Published in final edited form as:

Lab Anim. 2013 July ; 47(3): 175–183. doi:10.1177/0023677213480769.

Impact of anaesthetics and analgesics on fetal growth in the mouse

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Abstract

Common anaesthetic and analgesic agents used during pregnancy in mice have been observed to cause fetal growth restriction. We investigated the impact of therapeutic doses of three anaesthetics (ketamine/xylazine, isoflurane, and tribromoethanol) and two analgesics (buprenorphine and meloxicam) on fetal and placental growth. Pregnant mice were treated with one of these agents at fertilization (E0), attachment (E4), beginning of organogenesis (E6), end of organogenesis (E12), or during the logarithmic growth phase (E15), or they were placed into an untreated control group. At term (E18), fetal and placental growth were evaluated, morphological analyses were performed, and skeletal measurements were conducted. Fetal growth was reduced significantly ($P < 0.01$) by ketamine/xylazine treatment at E0, E4, E12, or E15, by isoflurane administered at E0 or E6, and by tribromoethanol administered at E6 or E12. Two-day treatment with buprenorphine beginning at E4 or E6, or with meloxicam at E0 also significantly reduced fetal growth ($P < 0.01$). Neither placental growth nor litter size was significantly affected by any of these agents. The occurrence of microphthalmia was nearly eight-fold higher ($P < 0.05$) in response to buprenorphine administration at E6 compared with controls. The length of the humerus was reduced at most gestation times in response to each of these agents and was correlated ($P < 0.01$) with fetal weight for ketamine/xylazine, tribromoethanol, and meloxicam. These data reveal patterns of acceptable and detrimental anaesthetic and analgesic use during fetal development and have refined our capability to provide recommendations for the use of these agents during pregnancy in the mouse.

Keywords

Anaesthesia; analgesia; fetal growth; microphthalmia; refinement

Fetal growth restriction can result from any of several causes including maternal malnutrition, infection, hypoxia, uteroplacental ischaemic insult, or genetic defects. In modelling the various pathologies that affect fetal growth, it is essential that the model be

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stable except for the single variable used within the experimental investigation. Models of fetal growth restriction almost invariably require surgery or other procedures that necessitate the use of anaesthesia and analgesia. In our laboratory's rodent models of fetal growth restriction,¹⁻⁴ we have observed that certain common anaesthetic and analgesic agents used during sham surgery can cause growth restriction equivalent to that observed in the fetal growth restriction induction model. A similar observation was made in our studies with pregnant rats treated with more than one dose of the analgesic buprenorphine.

Besides being a significant confounding variable in fetal growth restriction and premature birth models, anaesthetics and analgesics commonly employed in the development of transgenic mice and in vivo gene transfer to embryos⁵ could affect results of these experiments as well. The use of anaesthetics and analgesics has the potential not only to affect the development of embryos but also to affect litter size and postnatal health into adulthood.

There is, therefore, a need for reliable anaesthetic and analgesic agents that do not affect fetal development, as well as knowledge of the periods during fetal development when analgesic and anaesthetic agents have detrimental effects. It is possible that harmful consequences from specific anaesthetics or analgesics are realized only during specific phases of development and not others. Consequently, we chose to investigate the impact of anaesthetics and analgesics that act through different mechanisms in mice at several stages of fetal development. Our hypothesis was that standard anaesthesia and analgesia regimens negatively impact fetal growth at specific stages of development in the mouse. Our goal was to identify anaesthetic and analgesic agents that can safely be used during pregnancy without endangering the welfare of the fetus. To this end, we investigated the impact of therapeutic doses of three commonly-used but mechanistically distinct anaesthetics and two analgesics on fetal and placental growth in pregnant mice.

Materials and methods

Animals

Male and female C57BL/6 J mice (8–10 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Female mice in oestrus were mated overnight and mating was confirmed by the presence of a vaginal plug the following morning. Date of plug positivity was designated as day 0 (E0) with expected delivery during the night between days 18 and 19. Mice were maintained in 12 h light/dark cycles and allowed free access to a standard rodent diet (#2918, Harlan Teklad, Indianapolis, IN, USA) and water. All experiments in this study were approved (EH10–199) by the NorthShore University HealthSystem Research Institute Animal Care and Use Committee.

Anaesthetic and analgesic treatments

Three common anaesthetics and two analgesics were administered at times approximating fertilization (E0), attachment (E4), beginning of organogenesis (E6), end of organogenesis (E12), and during the logarithmic growth phase (E15).

