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Transplacental Transfer of Nitrosodimethylamine in Perfused Human Placenta

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ABSTRACT

Nitrosodimethylamine (NDMA) is a carcinogenic compound present in tobacco smoke and food such as cured meat, smoked fish and beer. The O^6-methylguanine formed in human cord blood in mothers highly exposed to such products implicates NDMA exposure of the fetus. Dual recirculating human placental perfusion was used to get direct evidence of the transplacental transfer of NDMA and DNA adduct formation in perfused human placenta. Eleven placentas from normal full-term pregnancies were collected immediately after delivery and an isolated lobule was perfused with 1 or 5 μM of ^{14}C-NDMA with a reference substance, antipyrine (0.1 mg/ml) added to the maternal circulation. Perfusate samples were collected from both maternal and fetal circulations every half an hour for the first two hours and once per hour from thereon. NDMA was analyzed by scintillation counting and antipyrine by high performance liquid chromatography. The transfer of NDMA was comparable to that of antipyrine and probably occurred through passive diffusion, with the concentrations in maternal and fetal sides equilibrating in 2–3 h. No indication of any effect by efflux transporters on NDMA kinetics was noticed in the experiments utilizing Caco-2 or MDCK- MDCKII-MDR1 cell culture monolayer in a transwell system, either. Furthermore, no NDMA-DNA-adducts were found after the perfusions and no DNA-binding of NDMA was seen in *in vitro* incubations with human placental microsomes from 8 additional placentas. Thus, our study demonstrates that the human fetus can be exposed to NDMA from the maternal circulation. According to this study and the literature, NDMA is not metabolized in full-term human placenta from healthy non-smoking, non-drinking mothers. It remains to be studied whether NDMA concentrations high enough to evoke fetal toxicity can be obtained from dietary sources.

1. Introduction

Nitrosodimethylamine (NDMA) is a highly toxic and carcinogenic compound found in smoked and nitrite-treated Asian fish, cured meats, bacon, sausages, beer and cheese [1]. *N*-nitroso compounds, including NDMA, can also be formed in the stomach during digestion of alkylamine containing foods. It has been estimated that a substantial amount, or even the majority of human exposure to *N*-nitroso compounds can be attributable to *in vivo* formation (reviewed in [2,3]). Total human dietary intake of volatile nitrosamines, of which NDMA represents more than 90%, has been estimated to be 0.2–1.0 μg/day [2,4,5]. However, the exposure is as much as two times higher in heavy smokers or in people who eat large quantities of cured meats or drink beer. The elimination of NDMA has been studied only in animals. After i.v. injection, the mean elimination half-life is 73 min in beagles [6] and about 10 min in rats [7,8]. It has been estimated that the elimination of half-life in humans is less than one hour, especially at low doses [8,9]. The physicochemical properties of NDMA resemble those of antipyrine; NDMA is miscible in water and the log octanol–water partition coefficient (log K_{ow}) for NDMA is −0.57 [10]. Furthermore, as far as we are aware, there is no evidence of NDMA binding to serum proteins.

The ability of NDMA to evoke liver toxicity has been known for over 60 years [11] and in food NDMA was first detected in sodium nitrite preserved herring already over 40 years ago [12]. IARC [13] has classified NDMA as probably carcinogenic to humans (category 2a). It has been shown that after metabolic activation of NDMA [for a review, see [14]], tumors can be induced in several organs of all studied animal species [13] including transplacental tumors [15,16]. Recent epidemiological studies indicate that NDMA and other nitrosamines may induce brain tumors in

Abbreviations: NDMA, nitrosodimethylamine.

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children if the mother has consumed large quantities of cured meats during pregnancy [17–19]. Preston-Martin and co-workers [17] calculated an OR of about 2 for childhood brain tumors when the mother ingested more than 1.3 mg nitrate daily from cured meat. It is thus a real possibility that fetal exposure during pregnancy may evoke fetotoxicity including transplacental carcinogenesis [for reviews, see [20,21]]

NDMA can be activated into a reactive methyl diazonium ion by CYP2E1 [22] forming *N*7-methylguanine and O^6-methylguanine in DNA, adducts that are not specific for NDMA [23,24]. Georgiadis and co-workers [25] observed a strong correlation between O^6-methylguanine adducts in cord blood with those in maternal blood indicating transplacental transfer of methylating agents, with NDMA being the most probable agent. However, since these adducts can originate from any methylating compound [23,25], little is known of the actual transplacental transfer of NDMA in humans. The study by Chhabra and co-workers [26] where O^6-methylguanine was found in fetal and placental DNA after administering NDMA to pregnant patas monkeys points to transplacental transfer of NDMA in primates.

