Distribution of an Analgesic Palmitoylethanolamide and Other N-Acylethanolamines in Human Placental Membranes

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Research Article

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Abstract

Human amniotic and amniochorionic membranes (AM, ACM) are the most often used grafts accelerating wound healing due to their anti-inflammatory, anti-microbial, anti-fibrotic, and analgesic properties. We assessed the distribution of endogenous fatty acid amides N-acylethanolamines (NAEs): palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and anandamide (AEA) in placental tissues, as they could participate in wound healing properties of AM/ACM grafts. Ten placentas were collected after caesarean delivery. NAEs were detected using ultra-high-performance liquid chromatography-tandem mass spectrometry analysis in fresh samples of AM, ACM, placental disc, umbilical cord, umbilical serum and vernix caseosa), and decontaminated samples of AM and ACM. NAEs were present in all studied tissue types, with mean concentrations in fresh tissues ranging: 76 – 350 (PEA); 31 – 220 (OEA); 7 – 30 ng/g (AEA). The highest mean concentrations were found in AM (PEA: 350 ng/g) or placenta (OEA: 220 ng/g; AEA: 30 ng/g), respectively. Low levels of NAEs were found in serum and vernix. Decontamination of AM, but not ACM, induced a significant (3 – 3.5-fold) increase in the levels of NAEs.

PEA is the first compound with direct analgesic effect detected in AM and ACM. We thus propose NAEs, especially PEA, as one of the possible factors responsible for the anti-hyperalgesic, anti-inflammatory and neuroprotective effects of AM/ACM grafts, stimulating their wound healing effect. The increase of NAE levels in AM and ACM after tissue decontamination indicates that tissue processing may play an important role in maintaining the analgesic effect.

Introduction

Profound wound healing efficiency and relatively high availability of the source tissue, the placenta, make the amniotic membrane (AM) and amniochorionic membrane (ACM) ones of the most widely used grafts worldwide. Their wound healing effect is given by the interplay of anti-inflammatory, anti-microbial, and anti-fibrotic components [1,2]. The presence of numerous growth factors, cytokines and other bioactive proteins in AM/ACM has been experimentally proven [1–5] and their therapeutic efficacy has been repeatedly confirmed at the clinical level in ophthalmology, surgery, and wound healing [6–9].

The application of an AM/ACM graft on the wound leads to the significant pain relief [10–12]. To date, no specific substances directly responsible for the analgesic effect of these grafts have been reported [13,14]. The pain-relieving effect of AM/ACM is usually explained by the mechanical protection of exposed nerve endings after a tight adherence of the graft to the wound surface, hydration of the wound bed and the presence of anti-inflammatory and anti-scarring components which may alleviate nociception indirectly [13–15]. However, it seems unlikely that such a strong and generalized analgesic effect is invoked without major contribution from a specific compound [13,14]. As specific proteins have been determined to be responsible for the anti-inflammatory and anti-microbial properties of AM, we hypothesized that a similar mechanism could be involved in the analgesic effect of AM/ACM. Therefore, we were interested in establishing whether some known substances that have analgesic properties are present in placental tissue, and thus explain the substantial pain relief generally reported in relation to

application of AM/ACM. After an extensive search for potential candidates we shortlisted a group of endogenous bioactive lipid-related signalling molecules N-acylethanolamines (NAEs), in which particularly palmitoylethanolamide (PEA) has been shown to have profound analgesic and anti-nociceptive effect [16,17].

PEA along with other endogenous fatty acid amides, oleoylethanolamide (OEA), and anandamide (arachidonoylethanolamid, AEA), are ubiquitous in organisms from plants to mammalian tissues [18–22]. In the human body, NAEs have been detected in most organs, tissues (e.g. brain, nerves, muscles, gastrointestinal tract, adipose tissue, skin, eye), and fluids (e.g. blood, breast milk, amniotic fluid, saliva) [18–25].

