1 # Perfluorooctane sulfonate affects

2 # proliferation and differentiation of

3 # pluripotent human teratocarcinoma cells

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16   **ABSTRACT** Perfluorinated compounds have raised concern due to their potential

17   association with detrimental postnatal outcomes in animals and humans. We tested the effects

18   of perfluorooctane sulfonate (PFOS) on a human pluripotent teratocarcinoma (known as

19   NCCIT) cells as an *in vitro* prototype for developmental toxicity in mammals. NCCIT

20   contains stem-cells able to differentiate into endoderm, mesoderm and ectoderm. We tested

21   our cell model using a teratogenic compound, retinoic acid (RA), a cytotoxin, nocodazole

22   (ND), and PFOS. We assayed cells proliferation, morphology and expression of stem cell and

23   germ layer marker genes. PFOS reduced NCCIT cell proliferation in a concentration-

24   dependent manner and induced morphological changes in cell cultures that resembled

25   ectodermal phenotypes. A tendency towards a differentiated state in NCCIT was confirmed

26   by real-time gene expression. PFOS triggered up-regulation of the gene nestin, indicative of

27   ectodermal lineage differentiation, and interfered with the expression of the pluripotency

28   stem-cell marker TERT. PFOS produced effects on both cells proliferation and differentiation,

29   although not as severe as those observed for RA and ND, at levels that fall within the range of

30   concentrations found in animal and human plasma. We discuss our findings in the context of

31   possible interference of PFOS with the processes governing the early development of

32   mammalian tissues.

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34   **Keywords** human stem-cells, PFOS, developmental toxicity, pluripotency, NCCIT,

35   teratocarcinoma, germ-cell marker genes.

INTRODUCTION

The ubiquitous presence in the environment of perfluorinated surfactant compounds such as perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) has resulted in considerable concern (Giesy and Kannan 2001; Houde et al. 2008; Jensen and Leffers 2008; Loos et al. 2007). Animal studies have indicated that PFOS and PFOA have the potential for developmental toxicity (Austin et al. 2003; Beach et al. 2006; Berger et al. 2009; Bjork et al. 2008; Giesy and Kannan 2001; Kennedy et al. 2004; Lau et al. 2004; Loveless et al. 2006). Human studies have presented epidemiological evidence linking birth defects and thyroid disease to blood serum PFOS and PFOA concentrations in the range of μg/L (Apelberg et al. 2007a; Apelberg et al. 2007b; Jensen and Leffers 2008; Melzer et al. 2010).

In this study we investigated the effects of PFOS at exposure levels relevant to animal and human sera using a stem cell-based test. Embryonic stem (ES) cells have proven to be a promising model to study developmental toxicity of chemicals (Whitlow et al. 2007). The use of ES cells, however, presents a number of challenges (see Supplementary Online Material). To avoid issues associated with the use of ES cells, we chose a human germ-cell line derived from a teratocarcinoma known as NCCIT (National Cancer Centre Immature Teratoma), which is analogue to a pluripotent ES line (Damjanov et al. 1993; Rohwedel et al. 2001; Teshima et al. 1988). Teratocarcinomas contain both undifferentiated stem- and differentiated cells that can commit to endoderm, mesoderm and ectoderm. NCCIT cells express markers of germ layers like ES cells, are capable of continuous renewal in minimal media like cancer cells (without the need of a feeder layer of primary cells) but do not show up-regulation or induction of typical tumour gene markers (Damjanov et al. 1993; Sperger et al. 2003; Taranger et al. 2005). Pluripotency in NCCIT has been proven by the expression of undifferentiated hES cells markers, including the telomerase reverse transcriptase gene

61   (TERT) (Sperger et al. 2003; Taranger et al. 2005). Here, we aimed at using NCCIT as a stem

62   cell model to test the effects of PFOS compared to known teratogenic and cytotoxic

63   compounds such as retinoic acid (RA) and nocodazole (ND). We found that PFOS was able to

64   interfere with the growth and differentiation processes of NCCIT cells.

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66   **MATERIALS AND METHODS**

67   *Chemicals*

68   Standard solutions were purchased from Sigma (Sigma-Aldrich, Dorset, UK). Experimental

69   concentrations of PFOS were selected based on previously reported levels in human plasma

70   (for details see (Apelberg et al. 2007a; Apelberg et al. 2007b; Melzer et al. 2010)). Each

71   chemical was suspended in ethanol and stored at -20°C. RA was suspended in ethanol

72   immediately prior to experimental testing, due to its high sensitivity to UV light and oxidizing

73   agents.

