Methylparaben-induced regulation of estrogenic signaling in human neutrophils

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A B S T R A C T

Parabens, including the most common methylparaben (MeP), are popular preservatives, which possess estrogenic activity. The aims of this study were to assess the impact of MeP on estrogen receptors (ERs) and/or NF-κB-dependent generation of IL-8 and production of nitric oxide (NO), and also to verify the hypothesis about the crosstalk of ERs with NF-κB in xenoestrogen-exposed neutrophils. Human neutrophils were incubated for 20-h with MeP (0.06 μM) and/or ER antagonist (1 μM) and/or NF-κB inhibitor (100 μM). After the isolation of cell lysates and cytoplasmic and nuclear fraction, the expression of ER+α, p-IKKα/β, p65 NF-κB, and inducible nitric oxide synthase (iNOS) was measured by Western blot analysis. The concentration of NO was evaluated by Griess reaction, and that of IL-8 was measured by ELISA. The results showed that MeP modulated the expression of ERs, but not ERβ. Exposure to paraben activated iKKα/β-dependent NF-κB pathway, but translocation of p65 NF-κB into the cell nucleus was inhibited by ERs. MeP also decreased the iNOS-dependent production of NO, but did not influence the secretion of IL-8 by neutrophils. The study indicates that MeP may affect the functioning of human neutrophils by modulating intracellular signal transduction pathways, including ERs and NF-κB pathway.

1. Introduction

Preservatives are substances added to cosmetics, pharmaceuticals, and personal care and food products to prolong their shelf-life and inhibit the growth of microorganisms. Parabens are one of the compounds used as preservatives because of their antibacterial and antifungal properties, of which methylparaben (MeP) is the most widely used. The presence of MeP in food must be marked on the product label (E218). Most recent studies have shown the presence of MeP in frozen fruits, meat, olives, chips, rice, and dairy products. It should be noted that any perfect antimicrobial agent should be effective, efficient, and relatively cheap, while at the same time it should not be harmful to humans and the environment. In the case of parabens, issues on their safety strongly polarize the scientific and consumer communities (Galvez-Ontiveros et al., 2021; Matwiejczuk et al., 2020; Nowak et al., 2018, 2020a).

Parabens have been regarded as xenoestrogens (XEs)—exogenous compounds mimicking the action of estrogens. Other compounds that are widespread in the human environment, such as bisphenols including bisphenol A (BPA), F, AF, and S, nonylphenol (NP), octylphenol (OP), and organochlorine compounds, are also categorized as XEs (Acconcia et al., 2017; Nowak et al., 2019). The main mode of action of XEs is their interaction with hormone receptors such as estrogen receptor (ER)-α (NR3A1) and ERβ (NR3A2). The natural ligands of ERs are estrogens, which on binding to these receptors lead to conformational changes and chaperon dissociation and result in the formation of homodimers (ERα+ERα and ERβ+ERβ) and heterodimers (ERα+ERβ). These dimers translocate into the cell nucleus, where they bind to estrogen response elements and control estrogen-regulated genes. They may also activate/inhibit other intracellular signaling pathways and affect the expression of nuclear transcription factors (Acconcia et al., 2017; Nowak et al., 2019; Paterni et al., 2017; Toporova and Balaguer, 2020).

ERs are found in various types of cells, which make these cells sensitive to estrogen’s action and therefore potential targets of XEs. ERα and ERβ have also been detected in human neutrophils—immunocompetent cells responsible for inducing immediate immunological response against pathogenic microorganisms (Garley and Jablonska, 2018; Janiuk et al., 2021; Martin-Millan and Castañeda, 2013; Molero et al., 2002; Nowak et al., 2019).

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Neutrophils are the first immune cells to arrive at the site of an ongoing inflammatory process. They can directionally migrate in response to a gradient of chemotactic factors such as IL-8. Another feature of neutrophils is their ability to generate cytotoxic nitric oxide (NO) in an enzymatic, inducible nitric oxide synthase (iNOS)-related reaction (Ratajczak-Wrona et al., 2020; Ratajczak-Wrona and Jabłońska, 2019). All these processes occur as a result of activation or inhibition of intracellular signaling pathways such as NF-κB. In neutrophils, the transcriptional factor NF-κB controls numerous transcriptional programs, such as the expression of genes involved in the generation of inflammatory mediators. Interestingly, scientists have identified ER dimers among the modulators of NF-κB (Nowak et al., 2019; Ratajczak-Wrona et al., 2019; Xing et al., 2012).