All three lipid mediators (PEA, AEA, OEA) are synthesized constitutively or on demand, i.e., after exposing cells to specific, predominantly harmful/non-physiological stimuli. They are released from cell membrane phospholipid precursors by phospholipase D, and then act locally in the cells to which they are transported by carrier-mediated transport [22,26,27]. PEA and OEA exert their action primarily by activating the nuclear peroxisome proliferator-activated receptor-α (PPAR-α), the transient receptor potential cation channel subfamily V member 1 (TRPV1) and the G protein-coupled receptors GPR55 and GPR119 [28–32]. PEA can indirectly activate cannabinoid receptors CB_2R [33]. AEA ligates cannabinoid receptors CB_1R, CB_2R, TRPV1, PPAR-γ and some evidence points to AEA binding PPAR-α also [34–36]. NAEs are implicated in multiple physiological (immunity, fertilisation, feeding and sleeping behaviours) and pathological conditions (pain, inflammation, allergy) [23,24,27,37].

PEA was first isolated from egg yolk, soybeans, and peanuts where its anti-anaphylactic activity was also reported [18,19]. Later on, anti-inflammatory, neuroprotective, analgesic and anti-nociceptive effects of PEA were shown in experimental animal studies [16,20,21,27,38,39], and confirmed in human clinical trials [40,41]. It has been reported in rodent models that PEA also inhibits lung, liver and retinal fibrosis [42–44]. PEA has also been shown to have a positive effect on viral respiratory infections [38,45], and was recently proposed as a promising nutraceutical in COVID-19 infection [46]. The anti-hyperalgesic effect of PEA has been utilized in the treatment of peripheral neuropathy and chronic pain, e.g. sciatic pain or pain from carpal tunnel syndrome [40,47,48], and its efficiency is independent of the etiopathogenesis of pain [47].

OEA has mostly anorexigenic properties [49], but its ability to reduce nociceptive responses and inflammation has also been shown [50,51]. AEA has been implicated in possessing anti-nociceptive, vasodilation, and anti-inflammatory effects [22,35,52–54]. Recently its role in physiological wound healing has been also suggested [22,53,54].

Binding of PEA to the PPAR-α receptor leads to attenuation of nociceptive and inflammatory responses, as confirmed in PPAR-α null mice [55,56]. The activation of PPAR-α can aid in the regeneration of mice peripheral nerves at the level of axon repair [57], mediated via satellite glial cells [57,58]. PPARα activation downregulates nuclear factor kB (NF-kB) followed by the decrease of proinflammatory proteins, such as

inducible NO synthase (iNOS), cyclo-oxygenase-2 (COX2), tumour necrosis factor-α (TNFα) or interleukin 1 and 6 or prostaglandin E2 (PGE2) [41,59], which all can contribute to the anti-inflammatory properties of AM/ACM grafts. PEA also stimulates macrophages to remove invading bacteria and apoptotic neutrophils [60,61].

To date, of the NAEs lipid mediators, only AEA has been reported in frozen placental tissues [25,62]. However, its concentrations were not measured in isolated AM or ACM [63], which are the most important placenta related tissues used for grafting. We therefore designed our study to determine whether NAEs are present in placental tissues and whether their concentrations can explain the analgesic effect of the AM dressing.

Results

All three NAEs were unambiguously detected in all tested samples with the exception of vernix where AEA levels were below the limit of quantitation. Mean PEA concentrations (ng/g) in fresh specimens were 277.7 ± 214.6 (AM1), 423.0 ± 256.2 (AM2), 262.3 ± 121.1 (ACM), 205.4 ± 104.1 (PL1), 153.2 ± 72.8 (PL2), 75.8 ± 26.3 (UC), 35.1 ± 13.4 (VX) and 3.9 ± 2.6 (US). A significant difference in PEA content was found between fresh samples of AM2 and PL2 ($p = 0.005$), but not between AM1 vs. PL1. As there was no significant difference between AM1 and AM2, or between PL1 and PL2, we established mean reference levels of PEA 353.3 ± 239.3 ng/g for AM and 179.3 ± 89.9 ng/g for placenta. After decontamination, a significant increase in PEA concentration was found compared to fresh tissue: 1193.9 ± 402.1 in AM1-d ($p = 0.001$), 1177.8 ± 253.2 in AM2-d ($p = 0.001$), and 408.5 ± 276.8 in ACM-d ($p = 0.445$). No difference ($p = 0.445$) was found between fresh and decontaminated ACM samples, Fig. 1A.