74

75   *Cell cultures and toxicity assays*

76   The NCCIT cell line was obtained from American Type Culture Collection (ATCC,

77   Manassas, VA; Catalog No. CRL-2073). NCCIT cells were cultured in Dulbecco's modified

78   eagle medium (DMEM) (Lonza BioWhittaker, Basel, Switzerland), containing 2 mM

79   glutamine, 4.5 g/L glucose, 10% fetal bovine serum (FBS) (Gibco-Invitrogen Corporation,

80   Carlsbad, CA, USA), and maintained at 37°C with 5% CO_2 in a humidified atmosphere. This

81   work was performed using NCCIT cultures at 60% confluence.

82   Prior to testing, stock solutions of chemicals were diluted in DMEM (0% FBS) to

83   obtain the maximum exposure concentration of 1 mg/L. One in three dilutions were

84   performed in DMEM (0% FBS) to obtain 11 dilutions for each chemical ranging from 1000 to

85   0.017 μg/L (comprising environmentally relevant exposure levels). Test dilutions of

4

86   chemicals were added to three $x10^3$ cells seeded across 96-well plates (final volume 100 μL

87   DMEM 5% FBS per well). Five untreated controls and five replicates for each concentration

88   were prepared for each 96-well plate in each experiment and incubated for 48 h at 37°C, 5%

89   CO_2. Microtitre tests were performed using the CellTitre-96 AQueous One Solution Cell

90   Proliferation Assay kit (Promega Corporation, Madison, WI). Absorbances were recorded

91   using a microplate-reader at 490 nm (optical density - OD of the dye) and 650 nm (OD for

92   background reference). To document cell morphology, 250 $x10^3$ cells were seeded in 6-well

93   plates including duplicated IC_{10} and IC_{50} concentration treatments together with untreated

94   controls (final volume 3 mL DMEM 5% FBS), incubated for 48 h at 37°C, 5% CO_2. Cells

95   were then stained with trypan-blue, enumerated and microscopically photographed. More

96   details on standard procedure for harvesting, stocking of cells, proliferation and

97   morphological assays are provided in Supplementary Online Material.

98

99   *Effective concentration analysis*

100   Proliferation data, expressed as percentage of MTS absorbance final values in treated cells

101   over untreated-controls, were analysed using PriProbit (PriProbitNM, 1998-2000 Masayuki

102   Sakuma). Concentrations resulting in 10% and 50% inhibition of proliferation (IC_{10} and IC_{50})

103   were derived from a log-logistic symmetric fit of the concentration-effect curves, with the

104   quality of fit to the mathematical model evaluated as an Akaike's Information Criterion.

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106   *RNA extraction and real-time PCR assays*

107   Cells were seeded in 6-well plates (250 x 10^3 cells per well) including duplicated untreated

108   controls and duplicated IC_{10} and IC_{50} treatments with RA, ND and PFOS. Total RNA was

109   extracted from cells 48 h after exposure to test chemicals using TRI-Reagent LS following the

110   manufacturer's protocol (Sigma). RNA was resuspended in RNase-free water (Invitrogen)

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111   pooling experimental duplicates for each treatment, and 1 μg of total RNA was retro-

112   transcribed (cDNA synthesis, Marligen Biosciences, Rockville, MD, USA) using random

113   nonamers and oligo-dT primers. Reverse transcription was performed in 3 replicates as

114   follows: 22˚C for 5 min, 42˚C for 90 min and 85˚C for 5 min.

115   Quantitative PCR targeted nestin (ectoderm), brachyury (mesoderm), alpha-fetoprotein

116   (AFP, endoderm), and TERT (maintenance of pluripotency). Glyceraldehydes 3-phosphate

117   dehydrogenase (GAPDH) and β-actin were chosen as housekeeping genes (see

118   Supplementary Online Material, Table S1). Analyses were performed using QuantiTect

119   Primer Assays and QuantiTect SYBR-Green PCR Master-Mix (Qiagen, Doncaster,

120   Australia), optimised for efficiency by the producing company. One hundred-fold diluted

121   cDNA samples were added to the real-time PCR reaction mix (final reaction volume of 25

122   μL) in three replicates for each cDNA sample. Two-step cycling was performed using the

123   Rotor-Gene 3000A system (Corbett/Qiagen): 50˚C hold for 2 min, 95˚C for 2 min, 40 cycles

124   at 95˚C for 15 s and 60˚C for 30 s. Expression levels and statistics for each transcript,

125   normalised to housekeeping genes and relative to untreated cells, were obtained by

