P-cresol, but not p-cresylsulphate, disrupts endothelial progenitor cell function in vitro

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Abstract

Background. Patients afflicted with chronic kidney disease (CKD) typically suffer from cardiovascular disease (CVD) which is a leading cause of patient mortality. It has been demonstrated that two distinct physiological events contribute to this disease state. These include the abundance of abnormally high levels of protein-bound uraemic toxins as well as functionally aberrant endothelial progenitor cells (EPCs). Specifically, it has been demonstrated that the uraemic toxin p-cresol (pC; 4-methylphenol) inhibits EPC proliferation and tube formation in previous in vitro studies. More recently, however, it has been demonstrated that circulating pC is actually conjugated and that p-cresylsulphate (pCS) is its main metabolite. Therefore, within the context of this study, we examined the in vitro effects of pC and pCS on cultured human EPCs.

Methods. Late-outgrowth EPCs were treated with physiological concentrations of pC or pCS (10, 40, 80, and 160 or 10, 40, 80, 160 and 320 µg/mL for up to 72 h, respectively) in the presence of 4% human serum albumin (HSA). Cell proliferation was determined using WST-1 assay, while migration and tube formation assays were used to evaluate EPC function in vitro. Cell cycle analyses were also performed to determine the effects of pC and pCS on cell cycle status.

Results. With regard to EPC proliferation, data demonstrate that pC in the presence or absence of HSA had an IC50 of 80.1 and 100.8 µg/mL 72 h post-treatment, respectively, while pCS-treated groups did not impair EPC proliferation. Similarly, pC-treated groups showed limited vessel formation and migration compared with controls and no detrimental effects were seen with pCS treatment. Lastly, pC treatment of EPCs caused cells to accumulate in the G2/M phase of the cell cycle with accompanied down-regulation of cyclin B1 and phosphorylated CDK1. pCS had no effect on cell cycle parameters.

Conclusions. Our data demonstrate that pC and pCS have different effects on EPC function. Since there is a dearth of data that have focused on the toxicity of pCS, further research should be performed to determine the exact biological toxicity of pCS on the cardiovascular system.

Keywords: cell cycle arrest; endothelial progenitor cells; p-cresol; p-cresylsulphate; uraemic toxins

Introduction

Chronic kidney disease (CKD) is a major contributing factor to various aspects of cardiovascular disease (CVD) including poor glomerular filtration rates and increased proteinuria leading to increased morbidity and mortality [1, 2]. Endothelial dysfunction has been shown to play a pivotal role in this disease progression [3, 4]. Recent studies have repeatedly shown that decreased numbers of endothelial progenitor cells (EPCs) as in the case of CKD patients leads to an increase in the number of cardiovascular events [5–8]. Serum from uraemic patients has been reported to impair EPC proliferation and migration in vitro through yet to be delineated mechanisms [9]. Among the factors that may influence EPC biology in CKD patients, the constituents of the uraemic milieu, especially protein-bound uraemic toxins, appear to play an integral role [10–12]. This is because they are difficult to be removed by dialysis therapy [13].

In previous studies, several protein-bound uraemic toxins have been shown to promote endothelial injury while simultaneously inhibiting endothelial repair [14, 15]. One such toxin, p-cresol (pC), has been found to be associated with the occurrence of CVD in CKD patients and elevated mortality in haemodialysis patients [16–18]. In-depth in vitro studies showed that pC could inhibit endothelial function [19–21]. Our previous in vitro study also found that pC could inhibit EPC proliferation and tube formation [22]. These data suggest that pC may be a contributing factor involved in the high incidence of CVD observed in CKD patients.
However, it has recently been demonstrated that pC is primarily conjugated via sulphonation in the liver leading to its metabolite, p-cresylsulphate (pCS), with a minor fraction that is glucuronidated \([p\text{-cresylglucuronide (pCG)}]\) [23–25]. Liabeuf et al. [26] have reported that free pCS is a reliable predictor of survival in CKD. Wu et al. [27] found high serum concentrations of free pCS to be independently associated with increased risk of general mortality and cardiovascular mortality in Asian elderly haemodialysis patients. In vitro studies have also revealed the negative impact of pCS on leucocytes and endothelial cells [28–30]. Specifically, pCS increased the percentage of leucocytes that expressed oxidative burst activity and altered endothelial function in patients with CKD. Therefore, it is of great clinical interest to study the effects of pCS on EPC function. Uraemic toxins such as pCS are >95% protein-bound and only the unbound fraction is assumed to be responsible for abnormal biological effects [23]. For this reason, the European Uraemic Toxins (EUTox) Work Group advises that 4% human serum albumin (HSA) be added to culture media for in vitro study of protein-bound toxins [31]. The addition of HSA provides stability to the conjugated molecules while simultaneously acting as a transport carrier. In the present study, we synthesized pCS and attempted to assess any differences in the effects of pCS and pC on EPC function in the presence or absence of 4% HSA. The results of this study may promote better understanding of the toxicity of pC and pCS on the cardiovascular system.

