



Original Article

Abundant migration of midazolam and 1'-hydroxymidazolam into the fetal brain following midazolam administration to pregnant mice in the second trimester

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ABSTRACT — Midazolam is used in pregnancy post the second trimester for the treatment of convulsive seizures or as an anesthetic during cesarean section. However, its safety has not been validated. If the migration and accumulation of midazolam into the fetal brain can be clarified, the extent of damage to the fetal brain can be predicted. Therefore, we investigated the migration of midazolam and its active metabolite 1'-hydroxymidazolam into the fetal brain. Midazolam was administered intravenously to pregnant mice in the second trimester (E14.5), and the migration of midazolam and 1'-hydroxymidazolam to the maternal brain and plasma as well as the fetal brain were analyzed over time. Fetal brain levels of midazolam and 1'-hydroxymidazolam were high at 44.7% and 44.5% of that in the maternal brain. Furthermore, both mothers and fetuses expressed 1:1 brain ratios of midazolam and 1'-hydroxymidazolam. In both mothers and fetuses, migration of midazolam into the brain was higher than the migration of 1'-hydroxymidazolam. In this study, approximately half of midazolam and 1'-hydroxymidazolam in the maternal brain was transferred to the fetal brain. During the second trimester, neural stem cells in the fetal brain differentiate into neurons and glial cells and higher brain functions are developed. In the present study, we observed increased migration of midazolam and 1'-hydroxymidazolam into the fetal brain during this period. Therefore, damage to neuronal development may still be observed in the fetus even with post-second trimester midazolam use.

Key words: Midazolam, 1'-Hydroxymidazolam, Pharmacokinetics, Pregnancy, Fetal brain, Maternal brain

INTRODUCTION

Intake of midazolam and other benzodiazepines during the first trimester has been associated with high frequencies of congenital malformations (Wikner *et al.*,

2007). Consequently, benzodiazepine-based drugs are rarely administered during the first trimester. However, compared to the first trimester, there is a reduced risk of congenital malformations in the fetus when administered after the second trimester. Thus, although limit-

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ed, benzodiazepine-based drug use is still prevalent during this period. For example, it is used in the management of gestational hypertension-induced convulsive seizures (eclampsia) and as an anesthetic for cesarean sections (Danielak-Nowak *et al.*, 2016; Dodawad *et al.*, 2016; Esmoğlu *et al.*, 2009). However, the safety of the fetus during midazolam use in pregnant women during this period has not been entirely clarified. As midazolam is a drug that is easily transported into the brain, there is particular concern regarding damage to neural development of the fetus.

During the second trimester, neurons are formed from the repeated asymmetric division of neural stem cells. The fetal brain is in a constant state of hypoxia compared with the adult brain. Therefore, hypoxia-inducible factor (HIF)-1 α is highly expressed in the fetal brain. HIF-1 α promotes the differentiation of neural stem cells into astrocytes (Yasui *et al.*, 2017). Recent studies have shown the inhibition of HIF-1 α function in the fetal brain of mothers administered midazolam in the third trimester (Matsuyama *et al.*, 2015). This study suggests that post second trimester administration of midazolam may inhibit the function of HIF-1 α and affect fetal neurogenesis.

Thus far, the use of the benzodiazepine-based drugs, including midazolam, during pregnancy and their effect on the fetal brain due to migration and accumulation have not been studied. Therefore, in this study, the levels of migration and accumulation of midazolam in the fetal brain when administered to the mother during pregnancy were investigated. If these parameters are determined, fetal brain damage in cases of post second trimester administration to pregnant women could be inferred.

First, the migration and accumulation of midazolam in the brains of fetuses of pregnant mice administered midazolam was studied. Midazolam is rapidly metabolized in the maternal liver and converted into metabolite, 1'-hydroxymidazolam, which has half the activity of midazolam. Therefore, we also examined the migration of 1'-hydroxymidazolam into the brain. Next, we analyzed the route of migration and accumulation of midazolam and 1'-hydroxymidazolam in the fetal brain. Midazolam, which is migrated from the maternal to the fetal blood, repeatedly recirculates to the mother, almost without being metabolized. On the contrary, 1'-hydroxymidazolam may be excreted into the amniotic fluid not only through maternal and fetal circulation but also through fetal urine. Therefore, we focused on the amniotic fluid, the circulatory system of the fetus and analyzed the migration mechanism of midazolam and 1'-hydroxymidazolam to the fetal brain.