Fifteen groups of seven randomly-assigned mice were mated for timed pregnancy and treated with anaesthetics at a dose that maintained a surgical depth of anaesthesia for approximately 30 min. Due to the failure of some of the mated mice to become pregnant, the final group sizes varied from 5–7. Differences in group sizes did not correspond with specific treatments. All mice received a single dose of anaesthetic at one of the five specific gestation days indicated above. Anaesthetic agents included (1) ketamine/xylazine in combination (100 mg/kg and 10 mg/kg, respectively, intraperitoneally (IP)); (2) tribromoethanol (250 mg/kg, IP; prepared fresh according to the methods of Lieggi et al.⁶); and (3) isoflurane (vaporizer set at 3%, delivered in 3L/min of 100% O₂ in an anaesthesia chamber for 30 min). Following injection of either ketamine/xylazine or tribromoethanol, the mice breathed room air for the duration of the anaesthetic time and thereafter.

Thirteen groups of seven randomly-assigned mice were mated for timed pregnancy and treated with buprenorphine (0.1 mg/kg, subcutaneously (SC)) or meloxicam (2 mg/kg, intramuscularly (IM)), beginning on one of the five specific gestation days indicated above. Final group sizes varied from 5–7, as was described above for anaesthetic treatment. The maximum number of doses for each agent was in keeping with the usual recommended number of postsurgical doses for that agent.^{7,8} Groups of mice at each time point received buprenorphine twice a day for two days, and an additional two groups, at E4 and E6, received buprenorphine twice a day, for one day after the two-day regimen at these times was known to adversely affect fetal growth. Separate groups at each time point received meloxicam, once a day for two days, and an additional group, at E0, received meloxicam once after the two-day regimen at this time was known to adversely affect fetal growth.

Seven untreated timed pregnant mice served as controls for all treatment groups. The total number of female mice used for these experiments was 203.

Fetal growth and morphological analyses

At E18, the dams were administered an overdose of ketamine/xylazine (200/20 mg/kg, respectively, IP), the fetuses and placentas harvested, and the dams euthanized by bilateral thoracotomy. Fetuses and placentas from all litters were then weighed. Euthanasia was completed within approximately 2 min and would not have affected growth parameters of the fetal mice. Detailed morphological analysis was conducted on the fetuses from three litters from each experimental group (total number of fetuses examined per group ranged from 17–23). Fetuses were prepared either for skeletal analysis by alizarin red staining⁹ or for general morphological analysis after fixation in Bouin's fluid using Wilson's methodology.¹⁰ The humerus, ulna, femur, and tibia were measured and compared among treatment groups and controls. Microphthalmia was diagnosed by gross examination (under a dissecting microscope at 5x magnification) of free-hand sections of the head through transverse planes.

Statistical analyses

All groups were compared with the untreated control group. Comparisons of fetal and placental weights among groups were made using an analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test. All tests were two-tailed, with $P < 0.05$

considered statistically significant. Linear regression was conducted to compare fetal weights with bone lengths. A chi-square analysis was performed on microphthalmia incidence data. Data are reported as mean $\pm$ SEM.

Results

Fetal and placental growth following anaesthetic administration

The effect of anaesthesia administered at specific times during pregnancy on fetal and placental growth is shown in Figure 1.

Ketamine/xylazine.—Fetal growth was reduced significantly by ketamine/xylazine treatment at E0, E4, E12, and E15 ($P < 0.01$). Only at E6 was there no significant effect of ketamine/xylazine on fetal growth. Placental growth was not significantly affected by ketamine/xylazine on any gestation day.

Isoflurane.—Isoflurane reduced fetal growth when administered at E0 and E6 ($P < 0.01$) but not on other gestation days, representing a significant advantage over ketamine/xylazine. Placental growth was not significantly affected by isoflurane on any gestation day.

Tribromoethanol.—Tribromoethanol did not adversely affect fetal growth on most gestation days tested, significantly reducing fetal growth only when administered at E6 or E12 ($P < 0.01$). Placental growth was not significantly affected by tribromoethanol on any gestation day.

Fetal and placental growth following analgesic administration

The effect of analgesia administered at specific times during pregnancy on fetal and placental growth is shown in Figure 2.

Buprenorphine.—Fetal growth was significantly reduced by a two-day buprenorphine treatment beginning at E4 and E6 ($P < 0.01$). Limiting the treatment to one day did not alleviate the impact of this analgesic on fetal growth. Placental growth was not significantly affected by buprenorphine treatment on any gestation day.