Materials and methods

Reagents

pC was purchased from Sigma-Aldrich Co. (St Louis, MO), while pCS was synthesized using the method previously described [32]. Briefly, pC was dissolved in pyridine solution and converted to pCS by the deliberate crystallization methods utilizing a 9:1 mixture of ethanol to water. The identity and purity of pCS was confirmed using 1H-nuclear magnetic resonance (NMR). A 30% aqueous solution of endotoxin-free HSA used throughout all experimental procedures was purchased from EMD Chemicals, Inc. (Gibbstown, NJ).

Endotoxin testing

Endotoxin levels of the synthesized pCS were measured using the endpoint quantitative chromogenic assay, Tachypleus Amebocyte Lysate Kit (Houshiji, Xiamen, China) as previously described [33]. UV absorbance at 545 nm was detected and a standard curve was generated. The endotoxin content of the sample was determined in EU per microgram based on the standard curve as described by the manufacturer.

Culture and assessment of human late-outgrowth EPCs

Culture and assessment of human late-outgrowth EPCs was performed as previously described [22]. Briefly, mononuclear cells (MNCs) were isolated from 20 mL of human peripheral blood by Ficoll density-gradient centrifugation (1.077 g/mL, Sigma, MO). This study was approved by the Institutional Review Board of Shanghai Jiaotong University School of Medicine. Recovered MNCs were then washed twice with phosphate-buffered saline (PBS) and resuspended in Medium 199 (Gibco, MI) containing 10% fetal bovine serum (FBS) and EGM-2 Single-Quots (Lonza, MD). Cells were cultured in 6-well culture plates under standard conditions at 37°C with 5% CO₂ in the air. Late EPC colonies appeared after ~2–4 weeks of culture. These cells were then harvested and cultured for later experiments. Cell characterization demonstrated that these cells could incorporate acetylated low-density lipoprotein and had a binding affinity for UEA-1. Fluorescence-activated cell sorting analysis revealed that these cells were CD34⁺, CD31⁺, VEGFR2⁺, CD45⁻ and CD133⁻, hence termed EPCs by definition [34].

EPC proliferation assay

The effects of pC and pCS on EPC proliferation in the presence or absence of 4% HSA were determined using the WST-1 assay (Roche Applied Science, Mannheim, Germany). Briefly, 5000 EPCs/well were seeded into 96-well culture plates and incubated for 24 h under standard conditions at 37°C with 5% CO₂. pC at the mean concentrations of 10, 40, 80 and 160 µg/mL and pCS at the mean concentrations of 10, 40, 80, 160 and 320 µg/mL were then added to EPC-seeded plates for 24, 48 and 72 h. For pC experimental conditions, the methanol equivalent to pC at the highest concentration served as control. For pCS experimental conditions, since pCS was obtained as a sodium salt and dissolved in DMSO, both DMSO and DMSO supplemented with sodium chloride at an equimolar sodium concentrations served as controls. The concentrations of pC and pCS were chosen after careful examination of previous studies [28, 30, 31]. Ten microlitres of cell proliferation assay reagent WST-1 were then added to each well and incubated for 4 h. The absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek, VT).