MATERIALS AND METHODS

Animal handling

Pregnancy ICR mice (gestational day 13.5) were purchased from Japan SLC, Inc. (Tokyo Laboratory Animals Science Co. Ltd., Tokyo, Japan). The mice were kept at room temperature ($24 \pm 1^\circ\text{C}$) and $55 \pm 5\%$ humidity with 12 hr of light (artificial illumination; 8:00-20:00). Food and water were available ad libitum. Each animal was used only once.

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Animal Care Committee of the Hoshi University (Tokyo, Japan). All procedures using animals were carried out according to protocols approved by the Animal Care Committee of the Hoshi University. Approval number; 29-119

Drug preparation and administration

A stock solution of 10 mg/mL midazolam was prepared by dissolution in Tween 80. Stock solution was diluted 50 times with physiological saline to prepare a midazolam solution (0.2 mg/mL). Mice were intravenously administered 100 μL of midazolam solution per 10 g body weight under isoflurane anesthesia.

Dissection

Following midazolam administration, at each period (10, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min), blood samples were collected from the inferior vena cava of the pregnant mice under isoflurane anesthesia. Next, uterine blood vessels were cauterized with an electrosurgical knife to prevent the attachment of the maternal blood to fetus. Amniotic fluid was collected from the uterus with the use of a 26G 1/2 needle and syringe. When the fetus was removed from the uterus, the umbilical cord was cauterized with an electrosurgical knife.

The fetus was dissected under a stereoscopic microscope to extirpate the brain. The chest of the mother was then surgically opened, and followed by perfusion with phosphate-buffered saline (PBS) through the heart to flush residual blood from the tissues. Following this, the maternal brain was extirpated. All samples were stored at -80°C until use.

LC-MS sample preparation

The fetus and maternal brain were homogenized with 4 mL of 2% phosphoric acid. And fetal brain was homogenized with 1 mL of 2% phosphoric acid. The resulting serum, amniotic fluid and homogenates were purified by the solid-phase extraction (SPE) columns Oasis® HLB

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PRiME 3 cc (60 mg) (Waters, Milford, MA, USA).

LC-MS analysis

LC-MS analysis was performed using a combination of an LC-20A high-performance liquid chromatography (HPLC) system (SIMADZU, Kyoto, Japan) and LCMS-2010 (SIMADZU). The analytical column that was used was an XBridge C18 (Waters, Milford, MA, USA). The analysis was performed at a column temperature of 40°C. Ultrapure water containing 0.1% formic acid was used as mobile phase A. Acetonitrile containing 0.1% formic acid was used as mobile phase B. The analysis was performed in binary gradient mode, as shown below. (Gradient condition (% of B); 0.00 min 10%, 10.00 min 70%, 12.00 min 70%, 12.01 min 10%, 16.00 min 10%) The flow rate was constant at 0.2 mL/min.

For the interface, positive mode electrospray ionization (ESI) was used. The nebulizer gas flow rate was set at 1.5 L/min. The CDL and heat block temperatures were 250°C and 200°C, respectively. The voltage of the detector was set at 1.5 V. Measurement was performed by the selected ion monitoring (SIM) method based on an *m/z* value of 326 for midazolam, 342 for 1'-hydroxymida-

zolam and 4-hydroxymidazolam, 346 for 1'-hydroxymidazolam-d4 (Internal standard).

The LCMSsolution Ver.3.40 (SIMADZU) was used for HPLC system control and MS chromatogram analysis.

The MS chromatogram and retention time during analysis of the standard products of each drug are shown in Fig. 1 and Table 1.

Statistics

Experimental values are expressed as mean \pm standard deviation. Outliers were calculated using the Smirnov-Grubbs outliers test. Significant differences were calculated using the Student's *t*-test. The analytical software Numeric Analysis Program for Pharmacokinetics (NAPP) was used to calculate various pharmacokinetics parameters (Hisaka and Sugiyama, 1998).

