitro preimplantation development of mouse embryos.

Materials and Methods

Study Design

Five-week-old CD-1 virgin female mice (Charles River Inc., Portage, MI) were housed in stainless-steel cages in a temperature- and light-controlled environment (14h reverse-light cycle) and fed a commercial chow diet (LabDiet Laboratory Rodent Diet #5001). After a week of adaptation, the mice were superovulated, mated overnight with males of the same strain, and two-cell embryos were collected as described above. Within a single experiment, we pooled all the recovered embryos prior to assigning them to an experimental treatment to preclude any residual maternal effect. The embryos were screened for degenerates and one-cell embryos, and then aliquoted to groups of 20–30 two-cell embryos/culture treatment. A small number of four-cell embryos were included in the culture. The presence of four-cell embryos is expected, and reflects variations in times of ovulation and fertilization between mice.

Table 6
Development of Two-Cell Mouse Embryos In Vitro After 72 h of Culture in Baseline T6 Medium and in Media Containing Graded Levels of BA[1]

Boric acid (μM)[2]	no. of replicates[3].	no. of 2-cells[4]	% morulae at day 1	% blastocysts at day 3	% degenerates at day 3
0/T6	8	149	60.8 ± 5.1	86.3 ± 4.2	7.6 ± 1.7
6	3	59	76.4 ± 4.4	90.8 ± 2.6	4.7 ± 2.9
12	3	82	72.1 ± 5.7	91.3 ± 3.1	5.2 ± 1.7
25	4	115	61.4 ± 6.0	84.4 ± 8.7	7.5 ± 2.1
50	3	77	68.9 ± 8.9	88.2 ± 2.0	4.7 ± 4.7
100	4	96	85.7 ± 5.5	81.2 ± 5.3	6.6 ± 2.9
200	4	98	72.1 ± 5.6	81.1 ± 6.9	6.6 ± 2.3
400	4	93	59.2 ± 18.5	88.8 ± 4.7	1.9 ± 1.2
800	5	95	46.3 ± 14.5	79.4 ± 6.3	10.7 ± 5.5
1000	3	85	59.9 ± 12.9	78.2 ± 4.7	10.8 ± 5.2
2000	4	80	34.6 ± 4.8*	66.1 ± 15.7*	19.6 ± 8.1*
4000	3	56	26.1 ± 10.7*	27.9 ± 17.6*	62.5 ± 15.5*
8000	2	42	0*	0*	92.9 ± 7.4*
10000	2	40	9.1 ± 0.0*	0*	86.4 ± 13.6*

[1]Values are means ± SE. * indicate boric acid levels resulting in significant reduction of performance compared to baseline T6 medium ($p < 0.05$, Kruskal-Wallis).
[2]Concentration of boric acid added to T6 culture droplets.
[3]Number of individual experiments.
[4]Total number of two-cells per treatments with an average of 19 to 28 embryos per replicate/experiment.

To evaluate BA toxicity, preimplantation embryos were cultured in 25-μL droplets of T6 medium containing graded levels of BA (6–10,000 μ*M*). Medium droplets were prepared fresh by appropriate serial dilutions of a stock solution of 1 *M* BA in T6 medium (Sigma, St. Louis, MO). T6 medium was previously analyzed and was shown to contain approx 10 ng/mL B. Seven 25-μL droplets were plated in a plastic culture dish (one baseline T6 medium droplet and six BA-containing droplets). Preimplantation development was evaluated by determining the number of embryos reaching the blastocyst stage after 72 h of culture and by counting the number of cells in fixed blastocysts *(25)*.