This study aimed to analyze the impact of MeP on intracellular signal transduction pathways in human neutrophils. In particular, it focused on investigating the following: (I) Does MeP affect estrogenic signaling involving ERα and ERβ? (II) Does MeP exposure directly cause NF-κB modulation? (III) Does potential MeP-induced modification of the activity of NF-κB pathway occur via ERs? The study also examined whether MeP exposure influences iNOS-dependent production of NO and generation of IL-8 by neutrophils and whether the generation of inflammatory factors is coregulated via ERs/NF-κB signaling pathways.

### 2. Materials and methods

#### 2.1. Neutrophils isolation

This study was approved by the Ethics Committee of the Medical University of Bialystok (approval no. R-I-002/329/2017, R-I-002/280/2018 and R-I-002/412/2019).

The research was conducted on 30 participants: healthy male volunteer blood donors from the Regional Centre for Transfusion Medicine, Bialystok, Poland. All donors gave written informed consent prior to blood donations. Donors were non-smokers and non-alcohol consuming at least 48 h before blood donation. All males were healthy, without any immunological disorders and not under any hormonal treatment. Donors white blood cell count was counted in Bürker hemocytometer after incubation with Türk’s solution (crystal violet and acetic acid) (AQUA-MED, Lodz, Poland). May-Grünwald-Giemza (AQUA-MED, Lodz, Poland)-stained blood smears were also assessed. Results are presented in Table 1.

Neutrophils were isolated from heparinized (10 U/ml heparin) blood by centrifuge in density gradient – Polymorphoprep™ (Axis-Shield Diagnostics, Dundee, United Kingdom) (Fig. 1). Using of density gradient enables separation of two peripheral white blood cell fractions: mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs – 93% pure). To obtain highly pure (99%) neutrophils fraction, PMNs were positively selected in Midi-MACS magnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) with use MicroBeads conjugated to monoclonal anti-human CD16 antibodies. Neutrophils survival was assessed in a light microscope after incubation with 0.5% solution of trypan blue (Lachema, Praga, Czech Republic) and were equal to 98%. After each step of isolation, PMNs/neutrophils purity was assessed in a light microscope in May-Grünwald-Giemza-stained so-called “thick drop” smears.

### 2.2. Neutrophils incubation

Neutrophils (1 × 10⁶ cells/mL) were suspended in Hank’s Salt Solution 1 × (Biomed, Lublin, Poland) with bovine serum FBS Good (PAN Biotech, Aidenbach, Germany) and antibiotics: penicillin + streptomycin (Gibco, Thermo Fisher Scientific, Waltham, U.S.A.). Cells were placed into the 96-well plates (Microtest III-Falcon, Corning Inc., Durham, U.S.A.). All of the neutrophil’s incubations were conducted at 37 ◦C in a 5% CO₂ incubator (Nuaire™US Autoflow, Plymouth, U.S.A.).

To assess the role of NF-κB or ERs, some neutrophils were 1-h pre-incubated with NF-κB-inhibitor: ammonium pyrrolidinedithiocarbamate (100 μM) (PDTC; Sigma-Aldrich, MERCK, Saint Louis, U.S.A.), or with ERs-antagonist: ICI 182.780 (1 μM) (ICI, fulvestrant; Sigma-Aldrich, MERCK, Saint Louis, U.S.A.), while other cells were cultured with both of them.

After 1-h pre-incubation with or without PDTC and/or ICI 182.780, neutrophils were 20-h incubated with or without methylparaben (methyl 4-hydroxybenzoate, MeP; Merck, Burlington, U.S.A.). The concentration of MeP – 0.06 μM – was established according to Sandanger et al., 2011 results, as average MeP concentration measured in human blood. In our previous study (Nowak et al., 2020b), we determined that 0.06 μM MeP is not cytotoxic for human neutrophils. MeP was dissolved in DMSO then the stock was diluted in PBS. The final concentration of DMSO in cell culture was up to 0.1%.

