

Teratogen Update: Methylene Blue[†]

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Methylene blue (MB) is a cationic thiazine dye with the chemical name tetramethylthionine chloride. It has a characteristic deep blue color in the oxidized state, but the reduced form, leukomethylene blue (LMB), is colorless. Methylene blue has been widely used in a variety of clinical settings to identify anatomic (Manhes et al., '94; Sills and Zinkham, '94; Barber et al., '95) and pathologic (Derom et al., '93; Canto et al., '96; Lee and Sharifi, '96) structures and to treat methemoglobinemia (Curry, '82; Ellis et al., '95; Yang et al., '95). It has also been used to inactivate viruses in fresh frozen plasma (Wieding et al., '93), to treat chronic periodontitis (Gibson et al., '94; Ower et al., '95), to aid in the diagnosis of and targeted therapy for cancer (Orth et al., '95; Link et al., '96), and, experimentally, to treat septic shock (Schneider et al., '92; Preiser et al., '95; Brown et al., '96; Driscoll et al., '96). Intraamniotic injection of MB during obstetric procedures has been associated with serious adverse effects among newborns (Table 1). More recently, teratogenic effects have been reported following the injection of MB during midtrimester amniocentesis (Table 2). This article summarizes what is known about the biological actions of MB, the results of its use in obstetrics, and the evidence for its teratogenicity.

MECHANISMS OF ACTION

At low concentrations in the body, MB and LMB exist in equilibrium and form a reversible oxidation-reduction system, which is the basis for MB's function as an electron donor/acceptor and free radical scavenger. As such, MB has the potential to affect a wide variety of physiologic reactions. Its summary effect on red blood cells is to reduce the heme from methemoglobin to hemoglobin. Paradoxically, at higher doses, this equilibrium is destroyed and an excess of methemoglobin is produced (Curry, '82). The formation of hydrogen peroxide as a by-product can lead to oxidation of the red-cell membranes, denaturation of hemoglobin, hemolytic anemia, and Heinz body formation (Kirsch and Cohen, '80; Salaris et al., '91).

More recent research has focused on the vasoactive properties of MB. In response to agents such as acetylcholine and bradykinin, the vascular endothelium produces nitric oxide (NO) through the action of nitric oxide synthase. NO activates soluble guanylate cyclase, which raises levels of cyclic guanosine monophosphate (cGMP), opening calcium-sensitive potassium channels

and producing membrane hyperpolarization, smooth muscle relaxation, and vasodilatation (Katsuki et al., '77; Moncada et al., '91; Hampl et al., '96). Methylene blue inhibits this NO-mediated vasodilatation in vitro in a variety of tissues, resulting in vascular constriction, although the exact mechanism by which it exerts this effect remains unclear. Early evidence indicated that MB prevents the activation of guanylate cyclase (Katsuki et al., '77; Gruetter et al., '79), but more recent findings suggest it may inhibit the action of NO synthase (Mayer et al., '93; Luo et al., '95) or inactivate NO directly, possibly through the generation of superoxide anions (Brüne et al., '90; Wolin et al., '90; Marczin et al., '92). However, the high oxygen tensions present under experimental conditions in vitro may promote the generation of these superoxide anions. Whether MB exhibits the same effects in vivo under physiologically low oxygen tensions and whether the doses of MB used in vitro can be administered in vivo without intolerable side effects remain unclear (Marshall et al., '88; Archer et al., '90; Fineman et al., '91; Young et al., '94).

A NO-cGMP system also exists in the uterus of rats; the stimulation of this system by progesterone relaxes uterine smooth muscle. It is speculated that elevated progesterone levels help maintain uterine relaxation during pregnancy through this mechanism and that MB may inhibit this uterine relaxation (Yallampalli et al., '94).