Meloxicam.—Fetal growth was significantly reduced by a two-day meloxicam treatment only at E0 ($P < 0.01$). Limiting the treatment to one day with this analgesic did not improve fetal weights. Placental growth was not significantly affected by meloxicam treatment on any gestation day.

Skeletal analysis

Anaesthetic and analgesic effects on the limb bones are shown in Figure 3. The humerus was the only bone of the four measured that was significantly different from controls in virtually every treatment group, both with anaesthesia and with analgesia (not significant only for ketamine/xylazine at E6; $P < 0.05$ for ketamine/xylazine at E0; $P < 0.01$ for all others). The ulna, femur, and tibia were all of a similar respective length among the mice in each of the treatment groups.

Linear regression (Figure 4) of the anaesthetic data showed that fetal weights and humerus lengths were significantly correlated for ketamine/xylazine ($P < 0.0001$) and tribromoethanol ($P < 0.01$) treatment, but not for isoflurane. The same analysis of the analgesic data showed that fetal weights and humerus lengths were significantly correlated for meloxicam ($P < 0.01$), but not for buprenorphine treatment.

Litter size

Litter size averaged 7.6 ± 0.1 and did not differ significantly among the groups; nor did it correlate with the fetal or placental weights recorded for any of the groups.

Microphthalmia

Morphology appeared quite normal among the fetuses in all of the treatment groups, with the exception of one trait that was remarkably affected. The incidence of microphthalmia was increased in response to buprenorphine treatment at E6. Microphthalmia was present more often in the right eye but occasionally bilaterally. The incidence of microphthalmia in control mice was 4.3%. Its occurrence was 5.9% and 5.3% in the ketamine-xylazine and isoflurane-treated animals, respectively. It was not observed in other groups; but in the E6 buprenorphine-treated fetuses it had an incidence of 31.6% ($P < 0.05$). The incidence of microphthalmia was not related to fetal gender.

Discussion

We have shown that specific anaesthetic and analgesic agents have a negative impact on fetal growth and development at discrete times during pregnancy. The study reported herein was designed to test these agents at times approximating fertilization (E0), attachment (E4), beginning of organogenesis (E6), end of organogenesis (E12), and the logarithmic growth phase (E15). In this way we have provided a valuable resource of information not only for our own studies of fetal growth restriction but also for the larger scientific community conducting research that necessitates anaesthetics and analgesics at various stages of pregnancy in mice.

We studied three anaesthetics that are used commonly in mice, and that act by different mechanisms. Ketamine and xylazine were used in combination, as is the case in animal surgeries. Ketamine blocks activation of N-methyl-D-aspartate receptors¹¹ and muscarinic receptors¹² while xylazine activates α_2 -adrenoceptors,^{13,14} the combined effect producing deep anaesthesia. Tribromoethanol acts by a mechanism that remains unclear. Isoflurane binds to gamma-aminobutyric acid, glutamate, and glycine receptors and inhibits potassium channels.¹⁵

We also studied two commonly-used analgesics in mice. Buprenorphine is a long-acting, partial μ opioid receptor agonist.¹⁶ Meloxicam is a non-steroidal anti-inflammatory drug that inhibits cyclooxygenases and is preferential for cyclooxygenase-2.^{17,18}

Based on our data, the anaesthetics of choice appear to be tribromoethanol at fertilization, isoflurane or tribromoethanol during attachment, ketamine/xylazine at the beginning of organogenesis but isoflurane at the end of organogenesis, and isoflurane or tribromoethanol

during growth phase. In preliminary experiments (data not shown), ketamine and xylazine were administered individually to pregnant mice at E15 to test their impact on fetal growth and it was determined that xylazine was the cause of the fetal growth restriction. Ketamine alone had no adverse effect when administered at that time at gestation. Since these agents cannot be used independently for anaesthesia, they were used only in combination in the study reported herein. Isoflurane anaesthesia was maintained for a length of time (30 min) equivalent to that achieved by ketamine/xylazine or tribromoethanol, therefore the time during which the mice were deprived of food and water while under anaesthesia was similar for the three treatments. This avoided any confounding influence of differences in food and water deprivation among treatments. It should be noted that although isoflurane is usually considered a safe anaesthetic, it has been shown by our results to be detrimental to fetal growth if used at E0 and at E6. Some of the effects of anaesthesia can linger throughout gestation, as is shown by the reduced fetal growth in response to either ketamine/xylazine or isoflurane administered at E0. The mechanism for this long-lasting effect is unknown but it would appear to be an effect on maternal tissues and not the fetal or placental tissue since the timing is well before attachment and placental formation.

