DB-ALM Method Summary n° 129 : Oocyte In Vitro Maturation Assay - Summary

Effects on the female fertility

The potential of a test compound to impair female fertility can be assessed by examining its effects on *in vitro* maturation of isolated oocytes, which are evaluated for their developmental stages after exposure (*part of the EU Integrated Project ReProTect*).

Objective & Application

The oocyte *In Vitro* Maturation (IVM) test was developed within ReProTect, a 5-years Integrated Project under the EU 6th Frame Work Programme, that finished in 2009 with the overall aim to develop an *in vitro* testing strategy for reproductive toxicity prediction to cover the entire mammalian reproductive cycle. The process of oocyte maturation is a possible target for chemicals affecting female fertility. This assay was developed as an *in vitro* test for the prediction of adverse effects of chemicals on the ability of oocytes to mature (Lazzari *et al.*, 2008). To detect adverse effects of chemicals on the follow-up process of *in vitro* fertilisation (IVF), the IVM assay can be combined with the IVF assay (a method summary on the IVF assay will be soon available in the DB-ALM) in a test battery (Lazzari *et al.*, 2008).

In general, alternative test methods for reproductive toxicity are intended to complement existing *in vivo* assays for screening purposes and to reduce or replace experimentation on mammals in future (Hareng *et al.*, 2005; Schenk *et al.*, 2010).

In fact, so far chemicals are tested for potential toxic effects on fertility with *in vivo* studies according to OECD Test Guidelines (415, 416, 421 and 422; OECD, 1983, 2001, 1995 and 1996), EU test methods (B.34, B.35; EC, 2008) and segment 1 studies (fertility and reproductive performance) according to the ICH guidelines (ICH, 2005).

Basis of the Method

Isolated bovine oocytes have been routinely applied for *in vitro* maturation and fertilisation procedures for assisted reproduction purposes in animal breeding, with high success rates at producing viable offspring, which indicates that these procedures can model *in vivo* reproduction processes (Bremer *et al.*, 2005; Lazzari *et al.*, 2008). *In vitro* Mechanistic studies of chemical effects (e.g. on spindle disruption, cell cycle delay) on isolated mice oocytes are performed for quite some time as well (Can and Semiz, 2000; Hou*et al.*, 2008; Rossi *et al.*, 2006; Tarín *et al.*, 1996).

Due to the observation that chemical exposure can affect the maturation of oocytes in a way that they are not able to reach the metaphase II, which is crucial for fertilisation, isolated oocytes have gained interest as a test system for *in vitro* evaluation of chemical effects on mammalian fertility (Bremer *et al.*, 2005; Eichenlaub-Ritter and Betzendahl, 1995; Hareng *et al.*, 2005; Krogenaes *et al.*, 1998; Prather and Racowsky, 1992). Known oocyte isolation, maturation and fertilisation techniques were found to be suitable to be adapted for developing a standardised protocol for an *in vitro* chemicals testing strategy (Bremer *et al.*, 2005).

It came that within ReProTect, a 5-years Integrated Project under the EU 6th Frame Work Programme, the herewith introduced protocol for an oocyte *in vitro* maturation assay with bovine oocytes was developed (Lazzari *et al.*, 2008; Luciano *et al.*, 2010).

Experimental Description

Biological and Endpoint Measurement:

Developmental stage: by evaluating the completion of meiosis up to “Metaphase II” done through the study of the nuclear morphology by observation in 200-400x magnification via microscope

Cell viability: by using Trypan blue exclusion test

Endpoint Value:

EC50: Concentration or dose that produces an alteration of an examined effect to 50% compared to the control value

Maturation rate: Percentage of matured oocytes vs. total number of cultured oocytes

Experimental System:

Oocyte culture: Isolated oocytes (bovine, mouse) or Cumulus-oocyte complexes (COC)

The experimental procedure as developed in the ReProTect project is described by Lazzari *et al.* (2008) and Luciano *et al.* (2010) as follows:

Experimental system

Bovine ovaries are obtained from abattoirs. Immature oocytes are recovered from follicles by dissection or aspiration. The oocytes are transferred to maturation medium (here TCM 199 with epidermal growth factor, FSH/LH, glutamine and sodium pyruvate). Maturation in culture is conducted for 24 h in a 5% CO_2 incubator in saturated humidity at 38.5 °C.