After cell culture, neutrophils supernatant was frozen in –80 °C. The cells’ precipitates were collected and immediately used in further steps of research.

### Table 1

| Characteristic of peripheral blood leukocytes' populations in blood donors qualified in to the research. | White cell count (per µL) | Neutrophils (%) | Eosinophils (%) | Basophils (%) | Lymphocytes (%) | Monocytes (%) |
|---|---|---|---|---|---|---|
| Mean | 5912.5 | 50.3 | 3.8 | 0 | 39.9 | 6 |
| Standard deviation | 1771.8 | 9.4 | 2.3 | 0 | 9.4 | 3.3 |
2.3. Isolation of whole-cell lysates

Neutrophils precipitates were collected from each well and suspended in a Protease Inhibitor Cocktail (Sigma-Aldrich, MERCK) and sonicated (SONICS Vibra Cell). Samples were centrifuged at 15 300×g. In cell lysate supernatants, the amount of protein was measured by Qubit™ Protein Assay Kit and a Qubit 2.0 Fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, U.S.A.). Then, samples were prepared for western blot procedure.
2.4. Isolation of cytoplasmic and nuclear cellular fraction

Neutrophils precipitates were collected from each well and suspended in PBS 1× without CaCl$_2$ and MgCl$_2$ ions (Gibco, Thermo Fisher Scientific, Waltham, U.S.A.). Isolation of neutrophils cytoplasmic and nuclear fractions was performed according to the NucBuster™ Protein Extraction Kit (Novagen®, Merck, Darmstadt, Germany) procedure. The first step of isolation was to add the extraction reagent, vortexing, incubation in ice and samples renewed vortexing. After high-speed centrifuge (16,000×g) at 4°C, the supernatant (cytoplasmic fraction) was held out, while precipitate was used for isolation of nuclear fraction. The protease inhibitors, reducing agent dithiothreitol (DTT), and extraction reagent were added to each sample. Then, cells precipitates were vortexing, incubated in ice and once again vortexing. High-speed centrifuge (16,000×g) at 4°C allowed to obtain of nuclear fraction (supernatant) of neutrophils.

The concentration of protein was measured by the fluorometric method in Qubit™ 2.0 Fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, U.S.A.). Use of Qubit™ Protein Assay Kit (Invitrogen™, Thermo Fisher Scientific, Waltham, U.S.A.) allowed quantifying protein concentration. Each sample of the cytoplasmic or nuclear fraction was frozen until Western blot analysis.

2.5. Assessment of proteins expression by western blot

Expression of ER$\alpha$, ER$\beta$, p65 NF-$\kappa$B, and $\beta$-actin in whole-cell lysates, ER$\alpha$, ER$\beta$, p65 NF-$\kappa$B, p-IKKa/β, iNOS, and $\beta$-actin in cytoplasmic fraction as well as ER$\alpha$, ER$\beta$, p65 NF-$\kappa$B, and PARP-1 in nuclear fraction was assessed by Western blot method. Samples were suspended with 4× Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, U.S.A.). Electrophoresis was carried out on Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Hercules, U.S.A.). Proteins were separated on SDS-PAGE. The concentration of protein was measured by the fluorometric method in Qubit™ 2.0 Fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, U.S.A.). Use of Qubit™ Protein Assay Kit (Invitrogen™, Thermo Fisher Scientific, Waltham, U.S.A.) allowed quantifying protein concentration. Each sample of the cytoplasmic or nuclear fraction was frozen until Western blot analysis.