In addition, the vascular endothelium also releases prostacyclin, which activates adenylate cyclase and produces vasodilatation. Methylene blue has been shown to inhibit prostacyclin production and thus may also inhibit vasodilatation independent of the NO-cGMP pathway (Martin et al., '89). Methylene blue has also been shown to release noradrenaline from sympathetic nerves in vitro and to inhibit its metabolism (Soares-Da-Silva and Caramona, '88). In contrast, other in vitro experiments indicate that MB may directly activate calcium-dependent potassium channels, which is thought to produce vasodilation (Stockand and Sansom, '96).

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TABLE 1. Adverse effects in newborns associated with methylene blue use

Reference	Hyperbilirubinemia	Hemolytic anemia	Heinz body formation	Skin staining	Methemoglobinemia	Respiratory distress	Skin photosensitization, desquamation
Plunkett, '73	+	+					
Cowett et al., '76	+	+		+		+	
Serota et al., '79	+	+		+		+	
Spahr et al., '80	+	+	+	+	+	+	
Crooks, '82	+	+		+		+	
McEnerney and McEnerney, '83	+	+			+	+	
Vincer et al., '87	+	+	+	+		+	
Troche, '89	+			+		+	
Fish and Chazen, '92	+	+		+		+	
Porat et al., '96	+	+		+	+	+	+

OBSTETRIC USES AND ADVERSE EFFECTS AMONG NEWBORNS

Atlay and Sutherst ('70) first described the intraamniotic injection of dye to detect premature rupture of the fetal membranes, the determination of which was helpful in deciding whether to induce labor. They injected dye into the amniotic sac and observed the vaginal canal for drainage of colored fluid. Young et al. ('74) first described the injection of dye during genetic amniocentesis to ensure sampling of both fetal sacs in diamniotic twin pregnancies. They inserted a needle into the amniotic sac of one twin, withdrew fluid for karyotype determination, injected a dye, and removed the needle. They then made a separate attempt to sample the second amniotic sac. If they withdrew colored fluid at this second attempt, they assumed they had probably reentered the first sac. If they withdrew clear fluid on the second attempt, they were reasonably sure they had entered the second sac independently.

Evans blue was the first dye used in both of these procedures, but other dyes were subsequently employed. Not long after the first intraamniotic injection of MB close to the time of delivery, case reports of associated adverse effects among newborns appeared in the literature (Table 1). Most frequently reported were hyperbilirubinemia, hemolytic anemia with and without Heinz bodies, blue staining of the skin, and methemoglobinemia. While respiratory distress was also observed in many of these infants, it was in some cases mild and may have resulted from factors other than the injection of MB.

EVIDENCE FOR TERATOGENICITY

The published reports from the English-language literature that provide evidence for the teratogenicity of MB are summarized in Table 2. The first suspicion that MB use during midtrimester amniocentesis might be teratogenic was prompted by a report from Amsterdam (Moorman-Voestermans et al., '90). In a 29-month period from March 1986–September 1988, 12 neonates with jejunal atresia were treated in two hospitals there, the same number that had been treated over the previous 9 years. Eleven (92%) of the 12 infants were

one of a pair of dizygotic twins, and 10 of the 12 mothers were age 35 years or older. Although maternal age of 35 years or older is the most common indication for amniocentesis, initial review of birth records indicated that only 4 of the mothers had undergone this procedure.

At the time, no explanation for the association of jejunal atresia, dizygotic twinning, and advanced maternal age was apparent. However, later that year, Nicolini and Monni ('90) reported on a series of 7 twin infants with multiple ileal atresias treated at three centers, one in Italy and two in Great Britain. In each instance, MB had been injected during amniocentesis at 15–17 weeks. Of the 4 affected twins born at the center in Italy, 3 were one of a pair of unlike sex twins, making it possible to verify that the sac injected with MB belonged to the twin who was born with atresia.