RESULTS

Time course changes and pharmacokinetic analysis of midazolam and 1'-hydroxymidazolam in maternal and fetal brains

A sedative state was induced in mothers on gestation-

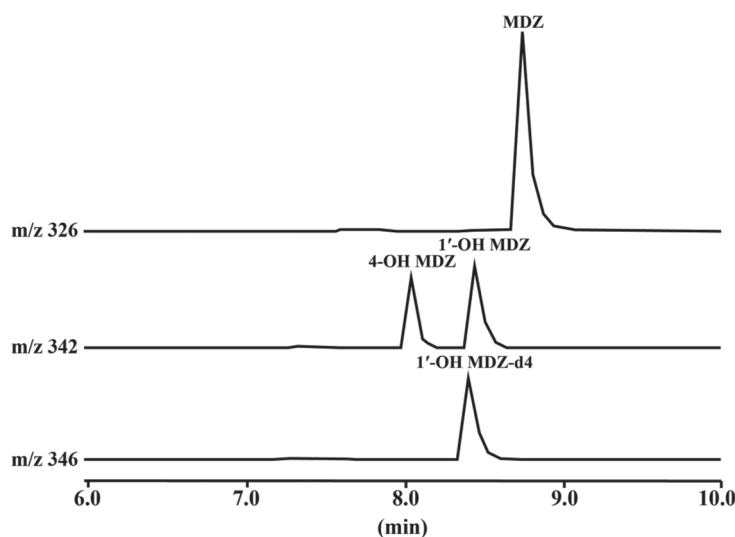


Fig. 1. MS chromatogram of each standard product. Midazolam, 1'-hydroxymidazolam, 4-hydroxymidazolam, and 1'-hydroxymidazolam-d4 were dissolved in the mobile phase to obtain concentrations of 100 ng/mL each, and 5 μ L was injected.

Table 1. Retention time of each standard substance.

Retention time (min)	Compound			
	Midazolam	1'-Hydroxymidazolam	4-Hydroxymidazolam	1'-Hydroxymidazolam-d4
	8.734	8.467	8.067	8.400

al day 14.5. Five minutes following the administration of midazolam (2.0 mg/kg) into the tail vein, the sedative state was induced for a 20 min period. After administration of midazolam, maternal and fetal brains were extirpated, and the concentrations of midazolam and 1'-hydroxymidazolam were measured. Maternal brain midazolam concentrations declined sharply and were below the detection limit 180 min following administration. In the case of 1'-hydroxymidazolam, however, detectable concentrations were observed in the maternal brain 10 min post-administration, followed by a trend similar to that of midazolam (Fig. 2A).

Conversely, in the fetal brain, maximal midazolam concentrations were observed 15 min after administration and were below detection limits after 120 min. Alternatively, maximal fetal brain 1'-hydroxymidazolam concentrations were observed 15 min after administration, and in contrast to midazolam were still detectable in the brain

even after 300 min (Fig. 2B).

The pharmacokinetic parameters (area under the curve (AUC)_{0-300 min}, AUC_{inf}, AUC_{inf} ratio (1'-hydroxymidazolam / midazolam), $t_{1/2}$) of midazolam and 1'-hydroxymidazolam in maternal and fetal brains are shown in Table 2. The abundance ratio (AUC_{inf} ratio) of 1'-hydroxymidazolam to midazolam in the brain was 1:1 in both mother and fetus. However, the abundance of midazolam and 1'-hydroxymidazolam in the fetal brain was 44.7% and 44.5%, respectively, of their abundance in the maternal brain. Thus, it was revealed that abundance of midazolam and 1'-hydroxymidazolam migrated into the fetal brain.

