

Final Report Summary

Section 1: PROJECT IDENTIFICATION Information to be provided for project identification		NOT CONFIDENTIAL
Title of the project Dysregulation of endogenous steroid metabolism potentially alters neuronal and reproductive system development: effects of environmental plasticisers		
Acronym of the project ENDOMET		
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Key words (5 maximum - Please include specific keywords that best describe the project.). Plasticisers, <i>in vitro</i> , endocrine disruption, brain ,thyroid		
World wide web address (the project's www address) http://endomet.bham.ac.uk		

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ENDOMET - Dysregulation of endogenous steroid metabolism potentially alters neuronal and reproductive system metabolism: effects of environmental plasticisers –FINAL REPORT

Objectives:

The overall goals of ENDOMET were to determine whether plasticisers, which are widespread environmental contaminants, could affect not only the human reproductive system but also human neuronal and thyroid development and function and which mechanisms might be involved. *In vitro* tests were to be developed to identify compounds with endocrine disrupting potential. The key objectives were therefore:

1. To determine the effects of plasticisers on the enzymes involved in steroid metabolism, using human cell lines.
2. To determine the effects of plasticisers on steroid receptors, signalling pathways and uptake mechanisms.
3. To determine how plasticisers may act as reproductive toxicants.
4. Correlation of the above objectives, using a proteomic/genomic approach, to give effective *in vitro* tests for endocrine disrupting potential.
5. Assessment of risk perception in EU populations.
6. Dissemination of results.

Scientific achievements

Steroids classically function via genomic pathways, binding to nuclear receptors and initiating a sequence of physiologically relevant reactions. However, it is clear from the literature that many compounds which act as endocrine disrupters (EDs), although active *in vivo*, have relatively weak potential as steroid agonists or antagonists at the receptors. Non-genomic mechanisms must therefore also occur. As well as genomic effects at the oestrogen, androgen, thyroid and AhR receptors, ENDOMET has investigated the non-genomic effects of plasticisers on steroid synthesis, steroid metabolism and alterations in cell signalling and cell transport. The project has used a proteomic/genomic approach to identify the interactions of plasticisers with biological systems.

The plasticisers investigated were :-

Bisphenol-A and bisphenol-A dimethacrylate, alkyl phenols (n-octyl, n-nonyl, tert-octyl), bis-ethylhexyladipate, phthalates (dibutyl, bis-ethylhexyl, di-isononyl, di-isodecyl, dioctyl, benzylbutyl) with 2-phenylphenol, 4-chloro-3-methyl phenol, resorcinol and 2,4-dichlorophenol.

Effects at steroid receptors

Plasticisers interacted with steroid receptors, 4-tert-octylphenol, 4-nonylphenol and bisphenol-A being active agonists at $<10^{-7}$ M at the oestrogen receptor in the human breast cancer cell line MVLN although only bisphenol-A was an agonist in the SK-N-MC human neuronal cell line. None of the tested chemicals reacted as agonists at the androgen receptor although nine of the sixteen elicited anti-androgenic effects. Of the test compounds, 4-nonyl phenol and di-butylphthalate were weak agonists at the arylhydrocarbon receptor (AhR) while in the presence of the dioxin TCDD, the test compounds showed a range of both synergistic and antagonistic effects.

Steroid synthesis and metabolism

The enzyme aromatase (CYP 2C19) controls the conversion of androgens into oestrogens, producing the aromatic D-ring which is an essential part of the structure. Of the tested compounds, 4-nonylphenol, bis-ethylhexyladipate, 4-chloro-3-methylphenol, di-butylphthalate, di-octylphthalate and 2,4-dichlorophenol were inhibitors of aromatase activity at $<10^{-8}$ M. Oestrogens are transported in the blood stream as their sulphated derivatives; these are inactive at the oestrogen receptor but are converted to the free steroid at the cell surface and re-sulphated in the cell by sulphotransferase enzymes (SULT isoforms) using PAPS (3'-phosphoadenosine-5'-phospho-sulphate) as cofactor. Compounds inhibiting the SULT enzymes will therefore act as

benzylphthalate and bisphenol-A were strong inhibitors of SULT 1A1 and 4-tert-octylphenol and butylbenzylphthalate inhibited SULT2A1 (dehydroepiandrosterone sulphotransferase).