The following year, Lancaster and Pedisich ('91) reported on a population-based cohort of 21 twin infants with jejunal and/or ileal atresia born in Australia between 1982–1989. Seventeen (81%) of these infants were born in New South Wales, 13 (76%) to mothers 35 years or older. The risk for intestinal atresia among twin infants of mothers 35 years or older was 26.74 (95% confidence interval (CI), 8.73–81.92) times that among twin infants of mothers less than 35 years old. Subsequent investigation revealed that midtrimester amniocentesis had been performed in 15 (88%) of the 17 pregnancies from New South Wales, and that MB had been injected in at least 12 of these procedures (Lancaster et al., '92).

The report by Nicolini and Monni ('90) led to closer examination of the records from Amsterdam, which revealed that, in reality, 10 of the 12 mothers of infants with jejunal atresia had undergone amniocentesis at a single prenatal center (Moorman-Voestermans et al., '92). This prompted examination of additional data from Amsterdam, the results of which provide the strongest evidence for the teratogenicity of MB (Van der Pol et al., '92). Between January 1, 1985–April 1, 1990, midtrimester amniocentesis was performed in 89 twin pregnancies at an outpatient prenatal center in Amsterdam. Methylene blue was injected during 86 of these procedures; no dye was injected during the other three.

TABLE 2. Reports of teratogenicity associated with methylene blue use

References	Moorman-Voestermans et al., '90; Van der Pol et al., '92		Nicolini and Monni, '90	Lancaster and Pedisich, '91; Lancaster et al., '92		Glüer, '95	
Study type	Retrospective cohort of twin pregnancies undergoing amniocentesis		Case series of affected twin infants	Population-based retrospective cohort of affected infants		Case series of affected twin infants	
Study period	January 1, 1985–April 1, 1990		1982–1990	1982–1989		1991–1994	
Type of intestinal atresia Exposure associated with intestinal atresia	Jejunal Twinning Amniocentesis with MB		Multiple ileal atresias Twinning Amniocentesis with MB	Jejunal and/or ileal Twinning Maternal age ≥ 35 Amniocentesis		Jejunal Twinning Maternal age ≥ 35 Amniocentesis with MB	
Location	Netherlands	Amsterdam	Italy and one center in Great Britain	Italy Alone	Australia	New South Wales	Hanover, Germany
Number of affected infants (rate per 10,000 live births)	67 (0.72)	17	7	4	176 (0.9)		4
Number of affected infants who were twins (%)	20 (30%)	17 (100%)	7 (100%)	4 (100%)	21 (12%)	17	4 (100%)
Number of affected twin infants with mother ≥ 35 (%)						13 (76%)	4 (100%)
Number of affected twin infants whose mothers had amniocentesis (%)	19 (95%)	17 (100%)	7 (100%)	4 (100%)		15 (88%)	4 (100%)
Attack rate of intestinal atresia among twin infants whose mothers had amniocentesis (%)		17/178 (9.6%)		4/42 (9.5%)			
Number of affected twin infants whose mothers had amniocentesis with MB injection (%)	18 (90%)	17 (100%)	7 (100%)	4 (100%)		12 (71%)	3 (75%)*
Number of affected twin infants whose sac was known to be injected with MB (%)		15 (88%)		3 (75%)			3 (75%)*

*The fourth affected infant was known to have received injection of indigo carmine during amniocentesis.

Of the resulting 178 twin infants, 17 (9.6%) had jejunal atresia. Each of these 17 infants was from a different pregnancy. For 15 of the 17 affected pregnancies, it was documented through sex determination and ultrasound records that the sac injected with MB belonged to the twin born with jejunal atresia; for the remaining two pregnancies, identification of the injected sac was not possible.

Despite this evidence, reports of intestinal atresia with MB use have continued to appear. Glüer ('95) described a series of 4 twin infants with small bowel atresia treated at a single hospital in Hannover, Germany, in a 3-year period from 1991–1994. Midtrimester amniocentesis was performed in all four affected pregnancies, with MB injected in three and indigo carmine dye in one. In all four instances, the sac that was

injected with dye belonged to the twin born with intestinal atresia.