Results

Addition of BA to the culture media in concentrations ranging between 6 μ*M* and 1 *mM* did not interfere with the development of two-cell embryos to the blastocyst stage; impaired development was observed only at the very high end of the spectrum of concentrations tested (between 2 and 10 *mM* boric acid; Table 6). At these concentrations, the two-cell embryos failed to replicate beyond the four-cell stage (Plate 2). When the frequency of blastocyst formation was expressed as percent of

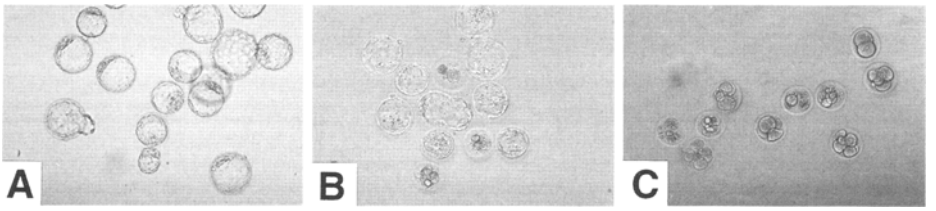

Plate 2. Photomicrographs of embryos taken after 72 h of culture under the folllowing conditions: Baseline T6 **(A)**; T6 + 1000 μ*M* BA **(B)**, and T6 + 10,000 μ*M* BA **(C)**. Note that under baseline conditions, most of the two-cell embryos formed blastocysts **(A)**; 1000 μ*M* is the highest BA concentration added to the culture medium that does not result in statistical difference from baseline **(B)**; at 10,000 μ*M*, embryonic growth is arrested at the four-cell stage or embryos become degenerate **(C)**.

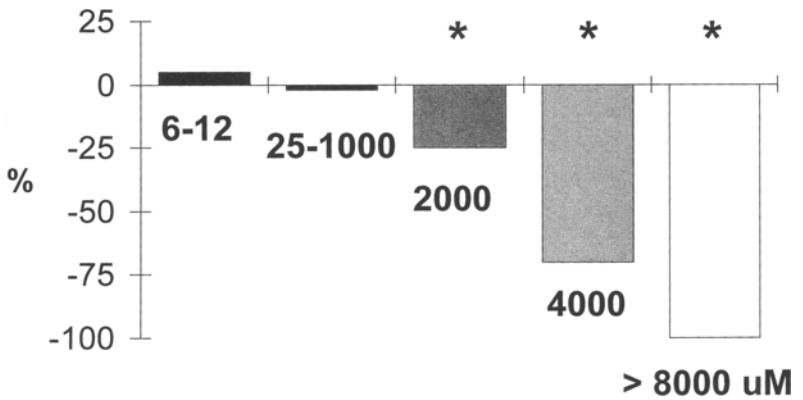

Fig. 7. Effects of varying concentrations of BA on the frequency of blastocyst formation expressed as percent of baseline T6 medium, indicated as 0. *Indicates statistical differences from baseline medium. Note the ameliorative effect of addition of low amounts of BA on early embryonic differentiation in vitro.

baseline T6 medium, addition of low amounts of BA to the medium (6–12 μ*M*) increased the frequency of blastocyst formation; between 25 and 1000 μ*M* boric acid, two-cell differentiation was not different from that of baseline; blastocyst formation was reduced by 25% at 2000 μ*M* and by 75% at 4000 μ*M* BA (Fig. 7).

Blastocyst cell number, an indicator of embryonic cell proliferation, was more susceptible to the effects of BA and may be a better marker for determining toxicity threshold levels. Compared to baseline T6, a significant reduction in blastocyst cell number was observed at 200 μ*M* boric acid and at concentrations >1000 μ*M* (Table 7). When expressed as percent of control, 50–1000 μ*M* boric acid decreased blastocyst cell number by 25% and 2000–4000 μ*M* by 50%; blastocysts did not form at concen-

Table 7
The Effects of BA Addition to T6 Culture Medium on Embryo and Blastocyst Cell Number[1]