During the last decades, it has become clear that there is a need for studies on developmental toxicology and fetal exposure (for a review see [20,21]). The possibility to conduct *in vivo* human studies is limited by ethical restrictions, insufficient knowledge and appropriate methods for exposure. Furthermore, there are functional differences in the anatomy and physiology of the placenta between different species [27–29]. *Ex vivo* perfusion of human placental cotyledon retains placental structure and functions for hours [30] and has proven to be useful for the analysis of transplacental transfer of drugs and other chemicals [31–34]. In addition to pharmacokinetic or toxicokinetic data, one can collect information of toxicity to placental tissue by the perfused compounds. At present, apart from metals (e.g. mercury and cadmium [35,36]) only a few studies on the transfer of environmental carcinogens in human placental perfusion have been carried out: transfer of acrylamide [37,38], PhIP [39] and phthalates [40].

Since there is no direct proof of the actual transfer of NDMA through human placenta and only a single study of NDMA metabolism in only two placentas [41], we have used human placental perfusion to clarify these issues. In addition, it was also evaluated whether NDMA could be activated to DNA-binding metabolites and whether NDMA derived DNA-adducts during perfusion could be formed in placental tissue. Furthermore, as far as we are aware, nothing is known of the role of transporter proteins in NDMA toxicokinetics. Therefore, we compared transfer of NDMA to antipyrine which is a passively diffusible drug [42,43]. To complement the perfusion data, we studied whether NDMA transport was affected by transporters in a transwell model with polarized cell-culture expressing either P-gp or both BCRP and P-gp in the apical surface of the cells. P-gp and BCRP are the major xenobiotic transporters in human placenta [44–46]. This is the first paper where placental transfer kinetics of NDMA and NDMA derived DNA adduct formation as well as NDMA metabolism has been studied in perfused human placenta.

2. Materials and methods

2.1. Chemicals

$^{14}C_1$-NDMA (specific activity 54 mCi/mmol, purity > 98%) was purchased from Moravek Biochemicals, Brea, CA, USA. Cold liquid NDMA (purity > 99%), *N*7-methylguanine and antipyrine were obtained from Sigma–Aldrich (St Louis, MO, USA). Perfusion medium consisted of RPMI 1640 cell culture medium (Cambrex, Verviers, Belgium) with dextran (2 g/l, Sigma–Aldrich), albumin SPR (2 g/l, The Finnish Red Cross and Sanquin, Finland), heparin (25 IU/ml, Leo Pharma, Malmö, Sweden), sodium pyruvate (1 mM, Cambrex), Non-essential amino acid–solution (10 ml/l, Cambrex), Penicillin–Streptomycin (25 U/ml, Cambrex) and L-Glutamine (8 nM, Cambrex). All reagents were of analytical grade, and the solvents were of HPLC grade.

2.2. Human placentas and ethics

In Finland, human placenta is disposable after delivery. The use of the placenta for this study did not affect the delivery or the treatment of the mother and child in any way. The official Research Ethics Committee of the University Hospital District of Kuopio region approved the study protocol (11.5.2005). Only placentas from healthy, non-smoking (according to their own information), Caucasian mothers after uncomplicated full-term pregnancies from the Kuopio University Hospital were used. Mothers received oral and written information before the midwife asked them to sign the informed consent form. The placentas were anonymized so that there is no way of tracking down the persons. Altogether 21 placentas were obtained after a normal delivery or a caesarean section.

2.3. Human placental perfusion and analysis of NDMA

A dual recirculating perfusion model with separate maternal and fetal circulations, based on the original method developed by Schneider and co-workers [31] with minor modifications was used [37,47–49]. The placentas were obtained within 10 min after the delivery. Krebs–Ringer–phosphate-bicarbonate buffer with heparin (25 IU/ml) was injected in the hospital into the fetal umbilical cord. The solutions were injected extremely slowly via a small needle, to prevent pressure formation. Also, if there was any damage, this became visible as an increased leakage (over 3 ml/h) during the perfusion, and these perfusions were not included in the final data. A similar buffer solution was used after cannulation of fetal vein and artery to flush the placenta. In the perfusions, a cell culture medium based perfusion fluid was used (see Chemicals). The flow rate during the perfusions was 3 ml/min on the fetal side and 9 ml/min on the maternal side. The maternal perfusate was gassed with 95% O_2/5% CO_2 and fetal perfusate with 95% N_2/5% O_2. Physiological body temperature (37 °C) was maintained during the perfusion.

Criteria for a successful perfusion (leak < 3 ml/h; antipyrine kinetics; glucose consumption; stabile pressure during perfusion in fetal circulation) were fulfilled in 12/21 placentas and these were used to study transplacental transfer of NDMA and putative DNA adduct formation in the perfused placental tissue (Table 1). Regardless of the careful checking of the placentas for lacerations before accepting them for perfusions, 8/21 of the placentas failed after adding NDMA and 1/21 before the perfusion with NDMA. NDMA and 0.1 mg/ml of antipyrine were added into the maternal perfusate simultaneously. Altogether, six placentas were perfused with 1 μM of ^{14}C-NDMA and six with 5 μM of ^{14}C-NDMA (final specific activities were 12.5 and 2.5 mCi/mmol, respectively). The perfusion time was 4 h in 11 perfusions and 6 h in one perfusion with 5 μM NDMA. During the perfusions, samples (1 ml) from both the maternal and fetal reservoirs were taken every 30 min for the first 2 h and once per hour from thereon. For the ^{14}C-NDMA analysis, 900 μl of scintillation cocktail (Hi-Safe 3, PerkinElmer, Waltham, MA, USA) was added to 100 μl of the sample and radioactivity was measured by 1450 MicroBeta Trilux liquid scintillation and luminescence counter (Wallac, PerkinElmer). The rest of the perfusion sample (900 μl) was centrifuged at 12 000 *g* for 15 min and the supernatants stored in the freezer (−20 °C) for antipyrine analysis. Tissue pieces from placental tissue before and after perfusions were snap-frozen in liquid nitrogen and stored at −80 °C. To determine NDMA recovery, the total amount of radioactivity was calculated after perfusion including maternal and fetal perfusion medium and the perfused tissue. A total of 0.5 ml of solubilizer (Soluene®, PerkinElmer) was added to a homogenized 0.1 g piece of perfused lobule and incubated at 60 °C for 6 h before adding 1.8 ml of