Mean OEA concentrations were 83.0 ± 63.7 (AM1), 140.9 ± 81.8 (AM2), 97.7 ± 39.2 (ACM), 241.1 ± 78.8 (PL1), 198.0 ± 95.2 (PL2), 30.5 ± 13.2 (UC), 6.7 ± 4.4 (VX) and 18.2 ± 9.6 ng/ml (US). A significant difference in OEA content was found between fresh samples of AM1 and PL1 ($p = 0.001$), but not between AM2 vs. PL2 ($p = 0.445$). Mean reference levels of OEA were determined as 102.2 ± 65.2 ng/g (AM) and 219.5 ± 86.3 ng/g (PL). Mean OEA concentrations (ng/g) in decontaminated specimens were 382.6 ± 167.8 (AM1-d), 349.2 ± 96.4 (AM2-d) and 249.4 ± 190.1 (ACM-d). Compared to fresh tissue, the statistically significant increase in OEA was between AM1 vs. AM1-d ($p = 0.001$) and AM2 vs. AM2-d ($p = 0.001$), but not between ACM and ACM-d ($p = 0.128$), Fig. 1B.

Mean AEA concentrations reached 13.2 ± 7.7 (AM1), 21.8 ± 10.0 (AM2), 19.2 ± 6.6 (ACM), 31.5 ± 7.8 (PL1), 28.6 ± 9.5 (PL2), 6.1 ± 0.4 (UC), 1.3 ± 0.8 ng/ml (US). A significant difference in AEA content was found between fresh samples of AM1 and PL1 ($p = 0.004$), but not between AM2 and PL2 ($p = 0.445$). Mean reference levels of AEA were 16.7 ± 9.2 ng/g for AM and 30.1 ± 8.5 ng/g for PL. Compared to fresh tissues, the statistically significant increase was found for AM1-d (64.5 ± 50.4, $p = 0.017$) and AM2-d (45.6 ± 24.3, $p = 0.017$), but not for ACM-d (37.6 ± 21.6, $p = 0.128$), Fig. 1C. All original values, and respective statistics are presented in **Supplementary Tables S1-S3**.

Discussion

To date, the anti-inflammatory, analgesic, and neuroprotective properties of AM and ACM have been associated with various proteins discovered in these tissues [2,5]. Although the effect of AM on pain relief was mainly attributed to wound dressing and anti-inflammatory effects, it has been suggested that such rapid action in pain relief is mediated by hitherto undefined analgesic mediators [13]. As far as we know, this is the first study to demonstrate the presence of direct-acting analgesic compounds in placental membranes.

We established reference concentrations of PEA, OEA and AEA in all tested tissues, including AM and PL, by averaging values from the central and peripheral part of placenta, which did not differ in their statistical significance (**Supplementary Table S4**). An observed trend in the distribution of NAEs through all measured specimens (PEA > OEA > AEA) is in accordance with previously published results [22,25,53]. From placental specimens, NAEs have been previously detected in umbilical plasma and amniotic fluid [25,63], and AEA concentration has been measured in unseparated frozen placental membranes and placenta, where higher levels of AEA were found [63]. Lower concentrations of AEA in amniotic and amniochorionic membranes compared to placental samples were also detected in our experiments. The same trend was observed for OEA. Opposite values (i.e. lower levels in placental samples relative to AM/ACM) were found for PEA. This observation may reflect a different function of PEA compared to OEA and AEA in placental tissue. To date, of the NAEs present in the placenta, only the function of AEA, which stimulates oxytocin levels in human placenta at term, has been elucidated [62].

Our results show that the 24-hour decontamination procedure leads to a significant increase in NAE concentration in AM (about 3-fold) and a slightly smaller (not significant) elevation in ACM samples. This shows that particular steps of AM/ACM processing, such as decontamination, can affect the amount of these bioactive substances in the tissue prepared for grafting. We additionally observed a comparable increase in NAE concentration in AM samples stored in two additional solutions (standard cell culture medium or saline) and under different conditions (**Supplementary Table S5**), which suggests that the enrichment of NAE in tissue samples is more likely to be affected by the length of storage than by the composition of storage solution.

A similar observation; increase of AEA concentrations with increasing tissue processing time was previously observed by Marczylo et al. 2009 [63]. The authors also found elevated AEA levels after storage at -80°C, which increased with the number of freeze-thaw cycles [63]. This indicates that overall NAE levels may be affected more by tissue degradation allowing a release of tissue lipids from cells than by continuing synthesis of NAEs by surviving cells, which is balanced by cellular enzymatic degradation of these highly lipophilic, sparingly soluble compounds [64]. In any case, our results indicate that decontamination, as a basal step in AM/ACM processing, influences the quantity of NAEs in prepared grafts.

