Toxicological and histological analyses for a stillborn delivered by a mother under methadone maintenance therapy

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Abstract

Purpose Based on autopsy findings and toxicological results, it was ascertained whether chronic fetal exposure to methadone (MTD) could have caused intrauterine death of a fetus born to a mother under MTD maintenance therapy.

Methods Complete fetal autopsy, placental examination and toxicological and histological analyses were performed on a 39-week stillborn fetus, whose mother had regularly taken MTD during the pregnancy. The MTD and its main metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) were quantified in fetal tissues (brain, liver and kidney) by ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). A fully validated toxicological analytical method was developed.

Results High levels of MTD and EDDP were present in all samples. The EDDP/MTD ratios were similar in the liver and kidney, but in the brain MTD was predominant. Placental histology showed a thrombotic, partially organised lesion in the first branches of the umbilical vein (chorionic venous vessels) and delayed villous maturation (DVM) was diffusely found in the parenchyma.

Conclusions High levels of MTD and EDDP correlate with chronic fetal exposure to MTD maintenance therapy in pregnancy. Placenta DVM can be associated with the MTD exposure, indirectly affecting glycaemic balance. Fetal cause of death seemed to be related to a global hypoxic-ischemic status as a combination of chronic fetal MTD intoxication, placental DVM and impaired placenta blood flow due to chorionic thrombosis.

Keywords Methadone, EDDP, Human fetus, Placenta, Stillborn, Histopathology

Introduction

Methadone (MTD) is currently the treatment of choice for pregnant opiate-dependent women even though buprenorphine has been recently considered a less harmful maintenance therapy. Buprenorphine resulted in fewer adverse events as follows: a lower incidence and better prognosis of neonatal abstinence syndrome (NAS) [1, 2], a longer gestation period with a consequent higher birth weight [3, 4] and a reduced risk of preterm birth [4]. However, MTD is commonly prescribed, because it is safe, and no increase in the rate of fetal deaths in MTD-maintained pregnancies has been observed [5, 6]. Only one case of miscarriage was recorded in a retrospective case series involving 101 pregnant opiate-dependent women in MTD withdrawal [7].

MTD crosses the placental barrier and can cause adverse effects, the most common ones are premature delivery, being small for gestational age, head circumference below the percentile expected and NAS; although these adverse effects develop in 60-90% of opiate exposed infants [8], no death in uterus has been reported so far. Thus, studies have focused on the toxicokinetics and toxicodynamics of MTD, particularly in fetuses/placentas and breastfeeding children.

A few cases of acute MTD intoxication in neonates or child deaths following intoxication due to the accidental consumption of MTD have been reported [9-11]. These cases provide information regarding MTD metabolism [12-14]; however, to our knowledge no reports are available on MTD-related fetal death in the uterus. Here, we describe a case of intrauterine death in a woman under MTD maintenance therapy.

Case report

A stillborn female fetus was delivered at 39 weeks of gestation by a 28-year-old mother under MTD maintenance therapy at a dosage of 80 mg/day. According to her clinical history, the pregnancy was uneventful until delivery. MTD treatment was stopped fifteen days before the fetal delivery. The mother was admitted to hospital, because fetal movements had been absent for a few hours. The ultrasonography demonstrated the intrauterine death of the fetus; the delivery was pharmacologically induced, and a stillborn female fetus was delivered vaginally. The fetus underwent a complete autopsy, and the placenta and its adnexal tissue were analysed accordingly. Due to the maternal clinical history, toxicological analyses were performed on the fetal tissues (i.e., the brain, kidney and liver). The biological fetal fluids (i.e., blood and urine) were almost absent.

Materials and methods

Chemicals and reagents

The standards for the target analytes and their deuterated internal standards (ISs) were supplied as methanolic solutions (1.0 mg/mL) by Sigma-Aldrich (Milano, Italy). All solvents and chemicals used for the sample preparation were of analytical grade (Carlo Erba, Milano, Italy). Individual stock solutions were

prepared by diluting with methanol. The combined working solutions were prepared by diluting the individual stock solutions with common working standards.