126   comparative delta-delta-Ct method using the Relative Expression Software Tool (REST[©])

127   (Pfaffl et al. 2002). More details on real-time PCR protocols are reported in Supplementary

128   Online Material.

129

RESULTS AND DISCUSSION

131   *Cell proliferation*

132   RA, ND and PFOS were able to reduced NCCIT cell proliferation in a concentration-

133   dependent manner (Fig 1). Relative to untreated cultures and after 48 h exposure, ND more

134   strongly inhibited NCCIT proliferation compared to either PFOS or RA. IC_{10} and IC_{50} values

135   derived for RA, ND and PFOS are summarised in Table 1. PFOS showed a lower IC_{50} than

136   RA. Viability of cells was assayed by trypan-blue staining for RA, ND and PFOS treated

137   cultures (data not shown), supporting the cytotoxicity assay results and suggesting induction

138   of cell death as mechanism of reduced cells proliferation. The IC_{10} value derived for PFOS

139   based on NCCIT cells proliferation tests falls within the range of concentrations previously

140   reported in animal and human blood sera (Apelberg et al. 2007a; Apelberg et al. 2007b;

141   Melzer et al. 2010). Serum collected from pregnant women and umbilical cords have been

142   shown to contain 16.2 µg/L of PFOS (Monroy et al. 2008), while concentrations of 12.8 µg/L

143   were obtained from general human blood serum samples (Olsen et al. 2003).

144

145   *Cell differentiation*

146   IC_{10} and IC_{50} levels were used to assess morphological changes in NCCIT cells following

147   treatment with RA, ND and PFOS. Cells treated with the chemicals formed smaller and flatter

148   colonies compared to controls (Fig. 2A, Supplementary Fig. S2-S4), with multi-nucleated and

149   more differentiated branching cells characterised by elongated cytoplasmic processes. In the

150   IC_{50} treatments a number of detached small rounded (apoptotic) cells were evident

151   (Supplementary Fig. S2-S4), with ND showing the strongest effects (Supplementary Fig. S3).

152   Flattened colonies and differentiating cells with branching processes were visible in both IC_{10}

153   and IC_{50} treatments for all chemicals (Fig. 2 B-C-D). In particular, PFOS induced changes in

154   NCCIT cell morphology that were indicative of both differentiation and cytotoxic effects (Fig.

155   2D, Supplementary Fig. S4). IC_{10} exposure levels resulted in smaller and flattened colonies

156   with differentiated cells and IC_{50} doses induced apoptosis in NCCIT, with surviving cells

157   expressing extended cytoplasmic processes (Fig. 2D).

158   The inner mass of a NCCIT cells colony represents the equivalent of pluripotent stem

159   cells, which normally differentiate into epithelial-like (more flattened) cells at the edges of

160   colonies (Damjanov et al. 1993; Teshima et al. 1988). IC_{10} and IC_{50} treated cultures showed

161   epithelial-cell features (Fig. 2-3-4 B-D) indicating a possible differentiation of NCCIT into

162   the ectodermal germ-layer (Damjanov et al. 1993). These morphological observations were

163   verified by the expression pluripotency / differentiation gene markers.

164   Exposure of NCCIT cells to RA resulted in a 8.6-fold and 2.7-fold up-regulation of

165   TERT (pluripotency), and a 10.9-fold and 33.45-fold up-regulation of nestin (ectoderm) in the

166   IC_{10} and IC_{50} exposures, respectively, compared to controls and relative to housekeeping

167   genes (Fig. 3). Weaker effects on gene expression were observed for the ND treatments, in

168   which TERT showed a 4.6-fold and 2-fold increased expression and nestin a 1.7-fold and 9-

169   fold increase in transcript accumulation for the IC_{10} and IC_{50} exposure levels, respectively

170   (Fig. 3). PFOS was the strongest inducer of TERT mRNA expression in NCCIT, with a 62.6-

171   fold and 11-fold up-regulation of the pluripotency marker for IC_{10} and IC_{50} exposure levels,

172   respectively (Fig. 3). PFOS had an effect on nestin expression in NCCIT cells similar to the

173   one exerted by ND, with 1.5-fold and 6-fold up-regulation for IC_{10} and IC_{50} exposure levels,

174   respectively (Fig. 3).

175   TERT and nestin were significantly up-regulated across all levels of chemical

176   exposure compared to the controls and relative to housekeeping genes (Fig. 3). IC_{50} doses,

177   however, consistently resulted in reduced TERT and increased nestin genes expression

178   compared to IC_{10} treatments (Fig. 3). Increased transcription of TERT in response to IC_{10}

179   doses can be attributed to other functions of TERT beyond its major role in telomere

180   maintenance (Karlseder et al. 2004) and may highlight an early response to cytotoxic effects.