PEA and AEA are the most interesting of the NAEs studied here because their anti-inflammatory, analgesic, anti-fibrotic and neuroprotective properties [17,27,40] strongly overlap similar features

described in AM/ACM [2,3,6,65,66]. Thus, they may be at least partially responsible for the positive effects associated with AM/ACM in the healing process. It is known that PEA operates primarily through PPAR-α receptor, which is expressed also in the skin [53], the site where AM/ACM grafts are applied. It has been shown that activators of PPAR-α receptor promote differentiation of human epidermal keratinocytes [67]. Furthermore, PPAR-α expression, which is restricted under normal physiological conditions to basal epithelium [68,69], is upregulated by the entire epithelial layer at the edge of the wound during wound healing [69,70]. We propose that PEA, released from AM after grafting may operate via this mechanism, and/or down-modulate mast cell activation [20], or regulate other blood-borne leukocytes (basophiles, macrophages) recruited to the injury site [27]. We suggest that the analgesic features of AM/ACM are mainly related to PEA, but anti-inflammatory and wound healing activities are linked to AEA [54]. Interestingly, Bauer et al. suggested PPAR-γ, which is activated by AEA (but not by PEA or OEA) [71], is the main target of amniotic membrane-mediated anti-inflammatory and antiapoptotic effects on macrophages of mice corneas [72].

Regarding clinical effect of NAEs, it has to be taken into a consideration that their overall wound healing effects depends on their half-life duration in tissue, which is contingent on the ratio between synthesis and degradation rates [53,73]. The observed differences in NAEs concentration in samples from individual subjects are likely to reflect current state of health of donors, such as the presence of obesity, stress, or inflammation [53]. On the other hand, the concentration ratios between tested tissue types (AM, ACM, etc.) from a single donor remain roughly constant.

In this work we detected PEA, AEA, OEA in placental tissues and determined their basal concentrations levels. To our knowledge these are the first directly acting analgesic compounds detected in placenta. It is plausible that other endogenous substances participate in the analgesic effect of placental derivative after grafting, however, they have yet to be determined. We propose that analgesic, anti-inflammatory and neuroprotective properties of PEA and AEA are involved in analgesic and anti-inflammatory activity of AM/ACM membrane, and substantially contribute to their wound healing effect. Our data indicate that NAEs levels (in AM, ACM) increase after a 24-hour decontamination period in antibiotic solution, suggesting that the tissue processing may play an important role in maintaining or modulating the analgesic effect of the tissue prepared for grafting.

Materials And Methods

Chemicals and reagents

The standard of palmitoylethanolamide (PEA ≥ 98%) and solvent ethyl acetate (for LC-MS) were purchased from Merck (Darmstadt, Germany). The standards of oleoylethanolamide (OEA ≥ 98%), arachidonylethanolamide (AEA, MaxSpec standard quality) and palmitoylethanolamide (PEA-d_4, with ≥ 99% deuterium incorporation) were obtained from Cayman Chemicals (Ann Arbor, MI, US). Acetonitrile (Optima, LC-MS grade), methanol (Optima, LC-MS grade) and formic acid (Optima LC/MS) were obtained from Fisher Scientific (Loughborough, UK). Physiological solution (0.9% w/v, Fresenius Kabi, Germany)

and tissue decontamination solution BASE 128 (Alchimia srl, Italy) were used as purchased. Water was prepared using a Milli-Q integral system (Merck Millipore, Burlington, MA, USA).

Material

The study was approved by the Ethics Committees of the General Teaching Hospital and the First Faculty of Medicine of Charles University, Prague, Czech Republic and adhered to the tenets set out in the Declaration of Helsinki for research involving human subjects. Human placentas were obtained after a caesarean section delivery in Motol University Hospital (Prague, Czech Republic), and the General University Hospital (Prague, Czech Republic) from donors with a normal pregnancy who signed a written informed consent. Only healthy donors (mean age 36 years), screened for hepatitis B and C, syphilis and HIV were involved. The placentas with evident pathologies or visible injuries, such as hematomas, were excluded.