Table 1
Perfusion conditions for nitrosodimethylamine (NDMA) perfusions.

NDMA perfusion	[C] (μM)	Perf. time (h)	Leak (ml/h)	pH ±SD	pO_2 (fetal) (kPa) ±SD	pO_2 (maternal) (kPa) ±SD	pCO_2 (kPa) ±SD	Gluc. cons. (mmol/l) ±SD
Successful[a] 1 μM ($n = 6$)	1	4	1–3	7.47 ± 0.03	19.8 ± 0.3	19.9 ± 0.3	2.8 ± 0.1	1.5 ± 1.4
Successful 5 μM ($n = 6$)	5	4, one 6	1–3	7.49 ± 0.04	20.0 ± 0.2	20.0 ± 0.2	2.8 ± 0.1	2.2 ± 1.8
Unsuccessful ($n = 9$)		mean 3	4–30	7.52 ± 0.08	20.1 ± 0.4	20.4 ± 0.4	2.7 ± 0.3	2.4 ± 2.8

[a] Leak from fetal to maternal side <3 ml/h, pressure in fetal circulation stable throughout the perfusion, glucose consumption measurable and antipyrine concentration in equilibration between maternal and fetal circulations within 2 h.

scintillation liquid (Hi-Safe 3, PerkinElmer) to 200 μl of the sample. The amount of ^{14}C-NDMA was measured by scintillation counting.

2.4. Analysis of antipyrine by HPLC

Antipyrine concentrations in perfusion medium were analyzed by an HPLC–UV method modified from Myllynen and co-workers [49]. Briefly, methanol (100 μl) was added to a 100 μl sample. Samples were centrifuged for 15 min at 12 000 *g*. The supernatant (150 μl) and acetonitrile (150 μl) were combined, and the samples were centrifuged again for 15 min at 12 000 *g*. A 10 μl aliquot of the supernatant was injected into the HPLC–UV system (Shimadzu 20A VP) with a reversed-phase column (Supelco C-14 2.5 × 250 mm; 5 μm). Isocratic elution with 20 mM KH_2PO_4 (70%) and acetonitrile (30%) at a flow rate of 1 ml/min was used and the UV detector was set at 255 nm. The column temperature was 40 °C. Calibration samples (5, 10, 20, 40, 80, 120 and 150 μg/ml of antipyrine in perfusion medium) and quality control samples (triplicates of 10, 40 and 150 of antipyrine in perfusion medium) were run on each day of analysis together with the perfusion samples. The method was linear and reproducible over the used concentration range of 5–150 μg/ml.

2.5. DNA-binding in perfused placental tissue

DNA from placental tissue perfused with ^{14}C-NDMA and from a piece of tissue taken from the same placenta before perfusion was isolated by the phenol–chloroform extraction modified from Gupta [50]. DNA concentration was measured by absorption at 260 nm and 1 mg samples were depurinated by heating at 100 °C for 30 min. Two nmol of 7-MeG were added as a UV-marker and samples were separated on a Luna C18(2) reversed-phase column (5 × 250 mm) (Phenomenex, Torrance, CA, USA). The flow rate was 0.7 ml/min and UV monitored at 248 nm. The HPLC system consisted of a model 116 gradient pump and a model 168 UV detector, all from Beckman Coulter (Fullerton, CA, USA). The linear gradient used started with 98% 50 mM ammonium formate and 2% methanol with the methanol level raised to 35% during 50 min. One min fractions were collected and mixed with 3 ml of scintillation cocktail (Ready Safe, Beckman Coulter) and radioactivity measured in an LC 6000 scintillation counter (Beckman Coulter). The fractions were counted for 40 min each, which gave a background of 30 ± 2 dpm. It was expected that 5–10 dpm (if eluting in one fraction) would be detectable. The detection limit was thus estimated to be about $1/10^8$ normal nucleotides.

Additionally, the radioactivity of purified placental DNA was analyzed by scintillation counting by adding scintillation cocktail directly into 200–4000 μg of DNA. DNA quenching was investigated by mixing radioactive NDMA separately with H_2O or up to 4000 μg of placental DNA. DNA extracted from placental tissue before the perfusion was used as a negative control.