THEORIES ON THE ETIOLOGY OF SMALL INTESTINAL ATRESIA

There are two theories as to the etiology of small intestinal atresia (White et al., '71; Guttman et al., '75). The first proposes that atresia or stenosis is a primary defect in intestinal development caused by failure of the bowel lumen to fully recanalize after its normal solid stage of development in the second month of gestation. This is thought to occur primarily in the duodenum. The second theory proposes that the blood supply to one or more parts of the normally developed bowel becomes

secondarily compromised, producing ischemia and/or necrosis. Atresia or stenosis results when the damaged area heals. This is thought to occur more commonly in the jejunum and ileum, presumably at any time during fetal life. This type of vascular disruption has been verified in animal experiments in which interruption of the fetal mesenteric blood supply in utero resulted in intestinal atresia (Louw and Barnard, '55; Abrams, '68; Koga et al., '75).

CRITERIA FOR CAUSALITY

Criteria for establishing the causal relationship between an exposure and an outcome on the basis of epidemiologic evidence were first proposed in 1964 by the Surgeon General's Advisory Committee on Smoking and Health (Hamill, '97) and later expanded and refined by Sir Austin Bradford Hill (Hill, '65). The criteria for causation fulfilled by the data on intraamniotic exposure to MB and jejuno-ileal atresia summarized in the reports in Table 2 are presented below.

Strength of the association

Only the population-based data from New South Wales, Australia, provide an appropriate comparison group for the quantitative assessment of the risk of twin amniocentesis. As noted earlier, Lancaster and Pedisich ('91) estimated the prevalence of jejuno-ileal atresia among twin infants of mothers age 35 years or older to be 26.74 (95% CI, 8.73–81.92) times higher than that among twin infants of mothers less than 35 years old. If maternal age of 35 years or greater is a reasonably accurate indicator of amniocentesis, this provides an estimate of the relative risk for intestinal atresia among twins resulting from amniocentesis.

In the Amsterdam data, 9.6% of the twin infants whose mothers underwent amniocentesis had jejunal atresia. While data from a comparison group were not presented, the general prevalence of jejuno-ileal atresia among twin infants in other populations has been estimated at 5 per 10,000 liveborn twin infants (Lancaster and Pedisich, '91; Cragan et al., '94). Comparison of the Amsterdam prevalence of 9.6% with this general prevalence indicates that the relative risk for jejunal atresia among twins whose mothers underwent amniocentesis may be as high as 191 (95% CI, 71.3–512.0).

In all four reports, the frequency of MB use during amniocentesis among mothers of affected twin infants ranged from 71–100%. In three of the reports, the authors documented (through sex determination and ultrasound records) that, in 75–88% of the cases, the twin whose sac had been injected with MB was the twin born with intestinal atresia. Compared with an expected value of 50% if there were no association, this represents an MB exposure rate 1.5 (95% CI, 0.5–4.7)–1.8 (95% CI, 1.1–3.3) times higher among the twins with intestinal atresia than among those without. For the remaining infants in each report, it was not possible to determine which twin's sac had been injected.

Specificity and consistency of the association

Each of the four reports implying an association between MB injection and jejuno-ileal atresia in twins was from an independent source and, in each, MB was the only dye frequently associated with intestinal atresia. A single affected infant in the data from the Netherlands (Van der Pol et al., '92) and one in the data from Hannover (Glüer, '95) were exposed to indigo carmine dye instead. The association with MB could be an artifact if MB injection during twin amniocentesis were a widespread practice and some other factor related to amniocentesis in twins resulted in intestinal atresia. However, the lack of an association between jejuno-ileal atresia and intraamniotic dye use reported from regions where dyes other than MB are commonly used argues against this (Dolk, '91; Cragan et al., '93).

No other structural defects besides jejuno-ileal atresia were mentioned in the four reports. Because evaluation of the Australian data was intentionally focused on intestinal atresia, the presence or absence of associated malformations cannot be assessed. However, data from the other three reports were apparently obtained through record review, and one would expect these reports to have noted additional defects if they were present. Of particular note is the fact that no other defects thought to result from vascular disruption were reported, indicating a very specific association between MB use and intestinal atresia.