Tube formation assay

The tube formation ability of EPCs was investigated utilizing the base-membrane Matrigel, as previously described [35]. Based upon WST-1 assay results, EPCs were incubated with 80 and 160 µg/mL pC, pCS or control for 72 h, in addition to 40 g/L HSA (a 4% solution) added to the medium. EPCs were then harvested and seeded onto 96-well glass slides pre-coated with Matrigel at a cell density of 2 × 10⁴ cells per well (BD Bioscience, CA). After 12 h of incubation, cells were examined in six random high-power (×100) microscopic fields and graded by two investigators who were blinded to the experiment protocol. Slides were graded as follows: 0, separate, individual cells, 1, cells beginning to migrate and align; 2, visible capillary tubes, but no sprouting; 3, visible sprouting of new capillary tubes; 4, formation of closed polygons; and 5, complex, mesh-like structures. The averages of these six fields were taken as scores for each experimental group. Experiments were repeated three times.

EPC migration assay

The migratory function of EPCs was evaluated using a modified Boyden chamber (Transwell, Corning Inc., MA) assay as previously described [36]. Briefly, EPCs were incubated with the aforementioned concentrations of pC or pCS with 40 g/L HSA added to the medium. EPCs were then harvested and placed in the upper chambers of 24-well Transwell plates with a polycarbonate membrane (8 µm pores) containing 20% FBS at a density of 4 × 10⁴ cells/well. VEGF (50 ng/mL) in medium was placed in the lower chamber. After incubation for 12 h, the membrane was washed briefly with Hank’s balanced salt solution (Gibco). The upper side of the membrane was wiped gently with cotton wool to remove non-migrated cells. The membrane was then stained using Hoechst 33342 (Invitrogen, CA) and removed. The migration of EPCs was evaluated by measuring the area containing migrated cells as a percentage of the total area in six random high-power (×100) microscope fields, and the averages of these six fields were taken. Experiments were repeated three times.

Cell cycle analysis

The decreased numbers of EPCs after in vitro incubation of pC may have been the result of several different effects. Our previous study demonstrated that pC-treated EPCs did not undergo apoptosis, nor did it induce cell necrosis [32]. In order to determine the effect of pC on cell cycling parameters, standard cell cycle analysis was performed. Based upon data gathered from the WST-1 assay results, 3 × 10⁴ EPCs were incubated with 10, 40 and 80 µg/mL pC, pCS or control for 72 h with 40 g/L HSA added to the medium, respectively. Cells were then collected and fixed in ice-cold 70% ethanol for 1 h at −20°C. Samples were washed twice in PBS and resuspended in a solution of PI (propidium iodide, 50 µg/mL) and RNase A (0.5 mg/mL) in PBS for 30 min in the dark. The stained cells were filtered through 40 µm gauze and the DNA content of the single-cell suspensions was determined using a flow cytometer. The experiment was repeated three times.
Preparation of cell lysates and western blot analysis

After EPCs were treated with varying concentrations of pC in complete medium 199 for 72 h, cells were rinsed twice with ice-cold PBS, and proteins were extracted using a ProteoJET Mammalian Cell Lysis Reagent (Thermo Fisher Scientific, MA) as previously described [37]. Protein concentration was measured using the BCA Assay (Pierce, Thermo Fisher Scientific). Eighty micrograms of total protein were then separated on 10 or 12% gels by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. EPC-derived proteins were incubated overnight at 4°C with the primary antibodies (anti-phospho-specific Cdk1, anti-Cdk1, anti-cyclin B1 and anti-cyclin D1) (Cell Signaling, CA) in TBST with 5% bovine serum albumin. The membranes were washed three times with TBST and incubated for 1 h at room temperature in hors eradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. The immunoreactive proteins were detected using an ECL western blotting detection system (Millipore, MA). The detection of β-actin was used as the protein loading control. Each experiment was repeated three times. A representative blot is shown from each experiment.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). All numerical variables were tested for normality using the Kolmogorov–Smirnov test. Statistically significant differences among different treatment groups at a single point in time were determined by one-way analysis of variance followed by two-tailed Student’s t-tests. Statistical significance was assumed for P < 0.05. Data were analysed using SPSS 13.0 software (SPSS Inc., IL).

