COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

MEETING REPORT ON THE DEVELOPMENT AND FUNCTION IN ADULTHOOD OF THE HUMAN MALE REPRODUCTIVE SYSTEM – POTENTIAL CHEMICAL-INDUCED EFFECTS

Introduction

1. In August 2004, the Committee issued a statement on adverse trends in the development of the male reproductive system focussing on the hypothesis that these effects were due to exposure to endocrine disrupting chemicals at critical developmental windows [1]. At that time, although the evidence of endocrine disruption in wildlife was convincing, the Committee noted that extensive international reviews had not provided direct evidence that exposure to endocrine disrupting chemicals has adversely affected the human male reproductive system.

2. One of the Committee's recommendations was that a scientific meeting be held to review the evidence of adverse trends in male reproductive health, which in 2004 was conflicting, particularly with regards to sperm quality. Although not within the terms of reference of the COT, it was also considered important that the mechanisms involved in the formation of developmental abnormalities be investigated.

3. Male reproductive tract development is primarily driven by fetal testicular production of a number of hormones and signalling factors [2,3]. Disturbance of this complex process, either by genetic mutation or by pharmaceutical or environmental interference is hypothesised to result in disorders of male reproductive health, including low sperm counts, hypospadias, cryptorchidism and testicular cancer. These disorders are common in Western Europe, incidence may still be increasing [4-7], and evidence increasingly supports that all are interrelated symptoms of an underlying hypothesised pathology, namely a testicular dysgenesis syndrome (TDS) [8]. However, low sperm counts, hypospadias, cryptorchidism and testicular cancer may arise independently and there remains considerable uncertainty regarding the etiology of TDS [9].

4. In order to evaluate the evidence produced since this subject was last reviewed, in February 2006 the COT held a one-day workshop on development and function in adulthood of the male reproductive system. Presentations considered a range of topics, including cross-sectional and case-control studies of sperm quality and congenital malformations, the TDS hypothesis, potential chemical causes of reported effects, including cumulative effects of *in utero* exposure to anti-androgens and alternative hypotheses to that of endocrine disruption. Although potential chemical causes of cancer fall within the remit of the Committee on Carcinogenicity (COC) the inclusion of testicular cancer in the TDS hypothesis required its consideration by the COT. Information from

the talks and subsequent discussions is summarised here. This statement is not a comprehensive review of the extensive scientific literature of relevance to this topic.

Evidence of a trend towards lower sperm quality and counts

5. In 2004, the COT considered that, given the conflicting reports of significantly declining sperm counts [10-17], the evidence was equivocal. It is likely that these differences were in part due to the fact that some studies suffered from subject selection bias. In addition, the Committee noted that measurement of the quality of human sperm (density, motility and morphology) was subject to a number of sources of uncertainty, e.g. semen analysis methodological differences.

6. In the International Study of Semen Quality in Partners of Pregnant Women, a coordinated cross-sectional study of men across four European cities (Turku, Edinburgh, Paris and Copenhagen) [18], significant geographical differences in semen quality were detected. This study also detected seasonal variations in sperm concentrations and total sperm counts highlighting the need for future prospective studies to factor this into their design. The study population consisted of male partners (aged 20 – 45) of pregnant women, inevitably not including infertile men and likely to under-represent sub-fertile men. However, for these four cities, the data may be considered as a reference point for future studies on time trends in semen quality.

7. The Study for Future Families (SFF) utilised a design consistent with the International Study of Semen Quality in Partners of Pregnant Women [18] and examined sperm quality and other reproductive parameters in fertile couples in four cities in the north, east, west and south-central USA [19,20]. Sperm concentration and motility were significantly lower in the Missouri cohort relative to the cohorts from New York, Minneapolis and Los Angeles. The study authors hypothesised that the Missouri cohort's proximity to intensive agriculture using agricultural pesticides may relate to the poor sperm quality characteristics and further conducted a nested case-control study within this cohort, measuring urinary concentrations of eight pesticide metabolites. Pesticide metabolite levels were elevated in cases compared with controls for the herbicides alachlor and atrazine and for the insecticide diazinon. The association suggested to the study authors that exposure to current-use pesticides may have contributed to the reduced sperm quality seen in fertile men.

8. The European Union-funded INUENDO Project (http://www.inuendo.dk) has recently published initial findings from a cross-sectional study in pregnant women and their partners in Poland, Ukraine and Greenland [21]. A cohort of Swedish fishermen and their spouses were included but recruited independently of current pregnancy. An association between lipid adjusted serum concentrations of the persistent organic pollutants (POPs) PCB-153 and *p,p'*-DDE and time to pregnancy, sperm motility and morphology was investigated. A geographical difference between cohorts in fecundability compatible with serum *p,p'*-DDE concentrations was identified. However, it was not possible for the study authors to control for residual confounding given the differences in sample population demographics.