Specimens were prepared from ten fresh placentas (P1 – P10), which were processed within two hours after retrieval. The tissue was repeatedly rinsed by physiological solution and blood clots were removed. Then the placental membranes (AM, ACM) were manually separated from the residual placental tissue, dissected, transferred to a mesh support (Sanatyl, Tylex Letovice, Czech Republic) for a better manipulation with tissue and divided into 4 cm^2 samples [6,65]. AM and ACM were processed as either fresh tissue (set 1) or tissue was decontaminated (AM-d, ACM-d) with BASE 128 for 24 h at 4–8°C (set 2), according to standard decontamination procedures used for placental membranes in clinical practice [6,65]. As concentrations of some proteins are not homogeneously distributed within placenta and AM [74], we collected samples from two different areas of placental disc (PL): from the area near the umbilical cord (AM1, PL1) and from the area in the periphery of the placenta (AM2, PL2). Specimens of approximately 0.125 cm^3 were prepared from placental disc (after removing AM) and from umbilical cord (UC). All tissue types were prepared in triplicates. Where possible, a respective vernix caseosa (vernix, VX) sample was collected from healthy newborns immediately after full term delivery and frozen at -80°C until processing.

In addition, umbilical serum (US) samples were prepared from each placenta. Umbilical cord blood was collected from fresh placenta immediately upon reception, precipitated for 2–3 hours at room temperature (RT), centrifuged (3 000 g, 15 min, 4°C) and stored at -80°C until NAEs measurement (two months at maximum). A list of all samples is given in Table 1.

Table 1
Specimens from human term placenta for NAEs analysis.

Specimens	Description	Size
AM1	Fresh amniotic membrane from the area neighbouring the umbilical cord	400 mm^2
AM2	Fresh amniotic membrane from the edge (periphery) of the placenta	400 mm^2
ACM	Fresh amniochorionic membrane	400 mm^2
AM1-d	Amniotic membrane from the area neighbouring the umbilical cord after 22-24h decontamination in BASE 128 (4°C)	400 mm^2
AM2-d	Amniotic membrane from the edge (periphery) of the placenta after 22-24h decontamination in BASE 128 (4°C)	400 mm^2
ACM-d	Amniochorionic membrane after 22–24h decontamination in BASE 128 (4°C)	400 mm^2
PL1	Placental segment (chorionic plate and intervillous space of placenta) from the area neighbouring the umbilical cord	225 mm^3
PL2	Placental segment (chorionic plate and intervillous space of placenta) from the edge (periphery) of the placenta	225 mm^3
UC	Umbilical cord segments from the base of umbilical cord	225 mm^3
US	Umbilical serum obtained from umbilical cord and placenta	500–1000 (µl)
VX	Vernix caseosa from healthy newborn subjects delivered at full term immediately after delivery	30–50 (mg)

Specimen preparation for N-acylethanolamines detection

For NAEs analysis, all tissue samples (fresh and decontaminated) were washed in saline and homogenised mechanically by scissors for 120 seconds in 1 ml of cold acetonitrile [75]. To all homogenates, 10 µl (1 µg/ml) of PEA-d_4 internal standard solution was added, and these were then allowed to shake at 4°C and 800 rpm for 22–24 hours. Insoluble material was removed by centrifugation (20 min, 15 000 g, 4°C); the collected extracts (900 µl) and remaining pellets were stored at -80°C until NAEs detection (up to two months). The extracted material (pellets) was dried in an evacuated centrifuge (Refrigerated CentriVAp Concentrator, Labconco Corporation, Kansas City, MO, US) and weighed (analytical balance).

The vernix (60–75 mg) was placed into 1.5 ml Eppendorf tubes and suspended in 1.5 ml acetonitrile/ethyl acetate (2:1). The volume of 10 µl (1 µg/ml) PEA-d_4 internal standard solution was added. The mixture was sonicated 30 min in ice-cold water, shaken 30 min at 4°C (Thermomixer, Eppendorf, Germany) and centrifuged 15 min at 16 000 g at 4°C (Centrifuge 5417R, Eppendorf). The supernatant was transferred to the new Eppendorf tube.

