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COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

Summary of COM's evaluation of Germ Cell Mutagenicity

The COM explored recent advances in germ cell mutagenicity testing and some theories and hypotheses regarding human germ cell mutagenesis. The review included ;

- testing strategies and new assays, including assays used in human fertility investigations
- the concept, theories and evidence behind recent claims of an association between increasing de novo mutations in male germ cells being associated with certain diseases in off-spring
- the suggestion that air-pollution is a germ cell mutagen

The purpose of this paper is to summarise the conclusion reached.

Questions for the committee

1. Do the members have any further comment on this summary in general?
2. Do members have any further comment on the section related to germ-cell testing?
3. Do members wish to review the relative risk of germ cell mutagenesis in male and female germ cells? If not what conclusion can be drawn.

COM Secretariat

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1. The COM explored recent advances in germ cell mutagenicity testing and some theories and hypotheses regarding human germ cell mutagenesis. The review included ;

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Germ cell testing

2. The first paper considered was a 'Review of current approaches to germ cell testing (MUT/2013/05) which reviewed the literature describing assays for the assessment of chemically induced germ cell mutagenicity covering the period 2008-2013 . Novel assays discussed included a number of variations of the sperm comet assay, the sperm fluorescence in situ hybridization (FISH) assay, the expanded simple tandem repeat (ESTR) assay and whole genome sequencing (WGS) techniques. The majority of the assays described utilise rodent test systems.

3. The sperm comet assay is a variation of the somatic cell comet assay for which a OECD test guideline is available (TG489; 2014). This guideline does not encompass germ cells and it is widely accepted that the assay requires modification before it is considered validated in this cell type (Speit et al 2009). In the review, a number of modifications of the sperm comet assay were examined – including the formamidopyrimidine DNA *N*-glycosylase/AP-lyase (Fpg) assay which has been utilised in human sperm (Sipinen et al 2010; Nixon et al 2012; Laubenthal et al 2012). A fluorescence in situ hybridization (FISH) assay has been developed to detect aneuploidy sperm and has also been adapted for the detection of both numerical and structural chromosomal abnormalities in human sperm (ref). This offers a potential application in studies of chromosomally abnormal sperm after environmental , occupational or medical exposures (best refs)

4. The expanded simple tandem repeat (ESTR) mutation assay was also examined (best refs). These and mini-satellite regions contain highly mutable GC rich regions and this high spontaneous rate gives rise to a system which in principle is capable of detecting changes in mutation frequency in a relatively small population. This capacity is utilised to study mutations in sperm from animals and

humans. The COM concluded that further validation work was needed before these newly developed germ cell assays could be incorporated into general genotoxicity testing. Furthermore, is it considered that there are a number of methodological difficulties involved in the analysis of germ cells compared to somatic cells such as DNA extraction and the importance of good study design was highlighted.

5. Since the COM review, a report of the 2013 IWGT workshop on germ cell assays has been published (Yauk et al 2015) and provides a comprehensive summary of current assays to assess germ cell mutations. This includes specific locus test (OECD TG 485); dominant lethal test (OECD TG 478), cytogenetic analysis of spermatogonial metaphases (OECD TG482), transgenic mouse mutation assay in germ cells (OECD TG 488). Limitations of the later assay were highlighted , for example the best sampling time for sperm which may differ from the optimum sampling time for somatic cells. The potential to utilise information from reproductive and general toxicity assays as signals of germ cell mutagenesis were described. New technologies examined in the COM review were discussed (ESTR, sperm MN assay, sperm comet assay, sperm chromatin assay). The importance of understanding changes such as copy number variants (CNV) in terms of germ cell mutagenesis was highlighted and they also considered the need for examining pharmacokinetics in the male germ cell compartment.

6. It is considered that technical developments such as whole genome sequencing and the identification of copy number variants will contribute to a greater understanding of the outcome of germ cell mutagenesis, de novo mutations and heritable mutational (Singer and Yauk 2010). Harnessing genomics to identify environmental determinants of heritable disease were discussed by the ENIGMA (environmentally induced germline mutation analysis) working group. The phenomenon of age-related increases in de novo mutations in the male germ line and the role new sequencing and genomic techniques may have in investigating the aetiology of these mutations in humans were addressed (Yauk et al 2013). The group recommended a tiered approach, comprising preliminary studies to characterize the background variability, followed by specific definitive studies in highly exposed families (e.g. cigarette smoke, radiation, chemotherapy, air pollution). It was concluded that these should be prospective studies utilising well established epidemiological data collection metrics (e.g. family medical histories), exposure characterisation and biomonitoring. COM commented that it was not known whether unique germ cell mutagens exist (i.e. chemicals that are germ cell mutagens but not somatic cell mutagens), partially because the underutilisation of the currently accepted tests for assessing germ cell mutagenicity and a lack of investigations examining the possibility.