Time course of midazolam and 1'-hydroxymidazolam in maternal plasma

Maternal plasma concentrations of midazolam and 1'-hydroxymidazolam were quantitatively analyzed. Similar to maternal brain concentrations, there was a sharp

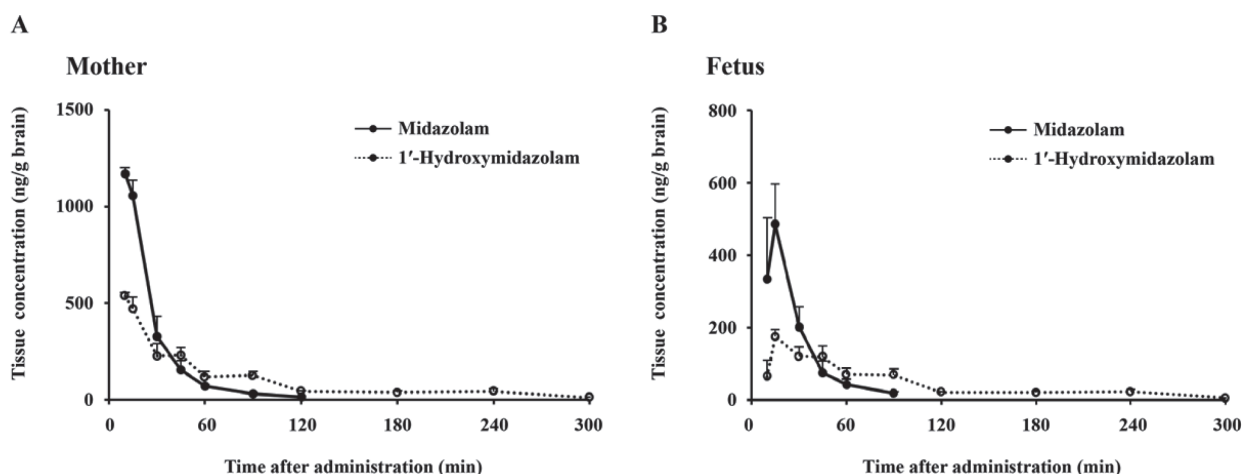


Fig. 2. Time course of midazolam and 1'-hydroxymidazolam in the maternal and fetal brain. Midazolam (2.0 mg/kg) was administered through the tail vein, and the concentrations of midazolam and 1'-hydroxymidazolam in maternal (A) and fetal (B) brain at each time point (10, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min) were measured by LC-MS. Changes in the brain midazolam and 1'-hydroxymidazolam concentrations are indicated by solid and dotted lines, respectively. The results are reported as median \pm standard deviation (Maternal brain: $n = 5$, Fetal brain: $n = 15$).

Table 2. Pharmacokinetic parameters of midazolam and 1'-hydroxymidazolam in maternal and fetal brain.

Sample	Compound	AUC ₀₋₃₀₀ (mg*min/g Tissue)	AUC _{inf} (mg*min/g Tissue)	AUC _{inf} ratio (1'-hydroxymidazolam / midazolam)	$t_{1/2}$ (min)
Maternal sample					
Brain	Midazolam	27697	28135	1.04	24.3
	1'-Hydroxymidazolam	27910	29287		69.5
Fetal sample					
Brain	Midazolam	12071	12573	1.04	21.3
	1'-Hydroxymidazolam	12531	13022		57.1

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decline in midazolam plasma concentrations immediately after administration (Fig. 3). In contrast to changes in the concentration of 1'-hydroxymidazolam in the maternal brain, plasma concentrations of 1'-hydroxymidazolam were the highest 15 min after administration, followed by a gradual decline. Comparison of plasma concentrations indicate higher midazolam concentrations until 15 min after administration, following which, 1'-hydroxymidazolam concentrations were higher (Fig. 3). However, 4-Hydroxymidazolam could not be detected in all samples.

The pharmacokinetic parameters ($AUC_{0-300 \text{ min}}$, AUC_{inf} , AUC_{inf} ratio (1'-hydroxymidazolam / midazolam), $t_{1/2}$) of midazolam and 1'-hydroxymidazolam in maternal plasma