Purification of the extracts

The extracts were evaporated to dryness in a vacuum centrifuge set at 0°C (Refrigerated CentriVAp Concentrator, Labconco) and re-dissolved in 1 ml of 30% (v/v) methanol/Milli-Q water. All extracts were purified by a slightly modified solid-phase extraction method [25] using a vacuum manifold (Agilent Technologies). The flow rate of solvent through the cartridge was pressure controlled to approximately 1 ml/min. Oasis HLB 1 cc, 30 mg cartridge (Waters) was washed using 1 ml of 100% methanol and preconditioned using 1 ml 30% (v/v) methanol. The extract was placed onto the cartridge. The cartridge was washed with 1 ml of 30% methanol, and the analytes were eluted with 100% acetonitrile to 2ml Eppendorf tube. Umbilical serum (0.25–0.5 ml) were spun at 13 000 g, enriched with 10 µl of PEA-d_4 (1 µg/ml) standard solution, made up to 1 ml with deionised water, vortexed and purified, as described above.

Liquid chromatography – mass spectrometry analysis

The ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC/MS) system was composed of the ExionLC UHPLC AD chromatography system and the QTRAP 6500 + mass spectrometer (both Sciex, Foster City, CA, USA) equipped with electrospray ionisation (ESI) ion source. The analysis was performed with VanGuard Acquity UPLC BEH C18 pre-column (2.1×5 mm, particle size 1.7 µm) connected to the analytical column Acquity UPLC BEH C18 (2.1×50 mm, particle size 1.7 µm) (both Waters). The temperature of the column and the autosampler were set at 40°C and 5°C, respectively. The volume of 5 µl of the solution was injected onto the column. Mobile phase A was water containing 2 mM ammonium acetate solution and 0.1% formic acid. Mobile phase B was acetonitrile containing 0.1% formic acid. The UHPLC gradient was programmed as follows: 0 min, 24% B; 0.3 min, 24% B; 1.5 min, 90 % B; 2.5 min, 100% B, 3.5 min, 100 % B then re-equilibrated at 4.5 min, 24 % B and hold 24 % B till 5 min; constant flow rate 700 µl/min.

ESI source operated in positive mode. The following ESI source parameters were used: curtain gas was set to 40 psi and collision gas to low; ion source was heated to 300°C; ion source gas 1 and 2 were both set to 50 psi, and ionisation voltage was set to 5500 eV.

Product ions were monitored in multiple reaction monitoring (MRM) mode. The following transitions were set: quantification transitions - PEA (m/z 300.17 / 62.10), OEA (m/z 326.30 / 62.10), AEA (m/z 348.30 / 62.10) and PEA-d4 (m/z 304.17 / 61.20); confirmatory transitions, PEA (m/z 300.17 / 57.20), OEA (m/z 326.30 / 55.10), and AEA (m/z 348.30 / 91.10). Declustering and entrance potential was set to 61 V and 10 V, respectively. For other parameters, see **Supplementary Table S6**. Eight-point combined AEA, OEA,

and PEA calibration curves spiked with internal standards PEA-d4 (10 µl of 1 µg/ml) were performed. The calibration curve was constructed for the analyte's relative signal intensity (for the area of the analyte peak divided by the peak area of the internal standard). Peaks integration, calibration curve construction and evaluation of analyte concentration were made using Analyst 1.6.3 (Sciex, Darmstadt, Germany). For tissues and vernix, the concentration was recalculated to the weight of extracted material.

Statistical analysis

Each type of sample obtained from one placenta was analysed in triplicate. The resulting mean + SD was always calculated from a minimum of 6 mean values calculated for each triplicate. The significance of differences in NAEs' concentrations between the fresh (control) and decontaminated samples of AM/ACM and placenta (PL1, PL2) were tested by Wilcoxon test. The descriptive statistics for each data set was calculated using R package [76]. The outliers were identified using the interquartile range (IQR) criterion. Outlier values were excluded from the Wilcoxon test. Only data with a p-value of ≤ 0.05 were considered statistically significant.

Declarations

Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions: A.S. and I.S. carried out the experiments under the supervision of V.V. and K.J. V.V. carried out part of the analysis. A.S. and K.J. prepared the manuscript. All authors reviewed and edited the manuscript.

Competing interests: The authors declare no competing interests.