Boric acid (μM)[2]	embryos[3]	no.of cells per embryos	embryos > 20 cells[4]	no. of cells per blastocysts
0/T6	90	77.8 ± 3.5	79	87.5 ± 2.5
6	39	78.7 ± 5.1	37	82.9 ± 4.4
12	28	90.5 ± 6.2	27	93.7 ± 5.6
25	64	93.6 ± 4.6	61	97.8 ± 4.1
50	20	59.8 ± 7.6	18	66.1 ± 7.0
100	55	68.2 ± 4.0	49	74.7 ± 3.5
200	50	53.5 ± 4.4*	40	64.3 ± 3.9*
400	53	74.5 ± 3.6	51	77.1 ± 3.2
800	46	70.6 ± 3.3	44	73.2 ± 2.9
1000	53	58.9 ± 3.9	47	65.2 ± 3.6*
2000	45	47.1 ± 3.8*	38	54.2 ± 3.4*
4000	40	33.1 ± 3.3*	29	42.6 ± 2.9*
8000	20	7.6 ± 1.4*	2	22.0 ± 2.0*
10000	22	6.2 ± 0.9*	0	0*

[1]Values are means ± SE. * indicate significant difference from baseline T6 ($P < 0.001$, ANOVA).

[2]Concentration of boric acid added to T6 culture droplets. Two-cell embryos were cultured for 72 h in each medium.

[3]Total number of embryos recovered after 72 h of culture; 1 to 8 replicates at each treatment group.

[4]Restriction of total embryos to those having greater than 20 cells; these were labelled blastocysts; 1 to 8 replicates at each treatment group.

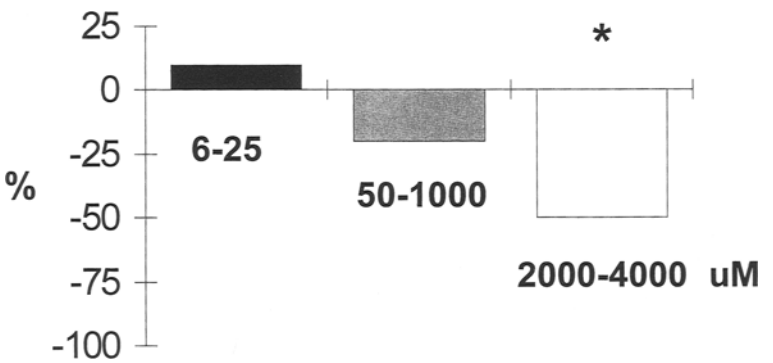

Fig. 8 Effects of varying concentrations of BA on blastocyst cell number expressed as percent of baseline T6 medium, indicated as 0. *Indicates statistical differences from baseline medium.

trations of BA >8000 μ*M*. Of note is the improved embryonic proliferation in response to addition of low amounts of BA to the culture media (6–25 μ*M*; Fig. 8).

Table 8
Comparison of BA Toxicity Thresholds for Preimplantation Development of Mouse Embryos with That of Other Metals[1]

Element	Blastocyst differentiation toxicity threshold (μM)[2]	Blastocyst proliferation toxicity threshold (μM)[2]	Toxicity threshold/Serum ratio[3]
As	1.5	0.1	0.5
V	25	3	150.0
Cu	50	12	0.7
Zn	25	25	1.4
Mn	25	6	300.0
$B(OH)_3$	2000	50	18.5

[1]Lowest concentration of element added to media which resulted in a significant reduction of mouse embryos preimplantation performance compared to control, in vitro.
[2]For metals, the ratio of threshold toxicity to serum concentrations are taken from Hanna et al. *(8)*, using embryonic cell number for toxicity. For boric acid, the ratio was calculated using blastocyst cell number over the average blood boron concentration of 2.7 μ*M* from Naghii and Samman *(26)*.

To evaluate the degree of toxicity of BA, we compared the threshold toxicity levels of this element with toxicity thresholds of other metals previously tested using this culture system *(8)*. As shown in Table 8, arsenic (As) has the lowest toxicity threshold (0.1–1.5 μ*M*), using either blastocyst differentiation or proliferation as markers; high toxicity was also observed with mercury (Hg) and silver (Ag). Vanadium (V), manganese (Mn), copper (Cu), and zinc (Zn) were shown to be toxic at concentrations ranging between 3 and 50 μ*M*. This contrasts with BA, for which no impaired embryonic differentiation could be observed at levels below 2000 μ*M*, and 200 μ*M* was the lowest concentration at which proliferation was significantly affected.