Sample preparation

Brain, liver and kidney specimens were collected during the post mortem examination for the toxicological analyses. All samples were stored at -20°C until use. The samples (0.1 g) were extracted using a previously published procedure after minor modifications [15]. All specimens were added with 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)-d_3 and MTD-d_3 (1000 ng/g final concentration), 1 mL of a mixture water/methanol 5:1 (v/v) and homogenized with a ball mill (two grinding cycles of 50s each, with 30s of pause, at 6000 rpm, using 6 steel balls in a sample holder of 2 mL). After centrifugation (14,000 rpm, 10 min), 50 µL are transferred to a new vial and finally a 1-µL aliquot was injected into the ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) system. As blank tissue specimens were collected from the bovine organs.

UHPLC-MS/MS method

Analyses of MTD and EDDP in the brain, liver and kidney were performed by injecting 1 µL of processed tissue sample into a Shimadzu Nexera 30 UHPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (Sciex, Darmstadt, Germany) with an electrospray ionization (ESI) in the positive-ion mode. For chromatographic separation, an Acquity UPLC 1 BEH C18 column (100 × 2.1 mm i.d. particle size × 1.7 µm (Waters, Sesto San Giovanni, Italy)), was used. The column oven was maintained at +45°C. Elution solvents were water/5 mM formic acid (solvent A) and acetonitrile/5 mM formic acid (solvent B). The linear gradient concentration of elution solvents (A:B; v/v) was 90:10 for 0.5 min and then to 30:70 at 4.0 min hold for 0.50 min, followed by isocratic elution for 2.0 min. The flow rate was set at 0.5 mL/min and the total run time was 6.5 min. Data were recorded at unit mass resolution in the selected reaction monitoring (MRM) mode, using nitrogen as the collision gas.

Calibration and validation parameters

The following validation parameters were evaluated for the UHPLC-MS/MS analysis according to recommendations in the literature: specificity, linearity, limit of quantification, limit of detection, accuracy, precision, recovery and matrix effect [16].

The calibration curve and quality control samples were prepared by adding appropriate volumes of working standard solution mixtures to 0.1 g of blank tissue obtained from bovine organs. To evaluate the specificity, five different aliquots of brain/liver/kidney samples were analysed to identify peaks that might interfere with the detection of the analytes. The specificity was found to be satisfactory when the chromatograms were free of coeluting peaks as follows: the signal-to-noise (S/N) ratio of the interfering peaks was less than 10. Calibration curves were constructed by plotting the peak area ratios of the selected ion species (for the analytes and ISs) versus the analyte concentration in a concentration range of 100-5000 ng/g for each

analyte. Accuracy and precision were calculated by analysing five separate spiked samples of the brain, liver and kidney at two different concentration levels (a low concentration of 100 ng/g and a high concentration of 5000 ng/g). The bias was calculated for each concentration using the following formula: [(grand mean of calculated concentration$_x$ – nominal concentration$_x$) / nominal concentration$_x$] × 100 [16].

Regarding the precision studies, the within-run precision and between-run precision coefficients of variation (CV%) were determined by analysing five replicates at two concentrations (low and high) and considered acceptable if resulted lower than 20%. Limits of detection (LODs) were estimated as the analyte concentration, the response of which provided a signal-to-noise (S/N) ratio of 3. The S/N ratio at the lowest concentration was used to extrapolate the theoretical LOD. The quantification limits were calculated as three times the LOD (Table 1). Whenever the effective drug concentration exceeded the calibration range, the samples were diluted to fit the quantitation interval considered in the curve. The dilution integrity was evaluated by spiking each matrix with the analytes at a concentration 1.5 times the highest calibration point and diluting the samples twice and ten times. These samples were analyzed along the standard calibration curve, and the accuracy was considered satisfactory within the interval ±20% around the expected value. The extraction recoveries and matrix effects were calculated by comparing the experimental results from two sets of solutions at the concentration of 1000 ng/g. The analytes' recovery was calculated by the ratio of the analyte tissue concentration determined after its extraction (first set) to that determined on the spiked extract (second set).