9. Following on from the Partners of Pregnant Women study [18], a "historically prospective cohort study" of Scottish male reproductive health was commissioned by the

Department of Health (with involvement of DEFRA and HSE). Two of the aims of this study are:

- To obtain a 1999-2000 estimate of (i) exposures to various factors suspected to adversely affect sperm quality and (ii) sperm quality, and of any association between these.
- To distinguish the effects of parental exposures (intra-uterine and perinatal effects mediated through maternal diet, smoking and potentially exposure to environmental chemicals) and direct effects (adult exposures and lifestyle such as smoking, scrotal heating and exposure to defined testicular toxicants), using a matched pairs design to study twin births.

This study is yet to report its main findings, but it would appear that gaining and meeting the terms of the ethical approval for this study proved a significant obstacle, and impacted negatively on the achieved response rate. However, when complete, data comparison with historical data for Scottish males [18,22] should provide an indication of sperm and semen quality trends in this population.

Testicular cancer and congenital genital malformations (cryptorchidism and hypospadias)

10. An increasing trend in the incidence of testicular cancer has been shown in Northern and Western Europe [23-26], Canada [27,28], New Zealand [29], Australia [30] and the US [31,32]. This was recently confirmed for northern European countries in an investigation using cancer registry data, although this study also highlighted large geographical variations and an attenuated incidence trend in Sweden from the early 1990s [5]. In this, and other studies [23,27,31,32], the increasing incidence of testicular cancer closely correlated with year of birth, i.e. a birth cohort phenomenon.

11. Cryptorchidism (undescended testis) is the best characterised and numerically most important risk factor for testicular cancer [33-35], but the etiologic fraction (proportion of cases of testis cancer explained by cryptorchidism) is only around 10% and cannot explain the observed temporal trends. Previous investigations and studies have consistently shown an increased risk of testicular cancer among fathers and brothers of testicular cancer patients [36-38]. Potentially relevant gene loci have been identified by association studies [39], segregation analysis [40], linkage, and microsatellite analysis [41,42]. Recently, a population-based case-control study in Germany showed that testicular cancer aggregates in families [43]. However, the study authors noted that such studies on familial disease aggregation require careful interpretation in order to attribute disease accumulation to genes when lifestyle, environmental factors and sibship sharing gestational characteristics are shared by family members.

12. Cryptorchidism and hypospadias (abnormally placed urethral meatus) are common congenital abnormalities. However, determining whether reported increases in incidence of these abnormalities are real has been confounded by differences in diagnostic criteria. Cryptorchidism has an established association with hypospadias [44].

13. In general, registry data on hypospadias are not reliable and not comparable between countries, and often cryptorchidism is not listed in registries of malformations. This was highlighted by a retrospective study in the Netherlands [45] that, using a carefully structured diagnostic procedure, reported a 0.7% incidence of hypospadias whereas the registry data for the same region reported a 4- to 6-fold lower incidence.

14. A significant increase in the incidence of cryptorchidism in the UK from the late 1950s to late 1980s was determined following time-trend analysis of two well-standardised and clearly reported studies [46,47].

15. Notable differences in semen quality between Denmark and Finland, with Denmark having poorer reproductive health [18,48], led researchers to undertake a synchronised and standardised cohort study to investigate the prevalence of congenital cryptorchidism in Denmark and Finland [6]. Significantly, researchers used the examination technique and definition of cryptorchidism developed by Scorer [46] allowing direct comparison of results with previous studies in the UK [46,47] and Lithuania [49]. Boisen and colleagues [6] reported a marked increase in the birth prevalence of cryptorchidism in Danish boys with normal birthweight (1.8% in 1959-61 compared to 8.5% in 1997-01). In addition, prevalence was four-fold higher in Denmark than Finland, which corresponds to a high incidence of testicular cancer in Danish men and a prevalence in Finnish men that is amongst the lowest in Europe [23,24].

16. In terms of hypospadias, the joint prospective cohort study of Finnish and Danish boys (recruited 1997-1999 and 1997-2002, respectively) [6,7] also showed significant differences in prevalence of hypospadias between the two cohorts [7]. A 1% birth-rate of hypospadias in the Danish cohort was detected which compared to a significantly lower rate of hypospadias (0.27%) in the Finnish cohort study. The etiology of hypospadias remains unclear, although this study suggested associations between hypospadias and fetal growth impairment, and hypospadias and elevated serum FSH levels at 3 months of age.