2.6. In vitro *incubations for DNA-binding of NDMA*

Placental microsomes were prepared as described earlier [51]. Samples were collected and snap-frozen from eight human term placentas immediately after birth. Placental microsomes were prepared by homogenizing placental tissue (5–7 g) into four volumes of buffer (0.1 M Tris-HCl, 1 mM K_2–EDTA, pH 7.4). The homogenate was centrifuged (4 °C, 10 000 *g*, 30 min) and the supernatant was collected and centrifuged (4 °C, 100 000 *g*, 1 h). The microsomal pellet was homogenized into about 1000 μl of buffer solution and the suspension was stored at −80 °C. The protein concentration was measured by EL×800 UV (Bio-Tek Instruments, Winooski, VT, USA) and bovine serum albumin (Sigma–Aldrich) was used as a standard.

The metabolic activation of NDMA to methyl diazonium ion and its binding to DNA catalyzed by human placental microsomes was examined in *in vitro* incubations. The incubation medium (final volume 500 μl) consisted of 1 mg/ml of microsomal protein as a final concentration (added in 10 μl) with 500 μg of salmon testes DNA (Sigma–Aldrich) dissolved in 315 μl of 100 mM phosphate buffer pH 6.8, 25 μl of 100 mM $MgCl_2$, 10 μM of ^{14}C-NDMA (in 100 μl of phosphate buffer, specific activity 54 mCi/mmol) and 50 μl of 10 mM NADPH. The incubations were carried out for 1 h at 37 °C. The negative controls were prepared without NADPH. Microsomes (100 μg/ml of protein as a final concentration) prepared from the liver of acetone treated rats to induce CYP2E1 were used as positive controls. The concentration of acetone induced microsomes was 10 times lower in the positive controls due to the high CYP2E1 activity.

After incubation, the reactions were stopped by the addition of 1 volume of phenol, which also served to purify the samples from proteins. After centrifugation at 4 °C for 12 000 *g* for 5 min, the supernatant was transferred to another tube and extracted twice with 1 reaction volume of chloroform:isoamyl alcohol (24:1). After centrifugation, 1 volume of cold 95% ethanol was added to the supernatant and stored overnight to precipitate the DNA. The DNA was collected by centrifugation at 12 000 *g* for 15 min at 4 °C and the pellet was washed once with cold 70% ethanol. The washed and dried DNA pellet was then dissolved in 100 μl of H_2O and the radioactivity was measured by liquid scintillation counting after the addition of 900 μl of scintillation fluid (Hi-Safe 3).

2.7. Transfer of NDMA through Caco-2 cell and MDCKII-MDR1 monolayers

Human colon carcinoma Caco-2 cells expressing various active transporters, such as P-gp (MDR1/ABCB1) and BCRP (ABCG2) [52,53] (from ATCC HTB-37, Manassas, VA, USA), in passage number 47, and canine kidney epithelial cells expressing transfected human P-gp (MDCKII-MDR1) [54] (from The Netherlands Cancer Institute, Amsterdam, The Netherlands), in passage number 30, were cultured and prepared for experiments as earlier described [53–55].

The Caco-2 cell monolayer transfer experiments were conducted as earlier described [55] with minor changes. Before the experiments the Caco-2 cell monolayers were washed twice with the transport buffer (Hanks' balanced salt solution buffered with 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH 7.4). Thereafter, the monolayers were allowed to equilibrate in transport buffer for about 20 min at 37 °C. After the equilibration period, transport buffer was added to the receiver chamber, the plate was placed on an orbital shaker (Titramax 1000, Heidolph, Germany) at 420 rpm and the experiment was started by adding the donor solution (1 μM or 5 μM of ^{14}C-NDMA in the transport buffer) into the donor chamber. The initial volumes added to apical and basal chamber were 600 μl and 1600 μl, respectively. 50 μl samples were withdrawn from both chambers at 10, 30, 60, 90, 120 and 180 min. No replacements were made and, therefore, there were no abrupt changes in concentrations. Transepithelial electrical resistance (TEER) was measured to ensure the integrity of cell monolayers before and after experiments and only data from monolayers with TEER higher than 300 Ωcm^2 were accepted. Additionally the possible effect of NDMA on Caco-2 monolayer integrity was controlled with a separate ^{14}C-mannitol transfer experiment with and without NDMA present in the donor solution. NDMA up to 50 μM did not compromise the integrity of the monolayers since mannitol permeability remained virtually unaffected (data not shown).

The MDCKII-MDR1 cell monolayer transfer experiments were conducted as earlier described [53,54] with minor changes. The initial ^{14}C-NDMA concentration in donor solution was 2 μM, the stirring rate was 420 rpm and the experiments were conducted for up to 60 min.