Discussion

The toxicity of BA on embryonic preimplantation development was evaluated in vitro. Our data show that culture media containing BA in concentrations between 2 and 10 *mM* significantly impaired embryonic cell proliferation and differentiation. This represents a low level of toxicity in contrast to other metals where toxicity is observed at doses ranging between 1 and 50 μ*M*. Furthermore, a conservative toxicity threshold of 50 μ*M* BA still represents a value that is 18.5-fold above the average blood B concentration *(26)*. In contrast, Cu, and Zn are toxic at concentrations below or at the normal physiological concentrations in human serum (Table 8).

Two-cell embryos exposed to high doses of boric acid (>8 *mM*) failed to progress beyond the eight-cell stage. In embryos growing from two to eight cells, key events occur that are essential for further embryonic development. The two-cell stage is the transition period when the embryo no longer relies on maternally derived mRNA and proteins, but switches to

its own genome *(27)*. Compaction, which occurs at the eight-cell stage, is a morphological event that confers cellular polarity to the embryo. Polarity, or the cleavage of the embryonic cells along specific axes, segregates the blastocyst into two distinct cell populations *(28)*. As a blastocyst, the embryo switches from lactate and pyruvate to glucose as its preferred energy substrate *(29)*. We can only speculate how high doses of B may interfere with any of these processes. High concentrations of BA could:

1. Result in a pH imbalance with subsequent alterations of cellular functions.
2. Directly compete with salts present in the culture medium and reduce their availability.
3. Interfere with the expression of cell-surface proteins and receptors.
4. Inhibit one or more metabolic reactions.

In a testicular germ-cell coculture system, Ku and Chapin *(30)* have observed a significant decrease in medium lactate and pyruvate levels, and a significant reduction in thymidine incorporation after a 72-h exposure to high levels of BA (3 and 10 *mM*), suggesting impaired metabolism and DNA synthesis. B in solution is likely to form an ester with hydroxyl groups present in simple alcohols and sugars, such as mannitol and sorbitol *(2,31)*. The preferential binding of B to cofactor nicotinamide adenine dinucleotide (NAD) over that of reduced nicotinamide adenine dinucleotide (NADH) may have implications for a number of dehydrogenase-based reactions *(32)*. A reduction in the NAD/NADH ratio would alter several energy-producing cycles; it would depress glycolysis and the Krebs cycle, but stimulate the ribose phosphate pathway. High levels of BA have been shown to inhibit certain enzymes, such as serine proteases *(32)*.

Although BA-induced embryotoxicity may involve several different processes, our data indicate that the early development of mouse embryos is highly resistant to large doses of this trace element. In this respect, our data obtained in vitro confirm the low levels of toxicity observed in animal and human in vivo studies *(33)*. Our data also suggest that the developmental defects observed in vivo may be secondary to the presence of maternal toxicity.

THE EFFECTS OF DIETARY B ON PREIMPLANTATION EMBRYONIC DEVELOPMENT (STUDY 4)

Materials and Methods

Animals and Diets

Weanling (4-wk-old) male and female virgin CD-1 mice (Charles River Inc, Portage, MI) were housed individually (males) or in groups of 2–3 (females) in suspended stainless-steel cages and adapted for a week

to the controlled environment (14-h reversed-light cycle; lights on: 0100–1500 h). Sixteen females and 6 males were fed the low B diet (0.04 μg B/g), and 15 females were fed the B-adequate diet (2.00 μg B/g). Diets were identical to those used in the rat studies. Mice were fed daily with fresh diet placed into suspended Plexiglas containers. The water was purified through a Sybron/Barnstead Nanopure system and supplied in plastic bottles to minimize B contamination. Additionally, 12 female and 20 male mice were fed a commercial diet (LabDiet Laboratory Rodent Diet #5001) and used to collect baseline data.