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Figures

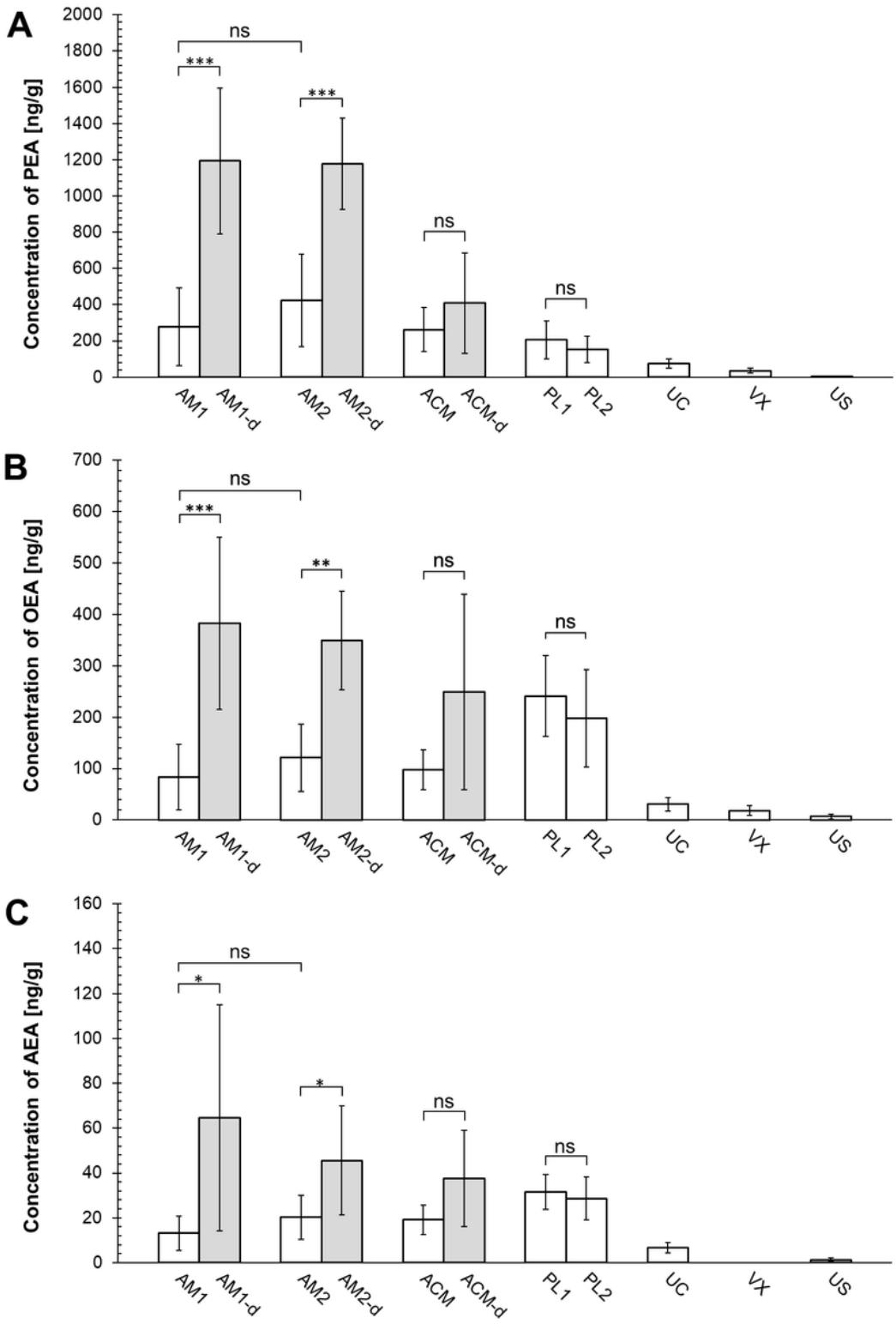

Figure 1

The concentrations of palmitoylethanolamide (PEA) (A), oleoylethanolamide (OEA) (B), and anandamide (AEA) (C) in placental tissues and fluids. Concentrations of N-acylethanolamines are expressed in ng/g, except for serum (US) - in ng/ml. P-value: < 0.05*; p < 0.01**; p < 0.001***; ns = non-significant; AM = fresh amniotic membrane, ACM = fresh amniochorionic membrane, PL1 = placental segment from the

area neighbouring the umbilical cord, PL2 = placental segment from the edge of placental disc, UC = umbilical cord, VX = vernix, d = decontaminated tissue

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