Previous work has compared the use of ketamine/xylazine and tribromoethanol for embryo transfer and transgenic mouse production.^{19,20} In part, these studies were conducted due to the continuing controversy over the use of tribromoethanol as an anaesthetic agent.^{6,21} These studies clearly demonstrate that anaesthetic agents can affect implantation, resorption and birth rates but the investigators only evaluated the use of anaesthetics during embryo transfer and did not consider their effect on fetal growth. Furthermore, these studies also included other variables (postoperative temperature variation and multiple transgene constructs in the transferred embryos) that confound the interpretation of the results.

Despite the controversy over the use of tribromoethanol, because it induces acute peritoneal inflammation, fibrinous serositis and necrotizing myositis when administered intraperitoneally in some strains of mice,^{6,20} this agent is still widely used because of its safety (i.e. low anaesthetic mortalities), because it is not a controlled substance, and because it requires no special equipment for administration. It is noteworthy that tribromoethanol has been shown to increase mouse uterine vascular permeability, at least in the non-pregnant uterus,²² and might be expected to be advantageous for maintaining strong oxygen-carbon dioxide and nutrient exchange with resultant normal fetal growth. Our data provide evidence that mouse fetal growth is negatively impacted by tribromoethanol when administered maternally at E6 or E12, the approximate timing of the duration of organogenesis. Whether or not oxygen-carbon dioxide and nutrient exchange are enhanced by the use of tribromoethanol, our study provides evidence that any such benefit it gives on these particular gestation days is outweighed by its adverse effects on fetal growth, and indicates that this agent has a high potential for impacting on study results when used during organogenesis.

Our data also reveal patterns of safe and detrimental analgesic use. The analgesics of choice for various stages of pregnancy in the mouse appear to be buprenorphine fertilization, end of organogenesis, and during growth phase; and meloxicam throughout pregnancy except during the time surrounding fertilization. Again, as indicated above for anaesthesia, the

effects of some agents are long-lasting, as demonstrated by fetal growth restriction at E18 in response to meloxicam treatment at E0. Consistent with our late pregnancy data showing no fetal growth restriction in response to buprenorphine administered either at E12 or E15, buprenorphine uptake by the placenta and fetus has been shown to be low at E13 (immediately following organogenesis) and E18 in mice.²³ Uptake on earlier gestation days was not investigated. Currently there are no opioid analgesics that are without risk of birth defects and that could be considered as alternatives during developmental times when buprenorphine is contraindicated.²⁴ Meloxicam has not been specifically tested for its effects on fetal development. Other nonsteroidal anti-inflammatory drugs have been tested in long-term repeated dosing regimens in mid to late pregnancy in both rats^{25–28} and rabbits.²⁹ With repeated doses of piroxicam over 10 times higher (based on milligrams of drug/body mass) than the meloxicam analgesic doses used in our study, fetal growth restriction,²⁵ decreased bone mineralization,²⁶ and skeletal variations²⁷ were observed. Lower doses of piroxicam actually increased bone mineralization in rat fetuses,²⁸ demonstrating a profound dose effect for this compound. The only period during which we found meloxicam at analgesic doses to be detrimental to mouse fetal growth was during fertilization, and this period has not previously been tested.

One-day and two-day regimens of analgesia had not been compared in mice, therefore they were investigated in these experiments. There was no advantage to fetal growth when an analgesic was used for only one day instead of two, indicating that whatever detrimental effects were caused by an analgesic were already established by a short exposure to the agent. This result differed from our previous observations in the rat in which multiple doses of buprenorphine at E14 caused fetal growth restriction but a single dose did not (unpublished observations).

Often growth-restricted neonates will experience accelerated growth postnatally. However, postnatal accelerated growth, or ‘catch-up’ growth, has been shown to be a major trigger of abnormal metabolic physiology during adulthood; it shortens the lifespan in mouse models of low birth weight and is associated with impaired cognitive performance.³⁰

The impact of anaesthesia or analgesia on overall fetal growth and specific bone growth may be through different mechanisms on different tissue types. Linear regression demonstrated a correlation between fetal growth and bone growth with only some of the treatment agents. Even though there was a consistent negative effect on bone growth in virtually every treatment group, inconsistent correlation between this parameter and fetal growth suggests that differential mechanisms are operative in different tissues throughout the fetus.