Study Design

The effect of B on preimplantation embryonic development was tested after 10, 12, and 16 wk of dietary treatment. At each time-point, a subset of females was superovulated by sequential hormone injections and mated for 24 h as described above. Low B-fed females were placed with males fed the low B diet; B-adequate- and commercial diet-fed females were mated with males fed the commercial stock diet. Two-cell embryos were collected on the fourth day following initiation of the hormonal treatment as described earlier. Embryo transfer was done by mouth-pipeting using pulled glass Pasteur pipets. Each pipet was coated with silicon (Sigma Coat; Sigma, St. Louis, MO) to minimize leaching of B from the glass pipet into the culture medium. The siliconized pipets did not contribute B to the media as shown by measurements of the B level in media prior to, and after, 10 min of constant pipeting. The two media used in the preimplantation culture system were prepared and stored in polypropylene tubes. ICPMS analyses indicated a B content of 6–9 μg/L for L15 and 10 to 14 μg/L for T6.

Within each dietary treatment, viable embryos were pooled from the dams, and two-cell embryos were then divided into groups of 10–30/25 μL medium droplet. Embryos from both diets were plated in separate droplets in the same culture dish that also contained embryos taken from commercial diet-fed mice as control. Developmental kinetics of the embryos were assessed daily during the 72-h culture period. Embryos were examined using phase microscopy, and the number of two-cell, four-cell, and eight-cell compacted embryos, morula, and blastocysts were recorded at each time-point; degenerate or dying embryos were also noted. The effect of B on embryonic differentiation was assessed by comparing the number of two-cell embryos reaching the morula stage at day 1 of culture, and forming blastocysts at day 3 of culture. At the end of culture, embryos were fixed on glass slides and stained with Giemsa stain *(25)*. The number of cells per embryos was determined by counting the number of stained nuclei.

Results

On average, the weanling mice fed the purified B diets gained 10 g of body weight during the 4 mo of dietary treatment, and there

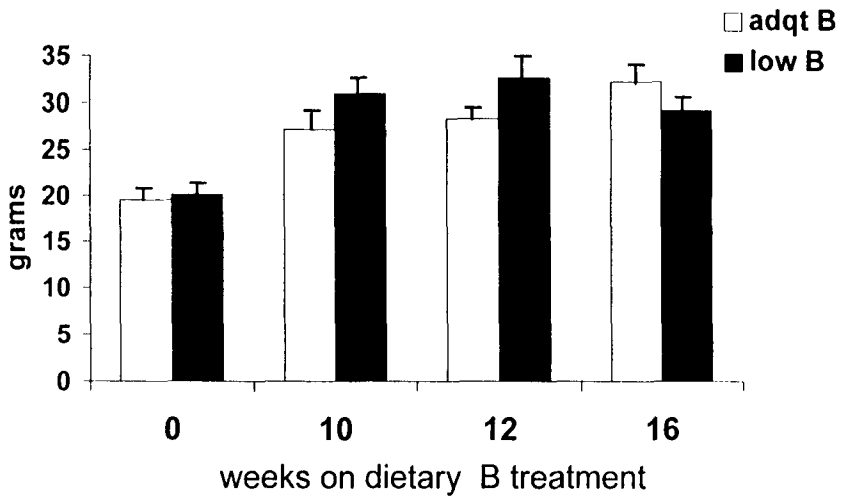

Fig. 9 Body weights of CD-1 female mice before and after 10, 12, and 16 wk of dietary B feeding. Bars are means ± SE. For low B, n = 17, 3, 6, and 8; for adqt B, n = 15, 3, 5, and 7 at 0, 10, 12, and 16 wk, respectively.

was no statistical difference between the low and adequate B diets (Fig. 9). After 12 and 16 wk of treatment, the B-fed mice had comparable body weights to mice fed the commercial diet of similar age (data not shown).