The flux of NDMA through MDCKII-MDR1 and Caco-2 cell monolayers was relatively rapid. Therefore, permeability coefficients were estimated using the following equation that takes into account the changes in the concentrations [56]:

$$C_{R,b} = \frac{M}{V_R + V_D} + \left(C_{R,a} - \frac{M}{V_R + V_D}\right) e^{-P_{app} \times A \frac{V_R + V_D}{V_R \times V_D} \Delta t}$$

where $C_{R,a}$ and $C_{R,b}$ are receiver concentrations at the beginning and at the end of time interval $a \rightarrow b$, M is the total amount of the compound at the beginning of time interval, V_D and V_R are the donor and receiver volumes, A is the area of the filter, P_{app} is the apparent permeability coefficient and Δt is the length of the time interval. Data from 0 to 90 min were used in least squares fitting that was undertaken with Microsoft® Excel software.

The experiments were conducted in both directions (apical to basal and basal to apical) to examine the possible polarization of the transfer. The sampling schedule in the Caco-2 experiments was designed to capture both the initial phase transfer rate (to calculate the apparent permeability coefficients) and the virtual steady state when there would be no net flux through the cell monolayer.

2.8. Safety of laboratory personnel

Since human placental tissue and blood were handled in this study, each worker was vaccinated against Hepatitis B. Carcinogen handling was carried out to minimize skin or inhalation contamination. Individuals working with radioactivity were protected by double gloves, goggles and a laboratory coat and they worked in a laminar hood behind a 10 mm Plexiglas shield. The amount of radioactivity and the duration of the work were minimized.

2.9. Statistical analysis

Microsoft® Excel 2002 and GraphPad Prism 4.03 2005 were used for calculating the statistical parameters. The results are shown as mean ± SD and the *p*-value was calculated using a paired, two-tailed distribution in *t*-test and $p < 0.05$ was regarded as statistically significant. The feto-maternal ratio (fetal concentration divided by maternal concentration) was calculated from the concentrations in fetal and maternal side at each time point. The transfer percentage was calculated using the following equation: $100 \times Fc \times Fv/[(Fc \times Fv) + (Mc \times Mv)]$, where Fc is fetal concentration, Fv is Fetal volume, Mc is Maternal concentration and Mv is Maternal volume. Differences between multiple groups were compared with non-parametrical Kruskal–Wallis rank order test and differences were considered significant when $p < 0.05$.

3. Results

3.1. Recovery of NDMA from human placental perfusion system

The mean total recovery of NDMA in perfusions (sum of NDMA in perfused tissue plus NDMA in maternal and fetal perfusates) was

90% (Table 2). In control perfusions without a placenta 3–13% of NDMA (1 μM) disappeared from the circulation within 4 h. The mean NDMA recovery from the perfusion medium was 76 ± 10% (the volume of samples during perfusion was taken into account). Various amounts of radioactivity (3–31%) were detected in placental tissue after perfusion pointing to considerable inter-individual variation in the accumulation of NDMA in placental tissue. No statistically significant difference was observed in the percent accumulation in placental tissue between the perfusions with different doses of NDMA (Table 2).

3.2. Transplacental transfer

In all perfusions, both the reference compound antipyrine and NDMA were detected in all samples taken after the addition of all compounds to the maternal circulation (Fig. 1A). The maternal concentration antipyrine was statistically significantly higher in the maternal circulation for up to 2 h ($p < 0.05$). At the end of the perfusions, the mean feto-maternal ratio of antipyrine was 0.93 ± 0.07 and the transfer percentage 31.0 ± 2.6. In successful perfusions, antipyrine kinetics was similar regardless of the concentration of NDMA (1 μM or 5 μM) suggesting that all perfusions were comparable with each other.

Radioactively labelled (^{14}C) NDMA crossed the placenta as easily as antipyrine (Fig. 1A). The feto-maternal ratio was even statistically significantly higher with 5 μM of ^{14}C-NDMA than that of antipyrine at 3–4 h. A small amount of radioactivity from ^{14}C-NDMA in the fetal circulation was found already in the first samples taken at 30 min after the addition of NDMA to the maternal circulation (Fig. 1B and C). At 30 min in the perfusions with 1 μM of NDMA, the mean fetal concentration was about one quarter of the maternal concentration (Table 3). With the lower concentration the difference between the mean concentrations of NDMA in maternal and fetal sides were statistically significantly different ($p < 0.05$) up to 2 h (Fig. 1B). At the end of these perfusions, the mean fetal and maternal concentrations were similar (Table 3), the mean feto-maternal ratio was 0.98 ± 0.05 and the mean transfer percentage 31.7 ± 1.7%.

Table 2
Recovery of nitrosodimethylamine (NDMA) in human placental perfusions.

Perfusion number	Concentration of NDMA (μM)	Perf. time (h)	Recovery, perfusates (%)	Recovery, tissue		Total recovery (%)[a]
				(ng/g)	(%)	
1	1	4	58	3	5	63
2	1	4	83	10	13	96
3	1	4	85	2	3	88
4	1	4	71	19	31	102
5	1	4	83	3	6	89
6	1	4	53	5	7	60
Mean			72	7	11	83
SD			14	7	10[b]	17[b]
7	5	4	83	39	13	96
8	5	4	76	58	28	104
9	5	4	81	58	13	94
10	5	4	72	55	10	86
11	5	4	81	50	22	103
12	5	6	81	23	24	105
Mean			79	47	18	98
SD			4	14	7[b]	7[b]
Total mean			76		14	90
SD			10		10	15

[a] Calculated based on concentrations in perfusates and in tissue at the end of perfusion.
[b] No statistically significant difference between the two doses.