Results

pCS purity and endotoxin contamination

Identity and purity (>95%) of pCS were confirmed by using 1H-NMR. The final preparation of the purified pCS was found to contain undetectable levels of endotoxin at a detection sensitivity level of 1 EU/µg.

Effects of pC and pCS on EPC proliferation

The effects of pC and pCS on EPC proliferation were analysed in the presence and absence of 4% HSA. For pCS, DMSO supplemented with a sodium chloride control solution showed no significant difference from the pure DMSO control (data not shown); therefore, only DMSO
control data are presented. In media without HSA, the incubation of EPCs with different concentrations of pC for 24, 48 and 72 h induced a significant reduction in the number of adherent EPCs. This inhibition was dose-dependent (IC$_{50}$ = 80.1 µg/mL after 72 h of incubation, Figure 1A). In the presence of HSA, a similar result was observed, but at a lower level of significance (IC$_{50}$ = 100.8 µg/mL after 72 h of incubation, Figure 1B). Among the pCS groups, no impaired EPC proliferation was observed either with or without HSA (Figure 1C and D).

**Effects of pC on pCS on EPC tube formation**

We used a Matrigel model to examine whether pC and pCS would decrease the ability of EPCs to differentiate into capillary-like structures. In the presence of 4% HSA, EPCs formed fewer capillary-like structures when incubated with different concentrations of pC than when incubated with controls for 72 h. This effect was found to be dose-dependent manner (P < 0.05 versus control). The tube formation index scores were as follows: 4.61 ± 0.16 in the control group, 3.23 ± 0.13 in the 40 µg/mL pC-incubated group, 2.45 ± 0.12 in the 80 µg/mL pC-incubated group and 2.12 ± 0.13 in the 160 µg/mL pC-incubated group. pCS had no observable effect on tube formation (Figure 2).

**Effects of pC and pCS on VEGF-induced EPC migration**

To determine the effects of pC and pCS on EPC migration, we counted the stained EPCs that migrated through an insert filter in a chamber filled with Medium 199 containing 0.5% FBS and 50 ng/mL of VEGF, a potent chemoattractant for EPCs [38]. EPCs exposed to pC showed significantly less migration than control groups (percentage of stained area was 23.31 ± 1.13, 16.35 ± 1.12 and 13.87 ± 1.13 in 40, 80 and 160 µg/mL pC-incubated groups compared with 35.32 ± 1.16 in the control group, P < 0.05 versus control). pCS had no effect on VEGF-induced EPC migration (Figure 3).

**Fig. 2.** Effects of pC and pCS on EPC tube formation. (A) Typical images of tube formation as measured by Matrigel assay, respectively (×100). (B) Accumulated data showed that pC significantly inhibited EPC tube formation in a dose-dependent manner (P < 0.05 versus control, #P < 0.01 versus control). In contrast, no significant differences were detected in any of the groups incubated with different concentrations of pCS. Data are expressed as mean ± SD of three independent experiments.
Effects of pC and pCS on EPC cell cycle status

EPCs incubated with pC and pCS were investigated for cell cycle analysis. Results demonstrated that pC could cause EPCs to accumulate in the G2/M phase in a dose-dependent manner (Figure 4A; Table 1). To confirm that pC can induce simultaneous G2/M arrest in EPCs, we then investigated the changes in cell cycle regulators. The results demonstrated that the levels of G2/M-specific cyclin B1 and phosphorylated Cdk1 both decreased after incubation with pC, and there was no change in G1/S-specific cyclin D1 expression (Figure 4B). pCS had no observable effect on cell cycle arrest in EPCs (Table 1).

Discussion

The crucial roles played by the endothelial lining in human vasculature have been the focus of multiple studies in the field of cardiovascular biology in normal and disease states [39]. Since mature endothelial cells possess limited regenerative capacity, there is a great deal of interest in circulating EPCs, especially in their purported role in the maintenance of endothelial integrity and function [40, 41]. Recent studies have repeatedly demonstrated the presence of decreased EPC counts in CKD patients, which may be directly related to the high incidence of atherosclerosis, arterial stiffness, vascular calcifications and neointimal hyperplasia in CKD patients [3, 5–7].