Increase in mutations in the aging male

7. The COM considered a scoping paper on germ cell mutagenesis, aging and disease in offspring (MUT/2015/11) which examined the recent interest in the contribution that *de novo* mutations in male germ cells make to human illness and disease in off-spring. This interest had arisen following publications of large datasets generated from high throughput, next generation sequencing methodologies which had enabled substantially more detailed analyses of human genome mutations, incidence and patterns, including those that occur from generation to generation

(McGrath et al 2014; Veltman and Brunner 2012). An investigation in the Icelandic population which sequenced the entire genomes of 78 parent offspring trios provided information on the importance of father's age on *de novo* mutation rates (Gudbjartsson et al 2015; Kong et al 2012). The frequency and diversity of single nucleotide polymorphisms (SNPs) increased with increasing age and it was estimated that 36 year old man will pass on twice as many mutations to his offspring as a 20 year old man (Callaway 2012). This increase in mutation rate has been associated with some diseases in children, particular focus on neurodevelopmental disorders, for example autism and schizophrenia (Goriely et al 2013; Malaspina et al 2015 Pedersen et al 2014).

8. An overview of the germ cell genome, meiosis, spermiogenesis and mutagenesis was provided. It was noted that an age related increase in spontaneous mutation rate in germ cells in rodents has been documented and that post-meiotic cells are more vulnerable to the effects of aging than pre-meiotic cells. COM commented that there are differences in meiosis and mitosis, and processes specific to germ cells (e.g. histone remodelling, multiple DSB's, unique repair), which means that it is possible that there are chemicals which affect only germ cells and raises the possibility that tests on somatic cells may not be relevant to gem cells. It is not clear what the contribution of the different endpoints (e.g. point mutations, chromosome aberrations, aneuploidy) to germ cell mutagenesis and paternal aging effect.

9. It was noted that the review focused on male germ cell mutagenesis and that the relative susceptibility, stage of division with greatest sensitivity and relative risk of germ cell mutagenesis could be different in male and female germ cells. For example, female germ cells are held in meiosis I, which increases the risk of aneuploidy – this was not considered further by the committee.

Human Germ cell mutagenesis

10. A number of significant publications commentating on, or collating current investigations and deliberations were reviewed. DeMarini from US EPA (2012) considered the past evaluation of human germ cell mutagenesis and notes the lack of chemicals being definitively classified as such. He notes that there are no international organizations for recognising germ cell mutagens if there were sufficient evidence to make these claims and suggests that there is a need to investigate and advise on the existence and implications of human germ cell mutagens, in a manner similar to that undertaken by IARC for human carcinogens. He suggested that there is sufficient information to classify the following as human germ cell mutagens: ionizing radiation, chemotherapy, smoking and air pollution changes. . COm commented that that DNA damage in germ cells can be associated with spontaneous abortions, infertility or heritable damage in the offspring/subsequent generations.

11. The claim that air pollution is a human germ cell mutagen, was substantiated by a number of studies in man and it was noted that these utilised routine semen characteristics/parameters (WHO parameters: semen volume, sperm concentration, sperm morphology, sperm motility) and assays of sperm DNA integrity, not evaluated by COM, as evidence of male germ cell DNA damage. The principle assays used

were the sperm chromatin structure assay (SCSA) and the TUNEL (terminal deoxynucleotidyl transferase mediated d-UTP nick end labelling) assay. These assays are most often used to investigate human fertility or those aiming to improve the outcome of assisted reproduction technologies (ART). It is suggest that DNA damage in the male germ line should be regarded as a risk factor for poor reproductive outcomes (Kleinhaus et al 2006). Prior to examining the claim that air pollution is a human germ cell mutagen it seemed appropriate to evaluated these assays. A paper, 'Assays used to evaluate germ cell DNA integrity in human fertility investigations' (MUT/2016/09) was considered by the COM.