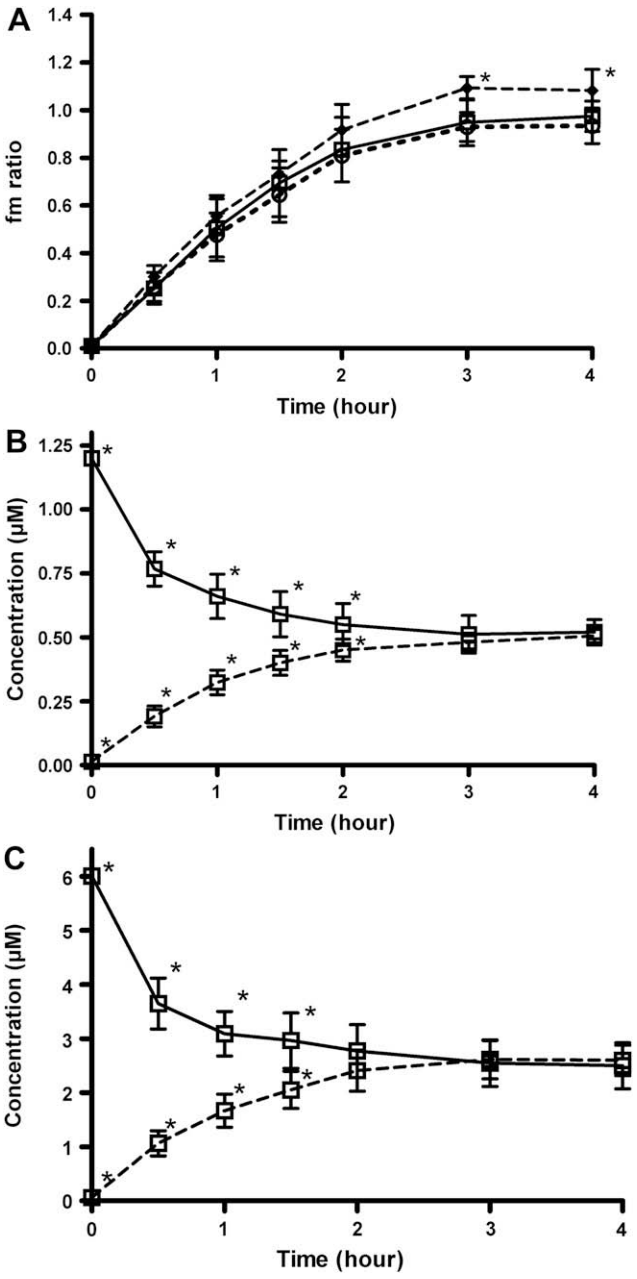

Fig. 1. Transfer of nitrosodimethylamine (NDMA) and antipyrine through the placenta in placental perfusion. A. Mean feto-maternal ratio of NDMA 1 μM (solid line, open squares; $n = 6$), NDMA 5 μM (broken line, black diamonds; $n = 6$) and antipyrine (broken line, open circles) in 12 successful perfusions. B. NDMA concentrations in maternal (solid lines) and fetal (broken lines) circulations in 6 successful perfusions with 1 μM of NDMA. C. NDMA concentrations in maternal (solid lines) and fetal (broken lines) circulations in 6 successful perfusions with 5 μM of NDMA. The results are given as a mean ± standard deviation (*$p < 0.05$).

In the perfusions with the higher concentration (5 μM), the equilibration between maternal and fetal NDMA concentration was achieved quicker than with the lower concentration of NDMA (1 μM) (Fig. 1A and C). As in the perfusions with the lower concentration, NDMA was already found in the fetal circulation 30 min after the start of the perfusions (Table 3). The difference between the mean concentrations of NDMA in maternal and fetal sides was statistically significantly different ($p < 0.05$) up to this time point. At the end of the perfusions, the mean feto-maternal concentration ratio was 1.07 ± 0.04, and the mean transfer percentage 34.6 ± 4.5%.

Table 3
The mean maternal and fetal nitrosodimethylamine (NDMA) concentrations ± SD at 30 min and in the end (at 4 h) of perfusions with initial calculated concentration of 1 μM ($n = 6$) or 5 μM ($n = 5$) of NDMA.

	NDMA 1 μM (range)	NDMA 5 μM (range)
Maternal concentration at 30 min	0.77 ± 0.07 μM (0.64–0.83)	3.54 ± 0.43 μM (2.95–4.01)
Fetal concentration at 30 min	0.19 ± 0.04 μM (0.14–0.26)	1.07 ± 0.26 μM (0.78–1.42)
Maternal concentration at 4 h	0.52 ± 0.05 μM (0.43–0.56)	2.41 ± 0.41 μM (1.91–2.89)
Fetal concentration at 4 h	0.51 ± 0.04 μM (0.46–0.54)	2.58 ± 0.31 μM (2.05–2.82)

No statistically significant differences between maternal and fetal concentrations at four hours with either of the concentrations.