The correlation between circulating EPC levels and uraemic toxins in CKD patients and the effects of uraemic toxins on EPCs merit further investigation [42].

There are two physiologically unique subsets of EPCs that can be isolated from human peripheral blood. These include the spindle-shaped EPCs (termed early EPCs), which demonstrate limited proliferative potential and are unsuitable for long-term culture in vitro. A second subset of EPCs consists of cobblestone-shaped (termed late EPCs) which are generated by prolonged incubation of peripheral blood MNCs in the presence of various growth factors. These cells have a greater proliferative capacity than their early stage counterparts and can achieve high cell numbers in a limited time-frame in vitro [43, 44]. Studies have demonstrated that early and late EPCs

Fig. 3. Effects of pC and pCS on VEGF-induced EPC migration. (A) Representative images of the migrated EPCs stained with Hoechst 33342 (×100). (B) pC significantly inhibited VEGF-induced EPC migration in a dose-dependent manner (*P < 0.05 versus control, #P < 0.01 versus control). In contrast, no significant differences were detected in any of the groups incubated with different concentrations of pCS. Data are expressed as mean ± SD of three independent experiments.
provide differing yet multiple facets with regard to vascular repair mechanisms [44]. Early EPCs contribute to vascular repair by preferentially secreting cytokines that aid in the recruitment of resident mature endothelial cells and induce their proliferation and survival. Late EPCs, on the other hand, enhance vascular repair by providing critical mass in the form of large numbers of localized endothelial cells as a consequence of rapid growth. We utilized late EPCs in this *in vitro* study for the aforementioned reasons. It seems that poor proliferation among late EPCs directly contributes to vascular dysfunction in CKD patients.

pC was previously described as a uraemic toxin [45]. The negative impacts of pC on the endothelium have been shown repeatedly *in vitro* including the concession of

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**Fig. 4.** Effects of pC on cell cycle status of late-outgrowth EPCs. (A) Flow cytometric analysis of EPCs incubated with different concentrations of pC for 72 h. In pC-incubated samples, the progression of the cell cycle at the G2/M phase was noted. The results shown are representative of three independent experiments. Dip G1, diploid G1. (B) Western blotting of cell lysate solution revealed that the protein levels of G2/M regulators cyclin B1 and phosphorylated Cdk1 both decreased after pC incubation, but there was no change in G1/S regulator cyclin D1 expression. Data are expressed as means ± SD of three independent experiments.

**Table 1.** Effects of pC and pCS on cell cycle status in late-outgrowth EPCs

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<tr>
<th>Cell cycle analysis</th>
<th>pC groups</th>
<th>pCS groups</th>
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<tr>
<td></td>
<td>Control + 4% HSA</td>
<td>10 µg/mL + 4% HSA</td>
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<tr>
<td>G1</td>
<td>80.08 ± 0.85</td>
<td>72.03 ± 0.84*</td>
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<tr>
<td>S</td>
<td>15.13 ± 0.50</td>
<td>21.32 ± 0.64*</td>
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<tr>
<td>G2/M</td>
<td>4.79 ± 0.39</td>
<td>6.64 ± 0.75*</td>
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<tr>
<td>pCS groups</td>
<td>Control + 4% HSA</td>
<td>10 µg/mL + 4% HAS</td>
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<tr>
<td>G1</td>
<td>80.08 ± 0.85</td>
<td>78.08 ± 0.45</td>
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<tr>
<td>S</td>
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<td>16.56 ± 0.76</td>
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<tr>
<td>G2/M</td>
<td>4.79 ± 0.39</td>
<td>5.45 ± 0.78</td>
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</table>

Values were the percentage of cells in each cell cycle phase. Data were expressed as mean ± SD of three independent experiments.

*P < 0.05 versus control.