17. In rodents, as perineal growth is dihydrotestosterone-dependent, anogenital distance (AGD) is a sensitive intermediate endpoint of anti-androgenic effects. Its measurement is included in the OECD Two-generation Reproductive Toxicity Test Guideline (TG 416). This measure of prenatal anti-androgen exposure has only recently been evaluated in human infants [50], and shown to be, as in rodents, sexually dimorphic and about twice as long in males as in females. A recently published study of AGD among human infants [51] reported shortened AGD and impaired testicular descent in boys whose mothers had elevated levels of prenatal phthalate exposure. The authors acknowledged that the reliability of AGD measurement in humans has not been established and the impact of shortened AGD at birth to male reproductive health in adulthood is unknown.

Testicular dysgenesis syndrome

18. The testicular dysgenesis syndrome hypothesis arose out of the findings that human male reproductive disorders in babies (cryptorchidism, hypospadias) or in young men (testis cancer, low sperm counts) are interrelated. This hypothesis proposes that maldevelopment (dysgenesis) of the fetal testis results in hormonal or other malfunctions of the testicular somatic cells, which in turn predispose to the disorders that comprise TDS.

19. A recent review [52] highlighted the evidence in support of the TDS, in particular for cryptorchidism, hypospadias and low sperm counts, identifying the points of vulnerability to endocrine disruption. For testicular cancer, although it is postulated that failure of

normal differentiation of fetal germ cells and their subsequent conversion to pre-malignant carcinoma-*in-situ* (CIS) cells is involved, the mechanisms are not fully established.

20. Considerable research effort has sought to establish causal links between TDS and environmental chemicals with endocrine disrupting properties. However, it has also been proposed that epidemiological evidence supports a contribution of environmental and genetic components [53,54]. Within this proposal is the hypothesis that exposure to a toxic agent would lead to a mutation, genetic damage or epigenetic process that increases the risk of one or more endpoints of TDS [55]. Survival of the mutation in subsequent generations will be dependent on its effect on health and fertility, and behavioural factors such as family size. However, candidate chemicals that fit into this hypothesis have yet to be identified.

21. A TDS-like pattern of disorders can be induced in male rodents by exposure *in utero* to high doses of certain phthalate esters [56-62]. This shows some clear parallels with human TDS (cryptorchidism, hypospadias, low sperm counts/low fertility, areas of focal dysgenesis and Sertoli cell-only tubules in the testis) including the relationship of these disorders to malfunction of the somatic cells of the fetal testis, which is hypothesised to underlie human TDS. For example, using dibutyl phthalate (DBP) it has been shown that *in utero* exposure induced focal dysgenesis in the rat testis by inducing aberrant migration/aggregation of Leydig cells in fetal life. This manifests in adult animals as focal dysgenetic areas, intratubular Leydig cells and focal occurrence of Sertoli cell-only tubules. However, so far, no animal model has been able to mimic all the symptoms of TDS, i.e. including testicular germ cell tumours (TGCTs), although CIS-like cells have been found in a spontaneous testicular neoplasm in a rabbit [63,64]. Although anti-androgens are able to reproduce the TDS-like changes in rodent testis, it remains to be established whether any associations between the symptoms of TDS in humans and exposure to external chemicals can be detected and are causal. The phthalate model of TDS in rodents offers the possibility to study the mechanisms that lead to TDS disorders. Although phthalates could theoretically contribute to TDS disorders in humans these studies cannot be regarded as providing evidence that phthalates cause TDS in humans.

Male reproductive system disorders and chemical exposure

22. Following the initial reports, in the early 1990s, of declining male reproductive capacity, Sharpe and Skakkebaek [65] redefined the ‘estrogen hypothesis’ originally proposed for testicular cancer [66-68], to implicate altered prenatal estrogen exposure in the increasing incidence of other male reproductive abnormalities.

23. It has been established that administration of diethylstilbestrol (DES; a potent synthetic estrogen) to pregnant women and rodents causes male reproductive tract malformations [69,70]. These effects were observed at pharmacologically active doses of DES. Such exposures may have little relevance to potential exposures to estrogens occurring in the environment, which are significantly less potent than DES and present only at low concentrations. As such, although evidence from animal studies shows that potent estrogens are capable of inducing the phenotype of TDS, the concentrations of less potent environmental estrogens required to induce such effects has brought into question whether estrogen exposure is an etiological factor in inducing TDS [71]. In fact, a

recent review of published epidemiological studies of male reproductive disorders and prenatal indicators of estrogen exposure [72], found, with the exception of testicular cancer, no strong evidence to indicate that prenatal exposures to estrogens are linked to disturbed development of the male reproductive organs. However, some estrogenic chemicals (e.g. bisphenol A and nonylphenol) have been shown to also exhibit anti-androgenicity *in vitro* [73,74] and *in vivo* [75] and it may be their anti-androgenic properties that are of importance.