The initial objective was to study 4 h perfusions using two concentrations. However, when comparing the feto-maternal ratios of antipyrine to those of NDMA, the ratio was higher in the perfusions with the 5 μM concentration compared to antipyrine or 1 μM concentration of NDMA ($p < 0.05$) during the third and fourth hour of perfusion (Fig. 1A). There was no difference in the radioactivity or in the recovery of NDMA between the 1 and 5 μM perfusions. Therefore, the difference in transfer between doses could not be explained by technical issues. However, one additional 6-h perfusion gave no indication of further accumulation of NDMA in the fetal circulation. Also, at the end of perfusions there were no statistically significant differences between the mean maternal and fetal concentrations (Table 3).

3.3. DNA-adducts in perfused human placenta

To study whether the radioactivity in tissue was due to DNA-binding, DNA was isolated from perfused placental tissue. However, there was no radioactivity associated with the UV-marker *N*7-methylguanine when separating the depurinated DNA by HPLC, followed by liquid scintillation counting of collected fractions (detection limit 1 adduct/10^8 nucleotides). There was also no radioactivity in DNA from perfused placental tissue when the total binding was analyzed by scintillation counting, indicating that no other adducts were responsible for tissue radioactivity, either.

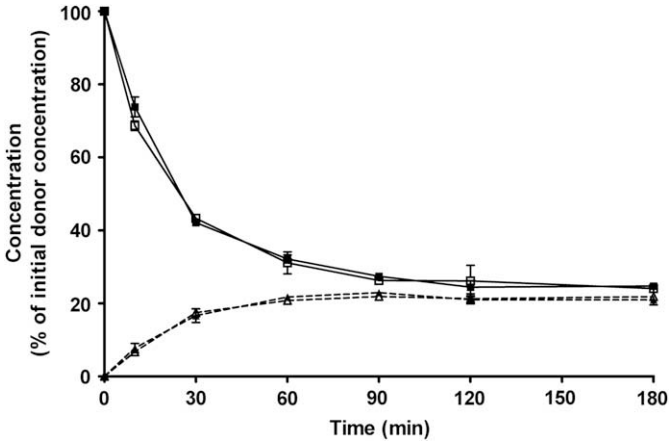

Fig. 2. Transfer of nitrosodimethylamine (NDMA) through Caco-2 cell monolayers in apical to basal direction. NDMA concentrations in apical (solid lines) and basal (broken lines) chambers with 1 μM (closed symbols) and 5 μM NDMA (open symbols). Experiments were conducted as triplicate. Results are given as a mean ± standard deviation.

3.4. NDMA binding into DNA *in* in vitro *incubations*

To further study potential NDMA activation into the reactive methyl diazonium ion in placentas from uninduced mothers placental microsomes from eight human placentas from non-smoking, non-drinking mothers were prepared. In incubation with rat liver microsomes, used as a positive control, NDMA was bound in the DNA after its metabolic activation by CYP2E1, and the radioactivity of the samples was 4.6 times higher than the negative control. However, no increased radioactivity was found in DNA incubated with placental microsomes. The radioactivity of the samples was 0.79–1.18 fold compared to negative controls, this being within the variation present in the controls. The fact that no increased radioactivity could be detected was confirmation that no NDMA at these concentrations had been activated into the reactive methyl diazonium ion in placentas from uninduced mothers.

3.5. Transfer of NDMA through Caco-2 cell monolayers

To study whether saturable transporters could be behind the small differences noted in fm-ratios between the doses, transwell system with tight cell monolayers was utilized. In the Caco-2 experiments, the concentration of NDMA equilibrated at 90 min and the concentrations remained virtually constant thereafter (Fig. 2). The time to reach equilibrium was unaffected by the NDMA concentration or by the direction of the experiment. Furthermore, the apparent permeability of NDMA through the Caco-2 monolayer was high and virtually the same in both directions with both of the used concentrations (Fig. 3). With 1 μM of NDMA, the apparent permeability (cm/s × 10^{-6}) was 226 ± 21 from apical to basal side, while it was 248 ± 11 from basal to apical side. The corresponding permeability values for 5 μM of NDMA were 232 ± 18 and 244 ± 3, respectively. The apparent permeability of a lower concentration of NDMA (2 μM) was also determined using the human P-glycoprotein expressing canine kidney epithelial cell line (MDCK-MDR1). The permeability was similar in both directions, being 297 ± 13 from apical to basal side and 289 ± 9 from basal to apical side.

4. Discussion

In this study, we have shown that in humans, NDMA is transported from the maternal to fetal circulation at a rate comparable to antipyrine. Similar results were observed in three perfusions in the laboratory of our collaborator in the NewGeneris project (Prof.