Dietary B did not statistically impair the development of the embryos in vitro, since >80% of the two-cell embryos matured into blastocysts. This rate was comparable to the frequency of blastocyst formation measured in the commercial diet-fed mice of the same age (92.9% in 12-wk-old; 89.3%, in 16-wk-old; Table 9). Importantly, there was a clear tendency for a lesser performance (although not statistically significant) in embryos explanted from dams fed the low B diet. A smaller number of the the two-cell embryos obtained from low B-fed dams attained the blastocyst stage compared to embryos from B-adequate-fed dams. This was noted as early as 10 wk of dietary treatment and was also observed at 12 and 16 wk (Table 9). In the low B group, the embryos either lagged in development or a high proportion became degenerate embryos (Table 9 and Fig. 10).

The low B diet also influenced cellular proliferation in vitro. At 10 and 12 wk, there was a lower average cell number in embryos from low B-fed dams compared to embryos taken from adequate B-fed dams. Because maternal B deficiency was associated with a lower rate of embryos reaching the blastocyst stage at the end of the 72-h culture period, we restricted cell counts on embryos that had reached the morula stage (>20 cells); even with this correction, we observed a lower average blastocyst cell number in the low B group on weeks 12 and 16 compared to the adequate B group (Table 10).

Table 9
Effects of Dietary B on Preimplantation Development of Mouse Embryos in Vitro[1]

Dietary Treatments[2]	no. of two-cells[3]	% morulae at day 1	% blastocysts at day 3	% degenerates at day 3
		10 week of dietary treatment		
low B (0.04µg/g)	28[3a]	64.3	82.1	17.9
adqt B (2.00 µg/g)	47[3a]	65.9	93.6	2.1
		12 week of dietary treatment		
low B (0.04µg/g)	9[3a]	55.6	88.9	11.1
adqt B (2.00 µg/g)	50[3b]	65.6 ± 9.7	91.9 ± 2.6	8.1 ± 2.6
commercial diet[2a]	77[3c]	82.3 ± 8.7	92.9 ± 4.3	6.2 ± 3.6
		16 week of dietary treatment		
low B (0.04µg/g)	63[3c]	58.6 ± 4.4	82.4 ± 3.7	12.1 ± 3.7
adqt B (2.00 µg/g)	57[3c]	51.8 ± 20.6	87.8 ± 3.6	9.4 ± 1.4
commercial diet	83[3c]	33.9 ± 12.7	89.3 ± 6.5	6.0 ± 3.1

[1]Values are means ± SE, when noted. No significant difference between dietary boron groups (Chi square analysis).

[2] Mice were fed either the low or adequate boron diet for a minimum of 10 weeks before collections of two-cell embryos for culture. [2a] CD-1 mice of similar age were purchased and kept for one week on commercial stock diet before collection of embryos to control for potential effects of aging.

[3]Indicate total number of two-cell mouse embryos cultured within each group. [3a] Represent a single replicate. [3b] Total number of embryos with 3 replicates. [3c]Represent 4 replicates.

Discussion

The results from this study strongly support the concept that B plays a critical role in early embryonic development. We consistently observed impaired embryonic growth and development in vitro following chronic maternal exposure to a low B diet. Thus, our data complement previous observations that B is an essential element to sustain growth in plants, frogs, and fish. Of note is the fact that our culture medium contains B. Medium B can be extracted by filtration through a B-specific resin (Amberlite IRA 743). This resin is an anion exchanger with hydroxyl groups that chelate B by forming esters *(34)*. We hypothesize that using a "B-free" culture medium will exacerbate the effects of maternal B deficiency on embryonic development.

Table 10
The Effects of Maternal Dietary B Treatment on Preimplantation Embryonic Proliferation In Vitro[1]

Dietary treatments[2]	embryos	no. of cells per embryos	embryos > 20 cells[3]	no. of cells per blastocysts
		10 week of dietary treatment		
low B (0.04 µg/g)	19	67.9 ± 10.4	13	94.0 ± 7.7
adqt B (2.00 µg/g)	32	77.8 ± 7.6	27	90.1 ± 6.6
		12 week of dietary treatment		
low B (0.04 µg/g)	6	69.7 ± 7.7	6	69.7 ± 7.7
adqt B (2.00 µg/g)	40	73.1 ± 4.3	36	80.4 ± 2.7
commercial diet	31	68.4 ± 5.9	26	79.2 ± 4.5
		16 week of dietary treatment		
low B (0.04 µg/g)	57	79.7 ± 3.5	57	79.7 ± 3.5
adqt B (2.00 µg/g)	37	79.9 ± 5.2	34	86.3 ± 4.1
commercial diet	58	74.6 ± 4.0	51	83.8 ± 2.6

[1]Values are means ± SE of embryos cell number after 72 h of culture. No significant difference between dietary treatments (ANOVA).