Morphological analysis, to determine the incidence of any teratogenic effects, revealed a high occurrence of microphthalmia in fetuses treated at E6 with buprenorphine. Eye development in the mouse begins at approximately E9-E10 and continues throughout gestation.³¹ C57BL/6 mice normally have a 1–9% incidence of microphthalmia.³² As was observed in the current study, microphthalmia is more commonly observed in the right eye. Our observation that fetuses treated at E6 with buprenorphine had a 32% incidence of microphthalmia is worthy of further investigation. Since this occurs well before the typical embryonic day when ocular development is known to begin, the reason for this action of

buprenorphine is unknown. A precise sensitivity of the forebrain where the optic vesicles will form is possible, but no specific sensitivity of this region of the embryonic brain in response to buprenorphine has been described.

The placenta contains receptors on which these anaesthetics and analgesics act; therefore these drugs could target the placenta. Our data, however, have shown no significant impact on placental growth of any of the anaesthetics and analgesics used. The only placental weights that appeared to approach significant reduction were in response to tribromoethanol at E4, E15 and meloxicam at E15, but the fetal weights at these stages with these agents were normal, giving no indication of reduced placental efficiency. We cannot rule out changes in placental efficiency which may occur apart from the observation of weight changes, as possible explanations of the observed fetal growth restriction. Other mechanisms, placental, uterine, or fetal, not reflected by placental weight changes, could be responsible for the observed fetal growth restriction even when placental growth remained normal.

The results reported for this study show the impact of anaesthetics and analgesics on fetal growth and skeletal development. By conducting a robust test of these agents at different gestational times, we have identified not only the compounds that may be harmful but also the specific stages of development at which various agents may cause harm. These results provide a foundation for the design of future studies to determine the molecular mechanisms by which common anaesthetics and analgesics may act to produce the pathophysiological outcomes we have observed. Haemodynamic data were not collected in this study and are unknown for these animals. It is conceivable that hypotension, hypoperfusion, or hypoxia during the time the mice were anaesthetized may have had a role in the outcomes reported herein, although the short time during pregnancy (once and for less than an hour) when these could have been factors minimizes concern. The longterm postnatal effects of these agents will also be an important area for future investigation as we seek to understand the impact of effective pain relief on experimental outcomes. More precise experimental design of the anaesthetic and analgesic regimens in experimental protocols should add significant refinement to the overall quality of experimental outcomes coming from investigations of fetal and neonatal development.

Acknowledgements

The American College of Laboratory Animal Medicine Foundation and Abbott Laboratories are gratefully acknowledged for their financial support.

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Figure 1.

Fetal weights (a) and placental weights (b) at term (E18) from pregnant mice treated with anaesthesia on the indicated gestation day. C=control group (untreated); data are presented as mean $\pm$ SEM; ** $P < 0.01$ compared with control mice, by analysis of variance (ANOVA).

Figure 2.

Fetal weights (a & b) and placental weights (c & d) at term (E18) from pregnant mice treated with analgesia on the indicated gestation day. Both two-day treatment (a & c) and one-day treatment (b & d) with these analgesics produced the same results. C=control group (untreated); data are presented as mean $\pm$ SEM; ** $P < 0.01$ compared with control mice, by analysis of variance (ANOVA).

Figure 3.

Skeletal analysis of fetuses from dams treated with the indicated anaesthetics (a) or analgesics (b) on different days of gestation. The values in the chart represent length measurements of the humerus. The data indicate that the growth of this bone was sensitive to treatment with anaesthesia or analgesia throughout gestation. Other major limb bones did not differ in length among the treatment groups. C=control group (untreated); data are presented as mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$ compared with control mice, by analysis of variance (ANOVA).

Figure 4.

Linear regression analysis of fetal weights and humerus lengths for mice treated with anaesthetics (a) or analgesics (b). The correlation coefficients and level of statistical significance for each treatment are indicated on each chart.

Table 1.

Anaesthetic and analgesic recommendations for mouse pregnancy.

	Gestation day and phase				
	E0	E4	E6	E12	E15
	Fertilization	Attachment	Begin organogenesis	End organogenesis	Growth phase
Anaesthetics					
Ketamine/xylazine	×	×	✓	×	×
Isoflurane	×	✓	×	✓	✓
Tribromoethanol	✓	✓	×	×	✓
Analgesics					
Buprenorphine	✓	×	×	✓	✓
Meloxicam	×	✓	✓	✓	✓

✓: acceptable; ×: not recommended