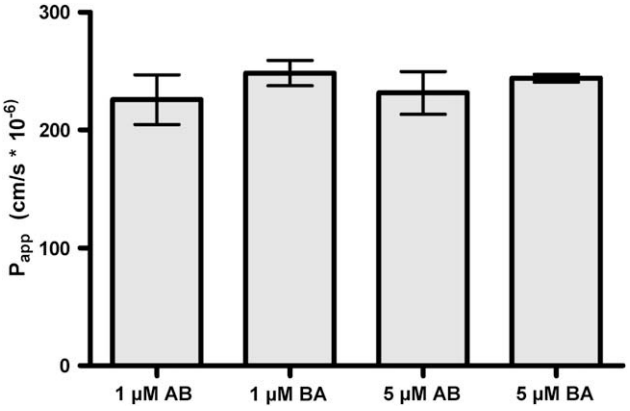

Fig. 3. The apparent permeabilities (P_{app}) of nitrosodimethylamine (NDMA) through Caco-2 monolayer from apical to basal (AB) and basal to apical (BA) directions. The experiments were conducted as triplicate. The results are given as a mean ± standard deviation.

Lisbeth E. Knudsen, University of Copenhagen, oral communication). The doses of NDMA which were used in our study, 15 μg (1 μM in perfusion) or 75 μg (5 μM), were clearly higher than the average daily exposure (1 μg) from the food [5]. However, the daily exposure may be more than 1 μg if estimated on basis of the total exposure from all sources including *in vivo* formation. The total daily exposure to all *N*-nitrosamines from food is probably as high as 80–120 μg/day [3]. To our knowledge, there is no information in the literature about the actual circulating levels of NDMA in maternal blood during pregnancy.

Towards the end of the 4 h perfusions the mean feto-maternal concentration ratio in the perfusions with 5 μM NDMA was slightly higher than in the perfusions with 1 μM NDMA. However, the difference was minor and no indication of further accumulation was seen in a longer perfusion carried out. The transfer from the maternal to the fetal side may be inhibited by saturable efflux transporters located in the apical surface of syncytiotrophoblast such as P-glycoprotein (MDR1/ABCB1) or breast cancer resistance protein (BCRP/ABCG2) [for a review see [57]]. Therefore, we carried out transfer experiments with human colon carcinoma Caco-2 cells and canine kidney MDCK-MDR1 cells expressing these transporters. Since the transfer from apical to basal direction did not differ from the transfer from basal to apical direction, this can be seen as evidence that NDMA is not a substrate for these transporters. Since NDMA is a very small and hydrophilic compound similarly to antipyrine, it could be transferred through the pores in membranes, but in human placenta, there are tight junctions in the syncytiotrophoblast layer and in term placenta may lack cell borders altogether forming one continuous layer [29,58]. All in all, these results with the rapid transfer of NDMA being comparable to that of antipyrine, are consistent with the transport of NDMA across the cells being most likely by passive diffusion.

NDMA is metabolized by CYP2E1 to the reactive methyl diazonium ion which may bind to DNA [22,23]. We found no DNA-binding in perfused placental tissue after the 4 h perfusions. Furthermore, no NDMA binding in 1 h incubations with placental microsomes was found, while the induced rat liver microsomes activated NDMA to DNA-binding metabolites. These results support the notion that NDMA is not activated into the reactive methyl diazonium ion in human placentas from non-smoking, non-drinking mothers. In the literature, there are several studies on CYP2E1 activity in human placenta from healthy mothers. According to the latest studies, it seems that CYP2E1 is not active in normal human placentas [59–62]. However, whether the enzyme is induced in the placenta by maternal use of ethanol during pregnancy leading to NDMA activation remains to be studied in more detail. Some indication for such an induction has been reported in human and patas monkeys [26,63].

In this study, no adducts were found after perfusion with NDMA and no metabolism to DNA-binding metabolites was seen in human placental microsomes. Thus, it seems that the O^6-methylguanine adducts found in the placenta and fetuses of monkeys [26] and in human cord blood [25] are not formed in the placenta. One explanation for finding O^6-methylguanine adducts in fetuses of monkeys and in human cord blood may be that the adducts are formed as a consequence of fetal metabolism. The other possibility is that these adducts are formed after metabolism of NDMA in the mother's liver to an active metabolite which is then transferred from maternal circulation to the fetus through the placenta. This hypothesis has not yet been tested, but since the carbonium ion formed from NDMA is extremely short-lived it seems likely that it would react close to its site of formation if not stabilized by protein binding in plasma. Because the O^6-methylguanine adducts are not specific for NDMA [23] another possibility is that the *in vivo* found adducts are not from NDMA.

In conclusion, fast transfer of NDMA in human placental perfusion indicates that maternal exposure to the mutagenic and highly toxic NDMA probably leads to fetal exposure and may thus endanger fetal health. In our study, no NDMA-DNA-adducts were detected in perfused placenta or in DNA incubated *in vitro* with NDMA and human placental microsomes. Thus based on these findings and the existing data in the literature of the lack of CYP2E1 activity in human placenta, it is likely that full-term human placenta does not participate in the activation of NDMA.

5. Conflict of interest

None of the authors have any conflict of interest concerning this paper.

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