The matrix effect was calculated as the percentage ratio of the analyte chromatographic peak area detected from the second set (tissue samples spiked after the extraction step) to that detected from the third set (spike after the extraction step on blank deionized water). The percentage difference highlighted matrix suppression (values below 100%) or enhancement (values above 100%).

Toxicological results

With regard to validation results, our data are presented in Table 1. A linear response was observed over the entire range of concentrations with a coefficient of determination (r^2) greater than 0.999 for both MTD and EDDP. At each concentration of both analytes, accuracy and precision showed satisfactory results (< 20%). The detection limits using the employed method in the liver, kidney and brain for MTD were 5, 3 and 1 ng/g respectively, while for EDDP they were 5, 5 and 6 ng/g respectively. In the dilution integrity studies, the precision was less than 10%, and the accuracy bias was within the range of 10% for all analytes. The matrix effects showed less than 5% of enhancement or suppression. These results revealed that authentic samples with concentrations higher than the highest calibrator could be diluted and reanalyzed.

The toxicological examination revealed the presence of MTD and its metabolite EDDP as reported in Table 2. The highest concentrations of MTD and EDDP were found in the kidney. The EDDP/MTD ratio was 0.08 in the brain, 2.60 in the liver and 2.09 in the kidney. A postmortem examination of the blood and urine was not performed, because these materials were unavailable.

External examination, autopsy, histology and their results

The autopsy showed a 39-week-old female fetus with a weight of 2350 g and a body length of 46 cm. The head circumference was 30 cm. Overall, the anthropometric parameters and organ weights were below the expected range for the gestational age, except for the spleen (11.8 g) and brain (428 g). In particular, the liver weight was 83.5 g (expected for gestational age 124 g) (Table 3), and the brain/liver ratio was 5.13 (cut off > 3 indicates a fetus/placenta pathological condition [17]). The fetal measurements and corresponding centiles are summarized in Table 3 (expected values of different organs at the full-term delivery are based on Archie et al. [18]).

External examination revealed purple lip discoloration consistent with cyanosis and conjunctival petechiae. Maceration was scarce with epidemolyisis involving less than 10% of the skin surface. No dysmorphic features or other anomalies were observed.

Internal examination showed several pleural and pericardial petechiae. Brain was edematous with pale cortex and dusky white matter, a condition also known as "ribbon effect".

The histology revealed acute/subacute brain damage with diffuse edema, eosinophilic neurons, glial karyorrhexis, reactive ramified astrocytes, mild reactive subcortical gliosis and capillary proliferation with a plump endothelium (Fig. 1). Fetal tissues were overall well preserved with mild autolytic changes. On the whole, according to scarce evidence of external maceration and mild autolytic changes in fetal tissues, fetal death might have occurred no more than 24 h before delivery. No other significant findings were observed in various organs.

The placenta weighed 477 g (25-50th percentile) (Table 3) and the fetal/placental weight ratio was 4.93 (< 3rd percentile) [19]. Gross pathology showed enlarged chorionic vessels and suspected thrombosis in the first branches of the umbilical vein and in its distal ramifications (fetal side) (Fig. 2a). Focal and superficial haemorrhage was observed at maternal side (Fig. 2b). Umbilical cord presented three regular vessels (Fig. 2c). Placenta was serially cross sectioned and no infarcted lesions were observed.

The microscopic analysis of the umbilical vein and its branching at the side of the cross sections indicated in the Fig. 2a confirmed the presence of thrombotic lesions that was not occlusive and partially organized. In the umbilical vein, at the point of insertion into the chorionic plate, a small parietal thrombus was observed (Fig. 3).