[2]See note Table 10.

[3]Represent number of embryos with greater than 20 cells and are called blastocysts.

B is efficiently absorbed and largely excreted in urine *(35)*. The amount of B retained by the body is inversely related to its concentration in the diet *(26)*. Though maternal status in response to the experimental diets was not evaluated in this study, results from study 1 showed that pregnant rats fed the same diets for at least 6 wk have depleted stores and a significant reduction in blood B. This depleted maternal status may in turn:

1. Reduce B availability to embryos.
2. Perturb the oviductal milieu, which in turn impacts the metabolism of the two-cell embryos.
3. Affect the transfer or expression of maternally-derived genes.

This last hypothesis is unlikely in view of our results showing that most embryos progress beyond the two-cell stage, at which point they no longer rely on maternal gene products. Of note is the observation that in plants, signs of B deficiency first appear in regions of growth and not in mature tissue *(2)*.

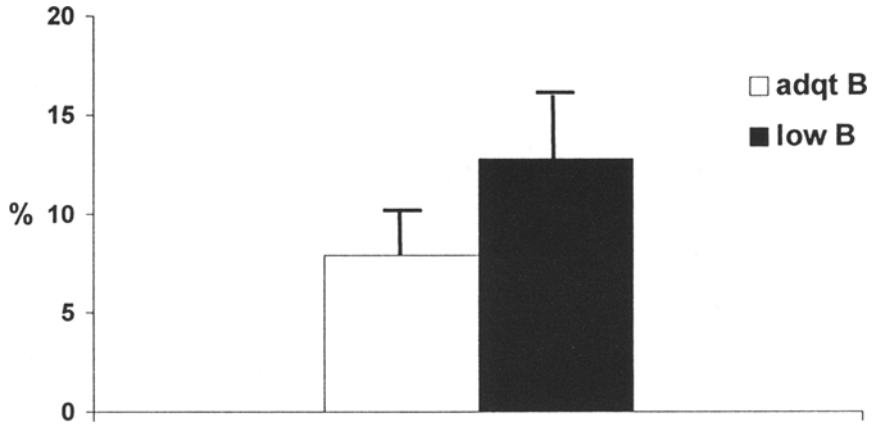

Fig. 10. Degenerate embryos (%) formed after 72 h of culture. Embryos were taken from dams fed either the low B or adequate B diets. Bars are means ± SE and represent data pooled for 10, 12, and 16 wk. Total number of embryos: 70 for low B and 157 for adequate B.

What makes B essential to early embryonic development is still the object of speculation. In plants, B is predominantly localized in cell walls and cell membranes; this specific spatial distribution suggests a predominant role of B in the structural integrity of plant cell walls by complexation with polysaccharides *(31)*. Alternatively, it has been proposed that the primary effect of B is at the membrane level, rather than having direct effects on cellular metabolism *(36)*. B deficiency has been associated with reduced transport of phosphate, K, indoles and sugars across plant membranes *(1,31)*. Also in plants, B is believed to play a role in membrane ATPase and redox systems *(37)*, important mechanisms for the initiation and sustenance of movements of nutrients across membranes. During preimplantation, the embryos undergo some major morphological changes via compaction and cavitation (Fig. 2). These events are associated with changes in the surface properties of the cells and nutrient requirements of the embryos. Whether B deficiency affects these events remains to be determined. Our initial series of experiments suggest that B impairs the early embryonic development in rodents.

ACKNOWLEDGMENTS

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