A supply of inorganic sulphate is essential for PAPS synthesis and steroid sulphation and is normally provided by oxidation of cysteine. Quantitative real-time PCR was used to determine effects of plasticisers on sulphate provision in human neuronal TE671 cells. Many of the compounds decreased mRNA expression of enzymes in the pathway, 4-nonylphenol, diisodecylphthalate and bis-ethyl-hexylphthalate acting at more than one point.

Cell signalling and cell transport

Indirect genomic effects on cell signalling are dependent on pathways which feed into GSK-3 β (glycogen synthase kinase) and on the mitogen-activated protein kinase (MAPK) pathway, both of which are oestrogen-modulated. Both bisphenol-A dimethacrylate and di-octylphthalate induced MAPK phosphorylation while resorcinol and 2,4-dichlorophenol treatment of GT1/7 cells resulted in GSK inactivation at low levels, showing that plasticisers can exhibit specific and selective effects on cell signalling in the brain.

Plasticisers are known to affect the thyroid; some cause changes in thyroid morphology while others inhibit binding of T3 to transthyretin and also inactivate thyroid peroxidase. In this study, phthalates in particular were found to affect the sodium/iodide symporter (NIS), altering iodine uptake. Several phthalates were found to regulate transcription of the NIS gene, di-isohexyl-, benzylbutyl- and dioctyl-phthalates increasing levels of NIS mRNA two-fold. Using T3-induced proliferation of GH3 rat pituitary cells, treatment with bisphenol-A, benzylbutylphthalate, 4-tert-octylphenol and 4-chloro-3-methylphenol gave positive results at $<10^{-6}$ M.

Effects of plasticisers on the reproductive system

Porcine ovarian granulosa cells were used as a test system to show effects of plasticisers on steroid hormone production. Treatment with bisphenol-A and other phenols induced stimulation of basal progesterone production while the alkylphenols were generally inhibitory; FSH-stimulated progesterone production was inhibited. Basal oestradiol synthesis was inhibited by 2-phenylphenol and most of the plasticisers inhibited the FSH-stimulated oestradiol production. Using porcine oocytes as a test system gave a complex picture but most of the plasticisers affected the number of completely mature oocytes formed, probably by affecting steroid synthesis. In vivo studies with rats confirmed that bisphenol-A and bis-ethylhexylphthalate had ED activity.

Genomics/Proteomics

Use of genomic arrays showed that plasticisers up-regulated genes including those for expression of amyloid beta(A4) precursor protein and down-regulated many more, including those coding for sulphated proteoglycan synthesis, protein kinases, zinc finger proteins and heat shock proteins. A mixture of 4-chloro-3-methylphenol and diisodecylphthalate had antagonistic effects, with fewer genes dysregulated. Use of lectin arrays showed that plasticisers at 10^{-7} M altered post-translational protein glycosylation patterns and hence protein activity, giving a further non-genomic mechanism of action.

In vitro test protocols

Combination of the results from the above in vitro tests using cluster analysis has given a series of simple assays with predictive capacity for ED potential. These are being patented.

Socio-economic relevance and policy implications

The work with ENDOMET (largely with human cell lines) has shown that plasticisers have endocrine-disrupting potential and has allowed the development of patentable tests which could be used in the future to identify compounds which might prove a human hazard. These tests are compatible with the objectives of the REACH programme and should reduce the number of live animals used for in vivo reproductive toxicology testing.

In addition, the results from the non-genomic tests have shown that this is an important mechanism of action and that it is not possible to describe EDs solely in terms of their action at steroid receptors. Phthalates in particular have strong in vivo ED activity but are weak agonists; nevertheless, they are strongly positive in non-genomic test systems. Further, EDs have been shown to affect the brain and thyroid as well as reproduction. Any policy decisions would need to consider these factors.

Analysis of risk perception of endocrine disruption in EU populations has shown considerable concern, with support for further research and clarification of any human risk.

Conclusions

Plasticisers can potentially affect human reproductive systems and also the developing brain and thyroid. They do this by genomic and non-genomic mechanisms. They are present in the environment at levels which are of the same orders of magnitude as those used in this study. ENDOMET has developed tests to identify compounds which may act as endocrine disrupters and these are being patented to provide validated in vitro methods for assessing human risk.