A parietal thrombus involving half of the circumference was observed in the first branching of the umbilical vein. Another parietal thrombus not occluding the lumen and partially organized was identified in a more distal chorionic vessel (Fig. 4).

The histological features of the placenta were as follows: diffuse delayed villous maturation (DVM) with plump villi and an irregular contour, increased stroma, abundance of avascular villi, scarcity of terminal villi and a lack of vasculosyncytial membranes (Fig. 5a). Inside the villi, the fetal vessels were far from the intervillous space, causing long-distance contact between the fetal and maternal blood (Fig. 5b); multiple areas showed fetal thrombotic vasculopathy with haemorrhagic endovasculopathy (Fig. 5c), fibroblastic septation in the stem villi and avascular villi. The cause of avascular villi is to relate to the downstream

ischemic effect of the vascular thrombosis. A small number of nucleated erythrocytes in the chorionic villi and in the fetal tissues were also found. DVM diagnosis was formulated in accordance to Khong et al. criteria [20] that defines this lesion in at least 1/3 of the whole placenta thickness (Fig. 6), while the extent of avascular villi can be classified as intermediate (that is 3 or more foci of 5 to 10 terminal avascular villi) [20].

Discussion

To the best of our knowledge, the present study is the first analysis of the postmortem concentration of MTD in different tissues of stillborn fetal organs. Additionally, the analysis of the histological characteristics of the placenta allowed the consideration of the effect of MTD on this organ. While the toxicology of MTD has been studied in children [21-24] and adults [15, 25-27], no investigation has been performed in fetuses.

As compared with adults, neonates and children are at an increased risk of MTD accumulation for various physiological reasons, such as a different body composition, immature enteric absorption, immature detoxification enzymes and slow glomerular filtration.

The lower body fat in neonates allows the accumulation of MTD in other lipophilic tissues such as the liver and brain; immature detoxification enzymes slow the drug inactivation and the minimal rate of glomerular filtration reduces the drug excretion [12]. All these mechanisms are responsible for the accumulation of MTD and prolongation of its half-life in neonates/children with a consequently higher toxic effect.

The combination of these physiological aspects, which have been observed in neonates and children, can occur in the fetus. Nevertheless, data interpretation in the fetus remains uncertain [12, 28, 29]. In the fetus, glomerular filtration is immature and the amniotic environment is generally alkaline; both situations reduce MTD excretion. Similarly, the enteric filter is immature, implying an alteration of the MTD assimilation/excretion. The fetus liver detoxification capacity depends on both genetic factors and liver mass. Fetuses have different cytochrome P450 metabolizer enzymes, such as CYP3A7 and CYP4A1 instead of CYP3A4, CYP2B6 and CYP2D6, which are specific to adult detoxification activity [12]. Neonatal clearance is similar to that in adolescents due to the increased levels of CYP3A7 and CYP4A1; the levels of these latter CYPs progressively decrease [12, 30] and are eventually replaced by the adult detoxification system. However, knowledge regarding the CYPs in the fetus is incomplete, and their function depends on the liver mass.

Thus, in infants and children, the liver volume can provide information regarding the liver detoxification capacity, which alone can account for certain differences in the drug metabolism efficacy [31, 32]; in fetuses, the liver detoxification capacity depends even on the liver mass that must be evaluated in relation to its mass expected for the gestational age. If the liver mass is less than the expected value, the MTD detoxification is less efficient.

However, in the case of chronic MTD exposure, it is highly important to analyse not only the absolute liver mass but also fetal growth during pregnancy as follows; if the fetal growth is delayed, the MTD metabolism will be slower, and its accumulation will increase.