181   TERT expression in response to cytotoxic stress can enhance cell survival with an anti-

182   apoptotic role (Cao et al. 2002; de Lange 2005). The subsequent decrease in TERT expression

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183 at increasing doses is consistent with loss in pluripotency and commitment of stem-cells to a

184 particular lineage after exposure to teratogenic and toxic chemicals (Adler et al. 2008;

185 Rohwedel et al. 2001). The hypothesis that NCCIT cells attempted to cope with chemical

186 stress at low doses and then differentiate at higher exposure levels was confirmed by the

187 detected patterns in nestin expression. An increase compared to control levels was observed at

188 IC_{10} and IC_{50} for all test compounds (Fig. 3). Our results indicated that exposure of NCCIT

189 cells to PFOS induced specialisation into ectodermal lineages.

190

191 *Conclusions*

192   Our data suggest that the environmental pollutant PFOS can interfere with the

193 functioning and differentiation processes of developing tissues *in vitro*. Given the mixed

194 population in NCCIT, comprising both differentiated and undifferentiated cells, the tendency

195 towards an increase in ectodermal cells after exposure to the tested chemicals may have been

196 the result of two processes: a commitment of stem cells to ectodermal linages or a preferential

197 selection of ectodermal cells among the population based on higher resistance towards toxins.

198 The overall effects of PFOS were comparable to those observed for known teratogenic and

199 cytotoxic compounds like RA and ND, at exposure levels that are relevant for risk

200 assessment.

201   Giesy and Kannan (2001) have reported the global distribution of PFOS with

202 measured concentrations in the environment and animal tissues that can exceed the IC_{10} levels

203 derived here, although free concentration of PFOS in our *in vitro* assay (5% FBS) were

204 probably higher than in animal sera where PFOS can bind to proteins (Jones et al. 2003).

205 Future work should further investigate the mechanism by which PFOS can induce adverse

206 effects on developing tissues *in vitro* and *in vivo*, considering both selection processes based

207 on cells sensitivity in a mixed population and induction of cells differentiation. In this study,

208　we did not detect NCCIT multi-lineage differentiation based on the expression brachyury

209　(mesodermal marker) and AFP (endodermal marker) (data not shown). The NCCIT cell-based

210　test presented here, however, which is accessible to any tissue-culture laboratory and can be

211　readily standardised, can be extended by the inclusion of ectoderm expression markers and

212　may contribute in the future to screening of pollutants for potential developmental endpoints

213　(for more details see Supplementary online Material).

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FIGURE LEGENDS

Fig. 1. Dose-response curves for the test chemicals on the proliferation of NCCIT cells expressed as percentage of effect over control (untreated) cultures. Boxplots represent the range of experimental data-points (n=10), grey dashed lines the derived response models (see Methods). Panels depict effects of RA (A), ND (B) and PFOS (C).

Fig. 2. Appearance of NCCIT cells treated with the test chemicals, colour of image differs for optimal contrast. (A) Control (untreated cultures): cell colonies showing a biphasic morphology with surface cells appearing tall, predominately columnar in shape (indicated by black arrows), whereas inner core cells are mostly rounded and closely packed (indicated by white arrows). (B) IC_{50} treatment with RA: close-up of cells with long cytoplasmic processes (black arrows). (C) Close-up of IC_{50} treatment with ND: cells exhibiting elongated structures (black arrows). (D) Close-up of IC_{50} treatment with PFOS: cells with cytoplasmic processes (black arrows).

Fig. 3. Expression profiles of TERT and nestin genes in NCCIT cells exposed to IC_{10} and IC_{50} levels of RA, ND and PFOS. Data are expressed as fold-change over the internal calibrator genes (GADPH and β-actin) and relative to control (untreated) cells. Bars on the graph correspond to the standard error of the mean (n=6). Statistical significance is reported at $p < 0.05$ with respect to control expression levels (*).

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Table 1. IC values (µg/L ± standard error) obtained with the NCCIT proliferation assay (48 h) for test chemicals (see Fig. 1).

Chemical	IC_{10}	IC_{50}
RA	0.07 ± 0.006	578.8 ± 43.4
ND	0.29 ± 0.06	86.6 ± 28.5
PFOS	1.91 ± 0.21	201.7 ± 28.2

Figure 1

Figure 2

Figure 3