Biological plausibility and coherence

Evidence of the vasoactive properties of MB in vitro suggests that exposure to the dye, possibly through systemic absorption by the fetus, might induce arterial constriction that impairs the blood supply to the small bowel, causing ischemic damage. Methylene blue has been shown to block the vasorelaxation effect of NO in the mesenteric vessels of dogs, monkeys, and rats in vitro (Okamura et al., '90). In addition, human umbilical and placental vessels release an endothelial-derived relaxing factor, presumably NO, in vitro, the vasodilatory effects of which can be inhibited by MB (Van de Voorde et al., '87; Myatt et al., '91; Chaudhuri et al., '91). If MB produces similar effects in vivo, such vasoconstrictions might impair a particularly vulnerable part of the mesenteric circulation supplying the jejunum and ileum. These effects would be consistent with the theory that jejuno-ileal atresia is caused by vascular disruption and with the observation that duodenal atresia, thought to have a nonvascular etiology, has not been reported with MB use. However, if jejuno-ileal atresia results from systemic absorption of MB by the fetus, one would expect that defects of other organs that are thought to result from vascular disruption would also be observed. To date, these have not been reported.

It is also reasonable to infer that the methemoglobinemia and hemolytic anemia observed in newborn infants after intraamniotic injection of MB prior to delivery may also occur in fetuses after midtrimester

injection. The resulting hypoxia with associated shunting of the fetal blood supply away from the small bowel could also lead to localized ischemia and intestinal atresia. In addition, MB may also have direct toxic effects on the bowel itself. Amniotic fluid is normally swallowed by the fetus, and thus dye contained in that fluid may come into contact with the bowel mucosa. Injection of MB into lesions in the colon during endoscopy has resulted in localized vascular necrosis, mucosal ulceration, mural necrosis, and extramural fat necrosis at the site of injection (Lane et al., '96). Direct exposure of the bowel to MB after intraamniotic injection might produce similar damage. However, such lesions would reasonably be expected throughout both the large and small intestines and not be confined to the jejunum and ileum.

Temporal relationship of the association

If intraamniotic injection of MB causes jejuno-ileal atresia, development of the fetal bowel prior to the time of injection would be expected to be normal. None of the reports mention whether the fetal bowel was evaluated by ultrasound before midtrimester amniocentesis was performed. However, it is clear that in 16 of the 17 affected twins from Amsterdam (Van der Pol et al., '92) and in 3 of the 4 affected twins from Hannover (Glüer, '95), the intestinal atresia was first diagnosed by prenatal ultrasound after amniocentesis was performed. In the other two twins, the intestinal atresia was first diagnosed after delivery. The timing of diagnosis was not mentioned in the other reports.

None of the reports specified the time period over which MB was used. However, the data from Amsterdam and from Hannover indicate clustering of the cases in time. Moorman-Voestermans et al. ('90) reported 11 twin infants with jejunal atresia born from March 1986–September 1988, but reported none from 1978–1986. Similarly, Glüer ('95) reported 4 twin infants with small bowel atresia from 1991–1994 but only 2 from 1977–1991. This implies that the increased frequency of intestinal atresia in twins may have resulted from an exposure not previously present, such as the use of MB in twin amniocentesis. Dye use during amniocentesis was discontinued in April 1990 in Amsterdam (Van der Pol et al., '92) and in 1988 in New South Wales, Australia (Lancaster et al., '92). Publication of subsequent data from these areas on the prevalence of intestinal atresia among twins for the years since the use of dye was stopped might be informative.

Dose-response (biological gradient)

Although the concentration of MB used at amniocentesis was cited in each of the studies, the actual amount injected was incompletely reported, and thus calculation of the dosage was not possible. However, because different concentrations of MB were used in different practices in Australia (Lancaster et al., '92), evidence of a dose-response effect can be inferred from these data. Twin infants of mothers injected with undiluted 1% MB at amniocentesis

were 3.97 (95% CI, 1.15–13.74) times more likely to be born with intestinal atresia than were twin infants of mothers injected with 0.25% MB (2.5% diluted 1 in 10).