Mitchell [17] proposed the following two parameters for analysing fetal growth: the ponderal index and fetal brain/liver weight ratio. The brain/liver ratio, which is independent of the fetus weight [33], is the most representative parameter of asymmetrical fetal growth. Fetuses with asymmetrical growth appear disproportionate, and brain development is spared at the expense of other somatic tissues, which is typical of intrauterine growth retardation (IUGR). IUGR is generally caused by placental insufficiency due to many conditions (such as maternal drug exposure or smoking, maternal hypertension, multiple pregnancies, and maternal malnutrition). Mitchell postulated that IUGR occurs when the brain/liver ratio is greater than 3. In the present case, the brain/liver ratio was 5.1, and the expression of IUGR had an asymmetrical aspect. Thus, the constant MTD exposure along with the delayed fetal growth provoked a further reduction in the metabolizing capacity and further drug accumulation.

In the present report, high concentrations of MTD and EDDP were measured in all tissues collected (Table 2). The high MTD accumulation in the liver and kidney is linked to both the specific functions of these two organs (the liver is the site of the first enzymatic MTD inactivation, and the kidney plays a role in MTD elimination from the amniotic fluid) and their weight. This fetus liver and kidney were small as compared with the expected sizes (the liver weighed 83.5 g, while the expected liver weight was 124 g; the combined weight of the kidneys was 18 g, while the expected weight was 25 g), and their size reduction must have diminished their detoxification/excretion competence.

While it is possible to speculate regarding the high concentrations of EDDP and MTD in the liver and kidney, the precise reason underlying the inverted ratio in the brain (in favour of MTD) is unclear. Other authors have observed a lower concentration of EDDP in the brain of adults. Jantos and Skopp [34] reported data on EDDP and MTD in both the brain and cerebrospinal fluid, and the EDDP/MTD ratio was less than 1; the authors hypothesized that this low concentration was associated with a slow and difficult passage of EDDP through the blood brain barrier (BBB). Holm et al. [25] reported similar data on the EDDP/MTD ratio in the adult brain showing a rate below 1. The present case showed the same inverted ratio but in a fetus. Our results seem to support the conclusions of Jantos and Skopp; however, further studies on this topic are necessary to confirm this hypothesis in human fetus.

The second interesting finding of this case is related to the MTD effect on the placental tissue. Here, we documented a DVM associated with MTD exposure, which is a condition that had never been previously histologically documented. DVM diagnosis was performed in accordance with the criteria proposed by Khong et al. [20]. The most important genesis of DVM was reported to be related to both gestational and pre-gestational diabetes mellitus [35].

The relationship between MTD exposure and disorders of the placenta was recently investigated by Serra et al. [36] in a retrospective cohort study. In that study, the outcome of pregnant women in maintenance therapy with MTD or buprenorphine was compared with that of pregnant woman who were opiate-free. Women treated with MTD were significantly more likely to be diagnosed with DVM than MTD-free women [36], but the mechanism underlying the association between the altered placenta maturation and MTD exposure

was unclear. DVM is mainly caused by an altered glycaemic metabolism in the case of uncontrolled diabetes; MTD also can causes glycaemic disequilibrium, which might be linked with DVM.

MTD has been reported to have both hyperglycaemic and hypoglycaemic effects. A high dose of MTD can induce a hyperglycaemic effect which is likely due to resistant hyperglycaemia following insulin therapy, as reported by Tiras et al. [14]. Giugliano [37] reported an impairment of the insulin effect at the tissue level or an insufficient insulin response to the glucose level in heroin addicts. Bartlett et al. [38] speculated that MTD had an inhibitory effect on pancreatic β Langerhans cells. In both rats and dogs, MTD exposure caused a hyperglycaemic effect as follows; in rats, MTD mimicked insulin-resistant diabetes [39] and in dogs L-MTD and R-MTD induced hyperglycaemia at different concentrations [40].