Fig. 2. Methylparaben (MeP)-induced protein expression. Cells was 1 h pre-incubated with NF-$\kappa$B inhibitor (PDTC, 100 μM) and/or estrogen receptors antagonist ICI 182,780 (ICI; 1 μM). Then, MeP (0.06 μM) was added for 20 h. Expression of proteins was measured in whole-cell lysates (A), cytoplasmic (B) and nuclear (C) fraction of neutrophils was assessed in Western blot method. $\beta$-Actin and PARP-1 was used as control bands. Relative band intensity of p-IKKα/β (D), iNOS (E), ERα (F), ERβ (G), and p65 NF-$\kappa$B (H) was quantified using ImageJ software and expressed in arbitrary units (A.U.). Results are representative of five experiments. Data are shown as mean (±SD). Value significantly different between: * – control cells (without MeP) (p ≤ 0.05); a – cells with MeP (p ≤ 0.05); b – cells with MeP and PDTC (p ≤ 0.05); c – cells with MeP and ICI (p ≤ 0.05); d – cytoplasmic and nuclear fractions (p ≤ 0.05).
The study assessed the influence of MeP (0.06 μM) on the expression of ERα and p65 NF-κB compared with nonexposed cells. The expression of ERα was equally distributed between the cytoplasmic and nuclear fractions. Cells incubated with MeP and PDTC to MeP-cultured cells reduced the total expression of p65 NF-κB compared with cells incubated with PDTC and MeP. However, the expression of ERα (not ERβ) and p65 NF-κB was lower in the cytoplasm fraction than in the cytoplasmic fraction of cells incubated with MeP and PDTC. Furthermore, the expression of p-IKKα/β was higher and the expression of iNOS was lower in these cells compared with neutrophils incubated without MeP.

The study analyzed the role of NF-κB pathway by culturing neutrophils with PDTC, an inhibitor of NF-κB pathway. Simultaneous incubation of cells with PDTC and MeP decreased the total expression of ERα in neutrophils lysates compared with nonexposed cells and cells incubated only with tested paraben. However, the expression of ERβ in MeP- and PDTC-exposed cells was higher compared to control cells. The addition of PDTC to MeP-cultured cells reduced the total expression of p65 NF-κB compared with neutrophils incubated only with MeP. Higher expression of ERα (but not ERβ and p65 NF-κB) was observed in the nuclear fraction than in the cytoplasmic fraction of cells incubated with MeP and PDTC.

To investigate the role of ERs in signal transduction, we incubated neutrophils with ICI 182.780, an antagonist of ER. Simultaneous incubation of cells with ICI 182.780 and MeP led to a decrease in MeP-induced total expression of ERα. Expression of ERα in neutrophils incubated with MeP and ICI 182.780 was significantly higher compared with cells incubated with PDTC and MeP. However, the total expression of ERβ in cells incubated simultaneously with MeP and ICI 182.780 was lower compared with the cells incubated with MeP and PDTC. Higher expression of ERα and p65 NF-κB in the nuclear fraction than in the cytoplasmic fraction of cells incubated with MeP and ICI 182.780 was observed, while ERβ was equally distributed between the cytoplasmic and nuclear fractions of these cells.

No differences in the expression of ERα, ERβ, and p65 NF-κB were observed in cells simultaneously incubated with PDTC and ICI 182.780 compared with nonexposed cells. However, cells incubated with PDTC and ICI 182.780 showed increased levels of ERα expression compared with cells incubated with MeP and PDTC and with MeP and ICI 182.780. To investigate whether MeP modulates the expression of cytoplasmic proteins such as p-IKKα/β and iNOS via NF-κB pathway and/or ERs, cells were cultured with paraben and PDTC (inhibitor of NF-κB pathway) or
with MeP and ICI 182.780 (ER antagonist). In neutrophils cultured with MeP and PDTC or with MeP and ICI 182.780, the expression of p-IKKα/β with MeP and ICI 182.780 (ICI; 1 μM). Then, methylparaben (MeP; 0.06 μM) was added for 20 h. Concentration of IL-8 was expressed in pg/mL. Data are shown as mean (±SD) of 20 experiments.

with MeP and ICI 182.780 (ER antagonist). In neutrophils cultured with MeP and PDTC or with MeP and ICI 182.780, the expression of p-IKKα/β was higher compared to cells incubated without MeP. In cells simultaneously incubated with MeP and PDTC and in those incubated with MeP and ICI 182.780, the expression of iNOS was decreased compared with the nonexposed neutrophils.