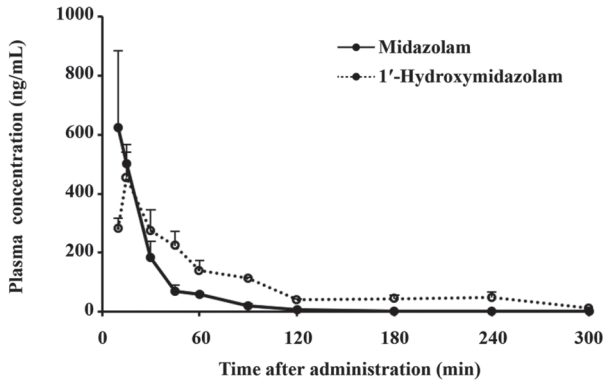


Fig. 3. Time course of midazolam and 1'-hydroxymidazolam in maternal plasma. Midazolam (2.0 mg/kg) was administered through the tail vein, and the concentrations of midazolam and 1'-hydroxymidazolam in maternal plasma at each time point (10, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min) were measured by LC-MS. Changes in maternal plasma midazolam and 1'-hydroxymidazolam concentrations are indicated by solid and dotted lines, respectively. The results are reported as median \pm standard deviation ($n = 5$).

are shown in the upper column of Table 3. The abundance of 1'-hydroxymidazolam in plasma was approximately twice that of midazolam, as the AUC_{inf} ratio was determined to be 1.92. However, comparisons of midazolam AUC_{inf} indicated that its AUC_{inf} in the maternal brain was 1.86 times higher than that in maternal plasma. In contrast, little difference was observed in the value of 1'-hydroxymidazolam AUC_{inf} between the maternal plasma and the maternal brain. Therefore, following maternal administration of midazolam, rapid metabolism results in the production of 1'-hydroxymidazolam, with a comparatively higher rate of migration between the maternal plasma and the maternal brain in case of midazolam. Additionally, fetal brain AUC_{inf} values for midazolam and 1'-hydroxymidazolam were 83.3% and 44.9%, respectively, of the AUC_{inf} values of these compounds in maternal plasma.

Time course of midazolam and 1'-hydroxymidazolam in the fetus

As we could not measure midazolam and 1'-hydroxymidazolam concentrations in fetal plasma, the concentrations of midazolam and 1'-hydroxymidazolam in the whole fetus was quantitatively analyzed. Fetal midazolam concentrations were the highest 15 min after midazolam administration to the mother, with a sharp decrease thereafter. Conversely, although 1'-hydroxymidazolam concentrations were the highest after 15 min, unlike midazolam, it was detectable in the fetus up to 300 min post administration (Fig. 4). As for 4-hydroxymidazolam, it could not be detected in the fetus.

The pharmacokinetic parameters ($AUC_{0-300 \text{ min}}$, AUC_{inf} , AUC_{inf} ratio (1'-hydroxymidazolam / midazolam), $t_{1/2}$) of midazolam and 1'-hydroxymidazolam are shown in the middle column of Table 3. The AUC_{inf} ratio of 1'-hydroxymidazolam to midazolam in the whole fetus was 2.46, which was higher than the AUC_{inf} ratio (1.92) in maternal plasma. Comparison of midazolam AUC_{inf} values indicat-

Table 3. Pharmacokinetic parameters of midazolam and 1'-hydroxymidazolam in maternal and fetal tissues.

Sample	Compound	AUC_{0-300} (mg*min/mL or g Tissue)	AUC_{inf} (mg*min/mL or g Tissue)	AUC_{inf} ratio (1'-hydroxymidazolam / midazolam)	$t_{1/2}$ (min)
Maternal sample					
Plasma	Midazolam	15071	15100	1.92	64.5
	1'-Hydroxymidazolam	27637	28973		62.4
Fetal sample					
Whole	Midazolam	7011	7033	2.46	40.0
	1'-Hydroxymidazolam	16697	17280		55.9
In Utero sample					
Amniotic fluid	Midazolam	846	865	2.65	21.1
	1'-Hydroxymidazolam	2171	2291		57.8