In contrast, MTD can have a hypoglycaemic effect. In a case report by Maingi et al. [41], the blood glucose levels were stable while the patient was treated with intravenous fentanyl; however, the patient experienced episodes of hypoglycaemia while on MTD, which were resolved once fentanyl was resumed. Recently, Li and Chu [42] reported a case of refractory hypoglycaemia following a massive MTD overdose.

Consequently, in the present case report, the long exposure to MTD could have played an important role in altering the placental villous maturation, which generally occurs in diabetic women, likely because of the alteration in the glycaemic balance.

Another particular finding in the placenta examination was the presence of thrombosis in the umbilical vein and chorionic vessels. Increased pressure in small vessels was likely responsible for haemorrhagic endovasculopathy described in stem villi, which consists of erythrocyte extravasation through the vessel wall. Therefore, haemorrhagic endovasculopathy in small vessels is a consequence of endoluminal thrombosis occurred in larger upstream venous vessels. The latter lesion described is part of the spectrum of fetal thrombotic vasculopathy, which also involves other features such as fibroblastic vascular septation and avascular villi.

To the best of our knowledge, there is also no literature evidence about the correlation between thrombosis in placenta vessels and MTD exposure. However, Saldeen et al. [43], studying placentas in a large number of diabetic mothers, discovered an increased incidence of thrombosis in fetus-placental circulation. Similarly, in our specific case, MTD might have altered glucose metabolism favouring vascular thrombosis.

Most of hypothetic MTD role in modifying glucose balance and in inducing thrombosis in fetus-placental circulation is still unknown and requires further investigations.

Conclusions

This paper reports the first analysis of the postmortem concentrations of MTD in tissues (i.e., the brain, liver and kidney) from a stillborn baby delivered at 39 weeks of gestation by a mother under MTD maintenance therapy at 80 mg/day. The highest concentrations of MTD and EDDP were observed in the kidney; the lowest concentrations were revealed in the brain; the EDDP/MTD ratio was <1 in the brain (0.08) and > 1 in the liver (2.60) and kidney (2.09). Major accumulation was observed in the kidney probably because of its small size, because of its immature glomerular filtration, which might result in excretion reduction and because of alkaline phase in the amniotic liquid, which reduced MTD excretion. Accumulation was observed even in the liver, because of its small size and reduced detoxification capacity. The inverted EDDP/MTD ratio in the brain is likely associated with the difficulty of EDDP in passing through the BBB, which has been previously documented in adults.

This fetus was small for 39 weeks of gestational age in terms of head circumference, weight, crown heel length and had an asymmetrical growth (typical of IUGR). IUGR has played a role in MTD intoxication provoking a further reduction in the metabolizing capacity and a further drug accumulation. Small for the gestational age signs are characteristic of chronic MTD exposure. Placenta DVM was also documented in the present case, being probably associated with the MTD exposure.

Therefore, fetal cause of death should be related to a global hypoxic-ischemic status as a combination of chronic fetal MTD intoxication, placental DVM and impaired placenta blood flow due to chorionic thrombosis. Certain forensic signs are coherent with hypoxic status: petechiae signs observed in the mucosal layer of the lungs and heart; edema revealed in the lungs and brain; the “ribbon effect” in the brain (i.e., presence of hypoxic-ischaemic damage). The petechiae signs have been previously described by Misty et al. [23] in a deceased 3-month-old infant due to MTD intoxication. The mucosal petechiae and “ribbon effect” in the brain likely describe the same aspects in different organs due to the global hypoxic mechanism. In particular, the ribbon effect is a characteristic sign of hypoxic-ischemic encephalopathy. It is due to severe hypoxia affecting both cortex and white matter. Furthermore, nucleated erythrocytes, found in fetal tissue and in chorionic villi, are associated to hypoxia.

Compliance with ethical standards

Conflict of interest The authors have no financial or other relations that could lead to a conflict of interest.

Ethical approval In Italy, there is no legislation that forbids the use of human tissues collected during an autopsy subject to being deprived of individual details/other references just linked a code.

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