### 3.2. Effect of MeP on NO production

The total concentration of NO in supernatants of neutrophils incubated with MeP and in cells cultured with MeP and PDTC was decreased compared with the concentration of NO in cells not exposed to MeP (Fig. 3). However, in neutrophils simultaneously incubated with MeP and ICI 182.780, no statistical difference was observed in NO production compared with cells cultured without MeP.

### 3.3. Effect of MeP on IL-8 generation

In this study, no observed any statistical difference was found in the concentration of IL-8 in the supernatants of neutrophils incubated with MeP compared with cells incubated without MeP (Fig. 4). Similarly, no statistical differences were observed in cytokine concentration in the supernatants of cells incubated simultaneously with MeP and PDTC or with MeP and ICI 182.780 compared with neutrophils cultured without MeP.

### 4. Discussion

This study investigated whether parabens induce a biological effect in neutrophils by modulating estrogenic signaling, including ERα and ERβ. The results showed that only ERα was involved in MeP-induced signal transduction in human neutrophils, which was confirmed by an increase in MeP-induced total ERα expression (Fig. 2). The expression returned to normal levels after the addition of ICI 182.780, an ER antagonist, to the neutrophils. Moreover, ERα is predominantly located in the cell nucleus which indicates that MeP is involved in ERα dimerization and activation. It was also observed that the total ERβ expression remained unchanged in cells treated with MeP and ICI 182.780. Furthermore, due to the predominant localization of ERβ in cytoplasm, we excluded the fact that ERβ is activated in paraben-exposed cells. The obtained results indicated that MeP may show estrogenic activity on human neutrophils by activating ERα but not ERβ. Crystallographic and computational experiments confirm that parabens act in a ligand-dependent way by binding ERs in the same ligand-binding pocket in which endogenous estrogens also bind (but with lower affinity than estradiol). Moreover, the affinity of XEs toward ERs may vary, which might be the reason for the preference of MeP to act via ERα (Delfosse et al., 2015; Sasaki and Terasaki, 2018).

Researchers have investigated the role of ERs in intracellular signaling in XE-exposed macrophages. Mice macrophage cell line RAW264.7 exposed to BPA, NP, and OP showed inhibition of LPS-induced NO production in an ER-dependent way. However, in cells incubated with XEs (DCP and PCP), ICI 182.780 did not show any modulation in the parameters studied. Based on these results, Yoshitake et al. (2008) suggested that XE selectively modulates ER expression in immune cells. However, Teixeira et al. (2016) observed that XE-induced signal transduction in macrophages involves various pathways, including ERα and/or ERβ. The signal transduction pathways differ according to the parameters and XEs evaluated in the study: BPA, diethyl-2-hexyle phthalate, and dibutyl phthalate. The present study used ICI 182.780 which binds to both types of ER monomers and prevents their dimerization, but Teixeira et al. (2016) selectively blocked ERs with its antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazoledihydrochloride and ERβ with 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol (Carlson, 2005; Wakeling, 2000).

In this study, MeP (0.06 μM) activated the classical NF-κB pathway by increasing the p-IκBα/β-dependent total expression of p65 NF-κB protein (Fig. 2). Classical NF-κB activation involves the action of IκBα/β, which phosphorylates IκBκB inhibitor, thereby causing its removal from NF-κB dimers. The released NF-κB dimers translocate into the cell nucleus, where, as a transcriptional factor, they bind to the promoter sequences of genes (Kandasamy, 2021). Use of the PDTC inhibitor prevents the ubiquitination of phosphorylated IκBκB and precludes the releases of NF-κB dimers (Schreck et al., 1992). In MeP- and PDTC-exposed cells, p65 NF-κB was accumulated in the cytoplasm. However, in MeP-treated cells, with inhibited ER signaling, the localization of p65 NF-κB changed in favor of the nuclear fraction and p65 NF-κB activation when ERs were blocked. Therefore, there may be some interaction, which inhibits the translocation of dimers into the nucleus of neutrophils and prevents their activation in MeP-exposed cells. Comparing the effect of paraben on ERs, but not on ERβ, observed in the present study with those of the previous studies, it could be postulated that MeP-activated ERα is responsible for blocking NF-κB translocation in neutrophils. The results of this study also support the previous reports with regard to the presence of crosstalk between ERs and NF-κB signaling in immune cells treated with XEs. The suppressive effect of estrogenic signaling on the regulator of inflammatory response, NF-κB, is one of the well-known mechanisms of the anti-inflammatory action of estrogens (Smart et al., 2020; Stein and Yang, 1995; Simkova et al., 2020). As demonstrated by Yoshitake et al. (2008), the decrease of p65 NF-κB expression in macrophages exposed to XEs depends on ERs. However, in MeP-exposed neutrophils, blockade of NF-κB signaling increased the total expression of ERβ, but its cellular localization was predominant, which indicates that ERβ signaling remains inactive.