24. The original hypothesis proposed (i) suppression of follicle stimulating hormone (FSH) secretion and (ii) impaired Leydig cell development as plausible mechanisms via which estrogen exposure could induce these disorders [65]. However, new findings implicating (i) suppression of testosterone and insulin-like factor 3 production, and (ii) inhibition of androgen receptor expression point towards the male reproductive disorders being caused by chemicals exhibiting anti-androgenic rather than estrogenic properties [71]. Genetic disorders affecting normal androgen production and action in fetal life (e.g. complete androgen-insensitivity syndrome) provide support for this hypothesised role of anti-androgens.

Cumulative exposure to similarly acting anti-androgens

25. Anti-androgenic phthalate esters, such as dibutyl phthalate, induce cryptorchidism, hypospadias, impaired spermatogenesis, and reduce male fertility in rats [56-59,61]. Although these findings have been supported by the recent development of a possible animal model for TDS [60], whether the level of environmental exposure to any single anti-androgen is sufficient to impact on human male reproductive health is questionable [76]. However, in practice, exposure is to multiple anti-androgenic chemicals, never to single agents, and this has motivated research into the question as to whether several anti-androgens are capable of acting together.

26. Researchers have begun to investigate the effects of binary mixtures of anti-androgens in order to establish whether cumulative effects are additive and predictable on the basis of knowledge of the dose-response relationships of the individual mixture components [77-79]. Endpoints that have been shown to be sensitive and relevant in rodents are anogenital distance, retained nipples, sex accessory organ weight and reproductive tract malformations. Results from these studies have largely indicated that joint effects are predictable and dose additive [77], even for two chemicals with apparently different mechanisms of action [78].

27. In addition, the COT was informed about ongoing *in vivo* studies with multi-component mixtures (with between three and seven chemicals) investigating whether joint effects occur when each individual mixture component is present at concentrations below that which induces a detectable effect (Hass, U. and Gray Jr, L.E.; personal communications). These studies have been designed to not only assess mixtures of chemicals shown to have common mechanisms of action but to also specifically investigate mixtures of chemicals which act via different mechanisms to induce the same toxicological effect. Interestingly, data on AGD in rats from experiments combining seven anti-androgens with various mechanisms of action indicate that joint effects are dose additive (simple similar action) rather than response additive (simple dissimilar action) [80]. If these findings are confirmed then this might indicate the need to review the

current assumptions relating to risk assessment of mixtures of chemicals with dissimilar mechanisms of action.

COT Discussion

28. The new epidemiology studies reported since the COT issued its statement on adverse trends in development of the male reproductive system, including those presented at the COT one-day workshop, provide further evidence that male reproductive health is declining in some populations. However, causal associations in humans have not been established, and in fact, it should be noted these studies were not designed to provide such evidence. The report that anogenital distance (the most sensitive marker of anti-androgen action in studies in rodents) is shortened and testicular descent impaired in human male offspring of mothers with elevated prenatal phthalate exposure [51] was considered of interest. The Committee agrees with the study authors assertion that follow-up of this cohort into adulthood, as well as confirmation of these findings in a significantly larger cohort, is necessary before any conclusions of regulatory relevance can be drawn. The COT would further recommend analysis of possible confounding factors (in addition to ethnicity and diet) and whether phthalate exposure is a marker of mixed chemical exposures.

29. The COT noted that in Europe new legislation on ethical approval for human epidemiological studies was likely to make conducting studies on semen quality and congenital genital malformations unfeasible. Considering the importance of monitoring trends in these endpoints and the need to establish any causal associations, the Committee would encourage changes to legislation that would facilitate the undertaking of such studies in the future.

30. The high quality animal studies reported at the workshop were considered to be identifying plausible mechanisms of action. In particular, the Committee agreed that the hypothesised causative role of exposure to anti-androgenic chemicals, supported by the data being produced in animal models, was more plausible than that postulated for environmental estrogenic chemicals.

31. The Committee awaits the publication of results from the ongoing studies in rats of multi-component mixtures of similarly acting and mixtures of dissimilarly acting anti-androgens where individual chemicals are administered at doses more relevant to the human exposure situation than previously published high-dose studies. It remains to be seen whether the tools used in these studies will be useful for risk assessment of chemicals that cause a common effect. Initial indications from these studies on the effects of binary mixtures of chemicals with the same mode of action support the default assumption of dose additivity, previously recommended by the COT [81,82]. Studies of mixtures of dissimilarly acting anti-androgens will add significantly to understanding of their joint action and how to conduct risk assessments of such chemicals. The COT supports the continued research on characterising dose-response relationships for mixtures of anti-androgens. Analysis of these studies may require a more detailed understanding of the toxicokinetics of these chemicals.

32. Even though a clear link between experimental data and epidemiology is still missing, it was considered that the new data continue to emphasise the importance of

this area of research, the need to actively investigate causation and for risk assessment to incorporate consideration of potential for combination effects.

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