FETAL DEATH

Further assessment of the data from Australia, prompted by anecdotal reports, has revealed a possible association between MB use in twin amniocentesis and fetal death (Kidd et al., '96). Between January 1, 1980–December 31, 1991, 303 women with a twin pregnancy in New South Wales underwent midtrimester amniocentesis. Because of incomplete documentation in the medical records, dye use in these procedures was classified according to physicians' reports of their usual practice during the study period. Among the 245 women with a twin pregnancy who did not undergo elective termination, those in whom MB was injected at amniocentesis had significantly higher odds of fetal death in one of the twins (odds ratio (OR), 8.52; 95% CI, 2.28–31.9) than did those who had amniocentesis without MB injection. Stratified by dye concentration, the odds of fetal death were 4.63 (95% CI, 0.93–23.13) times higher among mothers in whom 0.125–0.25% MB was injected, and 14.98 (95% CI, 3.40–66.08) times higher among mothers in whom 1.0% MB was injected than among mothers in whom MB was not used.

Because neither the actual cause of fetal death nor the time interval between amniocentesis and fetal demise could be determined from the available data, the reported association between MB use and fetal death cannot be considered specific. However, it is unlikely that confounding or misclassification could fully explain an increased risk of this magnitude. Although it seems plausible that constriction of the umbilical vessels or severe hemolytic anemia with methemoglobinemia following injection of MB could lead to generalized fetal hypoxia and death, verification of this association in other data with information about MB exposure is needed. For appropriate comparisons to be made, the methods used to ascertain pregnancy outcomes and the gestational age range of fetal deaths included should be similar across studies.

CONCLUSIONS

The epidemiologic evidence for the teratogenicity of MB is quite strong. Methylene blue should not be used during midtrimester amniocentesis. Warnings about the risks of its use should be included on package inserts and widely disseminated among healthcare providers who perform amniocentesis.

Alternative methods for distinguishing the sacs of twins during amniocentesis have been described. Since 1976, Beekhuis et al. ('92) have injected a hemolysate of maternal blood instead of dye in 63 procedures. Jeanty et al. ('90) have had success using ultrasound to identify the interamniotic membrane followed by a single needle insertion to sample both sacs. Chitayat et al. ('95) reported that spectrophotometric differences in the

optical densities of the fluids obtained may provide reassurance that both sacs have been sampled independently. The use of these or other techniques that do not require injection of dye should become standard practice.

Because severe adverse effects from the intraamniotic injection of MB to detect ruptured membranes prior to delivery continue to be reported (Porat et al., '96), the use of MB in these procedures should also be discontinued. Alternative dyes should be made readily available for use in circumstances where dye injection is felt to be necessary. For example, indigo carmine has been widely used in amniocentesis (Elias et al., '80; Goldstein and Stills, '83; Tabsh, '90) with few, if any, reports of neonatal problems in the literature. However, indigo carmine is structurally similar to serotonin and appears to inhibit endothelium-dependent vasorelaxation in vitro through interference with NO (Chang et al., '96). Its intravenous injection in adults has produced hypertension (Erickson and Widmer, '68; Kennedy et al., '68; Ng et al., '76; Jeffords et al., '77; Flewelling, '80), and its potential effects on the fetus have not been fully evaluated. There are a few reports in the literature of the use of Evans blue in amniocentesis (Atlay and Sutherst, '70; Young et al., '74; Wolf et al., '79; Tabsh, '90) and in the estimation of blood volume in newborns (Jegier et al., '64) without adverse effects. Although the biological effects of Evans blue have not been fully investigated, preliminary evidence indicates that this dye does not inhibit vasodilatation in vitro (Shoemaker et al., '96). It might represent a safer alternative for the detection of premature rupture of the fetal membranes prior to delivery.

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