#P < 0.01 versus control.
Effects of pC and pCS on in vitro EPC function

<table>
<thead>
<tr>
<th>In vitro studies</th>
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<tr>
<td>Inhibition of EC proliferation and wound repair [20]</td>
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<td>Increase in EMP release by EC [28]</td>
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<tr>
<td>Increase in EMP release by EC [46]</td>
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<td>No effects on ROS production in EC [47]</td>
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<td>Alteration of endothelial barrier function [19]</td>
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<tr>
<td>Inhibition of EPC proliferation and tube formation [22]</td>
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<td>Decrease in EC response to inflammatory cytokines [50]</td>
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<td>Predictor of mortality in patients at different stages of CKD [26]</td>
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<tr>
<td>Predictor of cardiovascular events in patients with mild-to-moderate kidney disease [18]</td>
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<td>Predictor of cardiovascular and all-cause mortality in Asian elderly haemodialysis patients [27]</td>
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<td>Predictor of mortality in haemodialysis patients [16]</td>
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Table 2. In vitro and clinical studies of the effects of pC and pCS on endothelial cells and EPCs

EC, endothelial cells; EMP, endothelial microparticles; EPC, endothelial progenitor cells; CKD, chronic kidney disease.

cellular integrity due to repeated toxin insult [19, 20, 46]. In the present study, we found that pC could inhibit proliferation, invariably decrease cell migration and tube formation, and arrest the cell cycle at the G2/M phase among late EPCs. However, it has recently been demonstrated that pC circulates mainly in the form of its sulphate conjugate, pCS and pCG [23–25]. This is of critical importance to in vitro research. Recent studies have started to focus on the biological toxicity of pCS on the cellular constituents of the cardiovascular system in vitro. Schepers et al. [30] first reported that pCS could increase oxidative burst activity of leucocytes at baseline levels. Although pC had no effect on the leucocytes at baseline, it inhibited leucocyte burst activity after stimulation. This was confirmed by the same team in a subsequent study [29]. Meijers et al. [28] later demonstrated that pCS could induce a dose-dependent increase in the shedding of endothelial microparticles by human umbilical vein endothelial cells (HUVECs). Itoh et al. [47] subsequently reported that pCS did not induce the production of reactive oxygen species (ROS) in HUVECs. The present study demonstrated that pCS had no negative impact on EPC function. The clinical and in vitro effects of pC and pCS on the cardiovascular system are summarized in Table 2. These findings suggest that pC and pCS might impose completely different types of biological toxicity. The toxicity of pCS on the cardiovascular system remains unclear and requires further investigation.

The present study focused on the different effects of pC and pCS on EPCs. Since pC and pCS have different molecular weight (pC weight 108 g/mole and pCS weight 210 g/mole), it is better to use equimolar concentrations of pC and pCS to allow for meaningful comparison [24, 48]. Although the concentrations of pC and pCS in the present study were chosen according to previous reports [28, 30, 31], we recommended the usage of equimolar concentrations when comparing the different effects of pC and pCS.

pC is a small phenolic compound (MW = 108.1 Da) with lipophilic properties [45]. It would easily penetrate the cell membrane and disorganize its function and as a consequence also the function of cells as a whole [49]. This might explain the biological toxicity of pC. The main difference in molecular structure between pCS and pC is the replacement of hydroxyl with sulphate. These structural variations make pCS a hydrophilic compound. Water-soluble compounds will have more difficulty to enter cells [49]. Thus, we strongly believe that the inhibitory impact of pC on EPC function is neutralized by its conjugation to pCS, producing a net detoxification effect.

One of the main limitations of our study is that we did not estimate the level of cross-talk between leucocytes and the endothelium. Studies by Schepers et al., as well as Meert et al., have found that pCS has a negative impact on leucocytes. It is plausible that pCS could also damage the endothelium via leucocyte activation [30]. Meert et al. also found that although pCG, the minor fraction of conjugated pC in circulation, takes no role in the production of free radicals by leucocytes, it adds to the pro-inflammatory effects of pCS when both are assessed simultaneously. In spite of the minor metabolite of pC and pCS conjugations, the effects of pCG on EPC function should also be studied in the future.

Data generated from our study revealed that pC disrupts EPC function with regard to EPC proliferation, migration, tube formation and cell cycle kinetics, while its sulphonated derivative pCS does not. Future studies will be performed to determine the exact toxicological effects of pCS on the cardiovascular system.

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Conflict of interest statement. None declared.

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