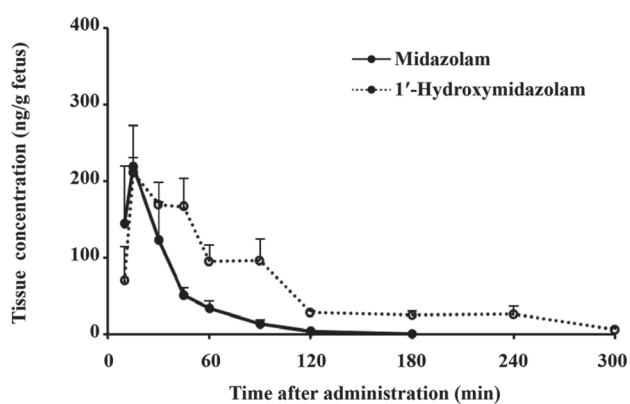


Fig. 4. Time course of midazolam and 1'-hydroxymidazolam in the fetus. Midazolam (2.0 mg/kg) was administered through the tail vein, and the concentrations of midazolam and 1'-hydroxymidazolam in the fetus at each time point (10, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min) were measured by LC-MS. Changes in the fetus midazolam and 1'-hydroxymidazolam concentrations are indicated by solid and dotted lines, respectively. The results are reported as median \pm standard deviation ($n = 15$).

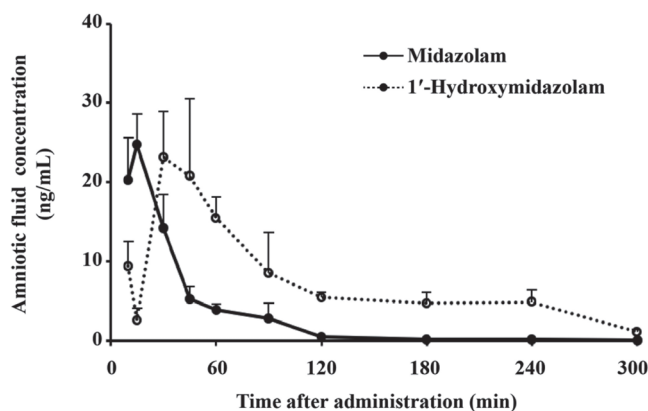


Fig. 5. Time course of midazolam and 1'-hydroxymidazolam in maternal amniotic fluid. Midazolam (2.0 mg/kg) was administered through the tail vein, and the concentrations of midazolam and 1'-hydroxymidazolam in maternal amniotic fluid at each time point (10, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min) were measured by LC-MS. Changes in maternal amniotic fluid midazolam and 1'-hydroxymidazolam concentrations are indicated by solid and dotted lines, respectively. The results are reported as median \pm standard deviation ($n = 5$).

ed that the fetal brain AUC_{inf} was 1.78 times higher than the whole fetus AUC_{inf} . This was similar to the relationship between the values of maternal plasma and maternal brain AUC_{inf} (1.86). Conversely, brain AUC_{inf} for 1'-hydroxymidazolam was 75.4% of that of the whole fetus. This value was smaller than the relationship of AUC_{inf} in maternal brain to AUC_{inf} in maternal plasma.

Time course of midazolam and 1'-hydroxymidazolam in maternal amniotic fluid

The AUC_{inf} ratio of midazolam and 1'-hydroxymidazolam in the maternal plasma and the AUC_{inf} ratio in the whole fetus were 1.92 and 2.46, respectively. Thus, we examined how this difference transpired. Consequently, we focused on the disappearance of midazolam and 1'-hydroxymidazolam from the fetus. In the mother, midazolam and 1'-hydroxymidazolam are excreted via urine with the latter reported to have a higher excretion rate (Heizmann *et al.*, 1983). When fetal excretion of midazolam and 1'-hydroxymidazolam occurs by the renal route, however, as opposed to the mother, the two compounds are migrated into the amniotic fluid. As the fetus ingests amniotic fluid, the drug is transferred back into the body of the fetus. This suggested that the fetal-amniotic fluid circulation is primarily responsible for the aforementioned difference. Therefore, the transfer of

midazolam and 1'-hydroxymidazolam to the amniotic fluid was analyzed.

In the amniotic fluid, maximal concentrations of midazolam were observed 15 min after administration followed by a rapid decrease. Conversely, 1'-hydroxymidazolam concentrations were maximal at 30 min after administration, followed by a gradual decrease (Fig. 5). There was a time lag of approximately 15 min for amniotic fluid transition of 1'-hydroxymidazolam compared to whole fetus transition of 1'-hydroxymidazolam.