12. The SCSA assay measures abnormal chromatin structure and involves staining with acridine orange which intercalates in double stranded DNA and stacks in single stranded DNA which causes differential shifts in fluorescence in a flow cytometer. Differences in ratios of red and green within the acceptable cell population represents susceptibility to DNA denaturation (Evenson et al 1999). The red population are considered to be those with denatured DNA and provide the DNA fragmentation index (%DFI) whilst the green coloured are high DNA stainable (HDS) sperm (Evenson 2006; Bungum et al 2011). COM examined a number of publications which had utilised SCSA to measure DNA integrity in infertility or ART scenarios. The use of SCSA in ART appear to assign a cut-off point of >30% DFI to designate infertility. However, on the whole, studies do not consistently show a clear association between fertility and DNA fragmentation.

13. The TUNEL assay is based on the labelling and detection of double and single strand breaks in DNA, using terminal deoxynucleotidyl transferase (TdT). Damage is quantified using fluorescence detection by microscopy or flow cytometry. The assay has been widely utilised to detect apoptosis but has also been developed for clinical investigations to examine sperm DNA damage with respect to infertility. During apoptotic cleavage, TdT activity principally detects double DNA strand breaks. This is considered to be a specific marker of apoptosis in somatic cells (Negoeso et al 1998). However, this has not been confirmed in germ cells, in which apoptosis is only one of the possible mechanisms causing sperm DNA fragmentation (Muratori et al 2010). Hence, the TUNEL assay is considered to have application in detecting genotoxic damage in sperm cells.

14. Members noted that as both the SCCA and the TUNEL were primarily assays for the detection of DNA strand breaks, they should only be considered as indicator assays as they do not inform on the consequences of the DNA strand breaks or downstream events (e.g they do not indicate whether DNA strand breaks lead to a mutation). It is unclear whether the observed reduced fertility was due to a genotoxic effect or a toxic effect. Members also agreed that the assays measured different types of DNA stand breaks and the observed DNA fragmentation could have arisen as a consequence of processes other than chemical induction (e.g oxidative stress, apoptosis) and it was not clear at what point in spermatogenesis the DNA damage occurs. Furthermore, the relatively high background level and range of DNA strand breaks present in sperm, would make it difficult to detect a chemically induced increase in DNA fragmentation.

15. Overall, the COM considered that that there was some lack of consistency and conflicting results reported in the data and papers provided (e.g. there were

conflicting reports on the correlation between the SCSA and the TUNEL assays). It was concluded that both the SCSA and the TUNEL assays would be difficult to interpret in terms of germ cell mutagenicity e.g. they were indirect methods for evaluating potential germ cell mutagenicity; there was a lack of consistency between some of the data and in the test methods used; uncertainty over the underlying biology leading to the formation of DNA strand breaks and downstream effects; a large variation in background levels and range of effects; and a lack of validation of the test methods.

Air pollution as a human germ cell mutagen

16. The claim that air pollution is a germ cell mutagen (DeMarini 2012) was examined by COM. The review was limited only to publications which directly addressed the impact of air pollution on human sperm. Several of these studies were based on a programme of work which analyzed effects of air pollution in a highly polluted area of the Czech Republic (district of Teplice) compared with a control, relatively unpolluted areas Sram et al 1999; Selevan et al 2000; Other studies investigated the impact of exposure to pollutants from traffic (Calogero et al 2011; Juerwicz et al 2015; Rubes et al 2010) or from an infertility clinic Ji et al 2013; A variety of endpoints and biomarkers were examined. For example, DNA damage markers/parameters in human sperm (e.g motility, morphology and increase in abnormal chromatin) or chromosomal (e.g X, x-y and or sperm 8 or 21 disomy) ; Pollution biomarkers included SO_2, NO_x, CO, PM_{10}, PM-TSP – total suspended particulates, PAH's. DNA damage was measured as fragmentation by the TUNEL assay or (DNS fragmentation index DI%) or SCSA. Some authors claim associations of changes in sperm parameters and air pollution.
COM were not able to corroborate the conclusion made by DeMarini , that air pollution is a human germ cell mutagen.

Questions for the committee

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5. Do members have any further comment on section related to germ-cell testing?
6. Do members wish to review the relative risk of germ cell mutagenesis in male and female germ cells? If not what conclusion can be drawn.

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