In previous research with MeP (0.06 μM) (Nowak et al., 2020b), no statistically significant differences were noted in the generation of NO by neutrophils that were incubated for 30 min with paraben. However, in the current study, the period of incubation of neutrophils with MeP was prolonged (20 h) and a decrease in the iNOS-dependent production of NO was observed. This shows that NO synthesized by neutrophils plays an important role in invading pathogens, and decreased levels of NO may be observed in people using a range of products that contain parabens as preservatives (Fig. 2). Based on the results obtained in the research on ER antagonist and NF-κB inhibitor, it could be suggested that MeP-induced decrease of NO production is independent of NF-κB and, at least partially, remains under the control of ERs.

In vitro, MeP suppresses the functions of neutrophils, including their ability of chemotaxis (Nowak et al., 2020b)—a process regulated by a gradient of chemotactic factors such as IL-8. Neutrophils not only respond to IL-8 but may also produce cytokines, thereby regulating the
immune cell influx to the site of inflammation. The results of this study showed no association of MeP exposure with IL-8 production, as well as with signaling pathways (Fig. 4), which are in accordance with the results obtained from previous in vitro studies. Based on the available scientific data (Aung et al., 2019; Berger et al., 2018; Watkins et al., 2015), the association between the exposure to parabens and intensification/inhibition of cytokine generation could be excluded. In in vivo studies on women participants Šimkova et al. (2020) did not find any correlation between the concentration of parabens and 27 cytokines measured in blood serum. Similarly, Watkins et al. (2015) and Berger et al. (2018) did not observe any correlation between the presence of parabens in the urine and concentrations of IL-1β, IL-6, IL-10, and TNF-α in the serum of pregnant women. However, Aung et al. (2019) demonstrated a correlation between the concentration of MeP in the urine and increased level of IL-6 in the serum of pregnant women, but the researchers did not observe any correlation between MeP concentration and other cytokines such as IL-1β, IL-10, and TNF-α. Moreover, no correlation was found between the concentrations of propyl- and butyl-paraben and levels of cytokines, whereas in the case of ethylparaben, its high concentration was linked with decreased levels of TNF-α.

5. Conclusions

To sum up, this study showed the impact of MeP on intracellular signal transduction in human neutrophils. MeP may cause multidirectional health implications, including disturbance in antibacterial immune responses in people using cosmetics, food, and pharmaceuticals that contain parabens as preservatives (Fig. 5). The obtained results confirmed that MeP affects ERα receptor but not ERβ, and, therefore, paraben cannot directly modulate the activity of NF-κB pathway. However, via ERα, MeP may disrupt the NF-κB pathway—a key pathway that regulates the functioning and survival of neutrophils. Exposure to paraben impairs the ability of neutrophils to produce NO—an important element in the elimination of pathogens. However, fortunately, this study showed that MeP did not affect the secretion of IL-8, a cytokine that plays a key role in regulating neutrophil chemotaxis, the deregulation of which could inhibit the recruitment of immune cells.

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Declaration of interest

Authors declare no commercial or financial conflict of interest.

CRediT authorship contribution statement

Karolina Nowak: Conceptualization, Methodology, Visualization, Funding acquisition, Writing – original draft, Investigation, Data curation, Writing – review & editing. Ewa Jabłońska: Writing – review & editing. Marzena Garley: Investigation, Data curation, Writing – review & editing. Piotr Radziwon: Writing – review & editing. Violette Ratajczak-Wrona: Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing, Supervision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmce.2021.111470.

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