The pharmacokinetic parameters ($AUC_{0-300\text{ min}}$, AUC_{inf} , AUC_{inf} ratio (1'-hydroxymidazolam / midazolam), $t_{1/2}$) of midazolam and 1'-hydroxymidazolam are shown in the lower column of Table 3. The AUC_{inf} ratio of 1'-hydroxymidazolam to midazolam in amniotic fluid was 2.65, which was higher than the AUC_{inf} ratios of both maternal plasma and the whole fetus. These results validated the amniotic fluid-fetal circulation of 1'-hydroxymidazolam. Moreover, this circulation was more extensive in the case of 1'-hydroxymidazolam than midazolam.

DISCUSSION

The use of midazolam during pregnancy is limited to its application during an eclamptic seizure or a cesarean section (Danielak-Nowak *et al.*, 2016; Dodawad *et al.*,

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2016; Esmoğlu *et al.*, 2009). Additionally, due to concerns regarding the safety of the fetus, its active use during pregnancy is not approved. Furthermore, studies on fetal pharmacokinetics are often focused on the permeability of the blood-placental barrier, and the fetal pharmacokinetics of midazolam has scarcely been investigated. We proposed that the study of fetal pharmacokinetics of midazolam would guide its appropriate usage during pregnancy. Therefore, this study especially focused on the brain, the site of action of midazolam.

The results of this study indicated an abundance ratio of midazolam and 1'-hydroxymidazolam in both maternal and fetal brains of approximately 1: 1 with little difference observed. Considering the above results and the AUC_{inf} of midazolam and 1'-hydroxymidazolam in maternal plasma, easier migration of midazolam into the brain compared to 1'-hydroxymidazolam was observed. However, fetal brain levels of midazolam and 1'-hydroxymidazolam were found to be approximately half that of the mother. This result may be attributed to the difference in permeability of the blood-placental barrier to midazolam and 1'-hydroxymidazolam. These results are consistent with previous studies that have reported 50–60% placental permeability to midazolam in humans (Bach *et al.*, 1989; Kanto *et al.*, 1983).

The AUC_{inf} ratio of 1'-hydroxymidazolam to midazolam in maternal plasma was 1.92. In contrast, the AUC_{inf} ratio of 1'-hydroxymidazolam to midazolam in the whole fetus was 2.46. Midazolam is rapidly metabolized in the maternal liver and converted into metabolite, 1'-hydroxymidazolam (Gandhi *et al.*, 2012; Johnson *et al.*, 2002; Reves *et al.*, 1985; Shi *et al.*, 2016; Williams *et al.*, 2002). We validated the metabolism of midazolam to 1'-hydroxymidazolam by cytochrome P450 (CYP) 3A in the fetal liver is negligible (Kitaoka *et al.*, 2018). Therefore, we investigated the reason for the higher abundance of 1'-hydroxymidazolam in the whole fetus compared to maternal plasma. The results demonstrated a higher fetal-amniotic fluid circulation of 1'-hydroxymidazolam compared to midazolam suggesting a reason for the high abundance ratio of 1'-hydroxymidazolam.

In conclusion, midazolam administered to the mother is rapidly metabolized and converted to 1'-hydroxymidazolam. The abundance ratio of 1'-hydroxymidazolam to midazolam in the brain was 1:1 in both mother and fetus. Furthermore, an abundance of half the amount of the two compounds in the maternal brain were observed in the fetal brain. As approximately half of midazolam and 1'-hydroxymidazolam found in the maternal brain is transferred to the fetal brain during the period of neuronal development, an effect on neuronal development can-

not be excluded. Consequently, adequate consideration is required even in cases of post second trimester use in pregnancy.

Additionally, it was also found that AUC_{inf} of fetal brain midazolam and 1'-hydroxymidazolam were 83.3% and 44.9%, respectively, of that found in the maternal plasma. This result suggests that fetal brain midazolam and 1'-hydroxymidazolam AUC_{inf} values can be predicted if maternal plasma AUC_{inf} is known. This area of research needs to be further explored.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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