Chemical treatment

15-20 oocytes are matured per experiment in the maturation medium with the dissolved test substance. Several test substance concentrations are tested. A control group of non-exposed oocytes and a group exposed to a substance for positive control are matured as well. A reference substance can be added as an internal control. After 24 h exposure, the oocytes are removed, washed, placed on a slide and prepared for microscopy by fixing and staining.

Data analysis

Nuclear morphology is evaluated by observation at 200-400x magnification. The evaluated endpoint is the completion of meiosis up to the metaphase II stage. This developmental stage is characterised by extrusion of the first polar body. Oocytes which are not at the metaphase II stage are classified as not matured (Lazzari *et al.*, 2008; Luciano *et al.*, 2010).

An SOP is available in DB-ALM as Protocol No. 129: "Toxicity Test on In Vitro Maturation of Bovine Oocytes"

Data Analysis/Prediction Model

The maturation rate is expressed as the percentage of metaphase II oocytes relative to the total number of cultured oocytes in the maturation medium (González *et al.*, 2010; Lazzari *et al.*, 2008). A dose-related response is considered as an evidence for a positive effect (Schenk *et al.*, 2010). Consequently, an EC50 value can be calculated from the dose-response data by non-linear regression analysis (Lazzari *et al.*, 2008; Luciano *et al.*, 2010).

No prediction model has been published up to now, but according to the DB-ALM Protocol No. 129 a preliminary prediction model was set up with the results from Lazzari *et al.* (2008) for the maturation endpoint (reaching of metaphase II): EC50 values below 50 µM correspond to a positive effect, EC50 values above 50 µM to a negative effect on the *in vitro* maturation of oocytes (DB-ALM Protocol No. 129, 2010).

Test Compounds

Some of the chemicals tested with the IVM assay, are: Organochlorines and polychlorinated biphenyls, fungicides, glucocorticoids, nicotine, insecticides, herbicides, heavy metals, endocrine disruptors.

The IVM assay has been challenged with 15 chemicals by Lazzari *et al.* (2008), including drugs, preservatives and agricultural chemicals. Schenk *et al.* (2010) tested 10 chemicals with the bovine oocyte IVM assay: methyl acetoacetate, glufosinate ammonium, methoxyacetic acid, sodium chloride, cadmium chloride, carbendazim, nitrofen, nitrobenzene, vinclozolin, bisphenol A.

Modifications

Several other research groups use experimental protocols similar to that reported in “Basic Procedure”; some of them use mouse oocytes (Chan, 2008; Lefèvre *et al.*, 2000; Pocar *et al.*, 2006; Rossi *et al.*, 2006). The oocytes can be cultured with their surrounding cumulus cells as complete cumulus-oocyte complexes (COC) (Chan, 2008; Rossi *et al.*, 2006) or the cumulus cells are removed before IVM (Lazzari *et al.*, 2008; Luciano *et al.*, 2010).

Oocytes are used by various investigators (not specifically referring to the protocol for the oocyte IVM assay developed in ReproTect) to study the effects of chemicals on oocytes for research purposes. Several other endpoints different from the maturation rate have been assessed in these studies. Cell viability (Casas *et al.*, 2010), gene expression (Bonilla *et al.*, 2008), alterations in meiotic spindle formation (Hou *et al.*, 2008; Rossi *et al.*, 2006), expansion of the cumulus shell (González *et al.*, 2010; Li *et al.*, 2009; Talbot, 2008) and if COCs are used cumulus cell apoptosis (Pocar *et al.*, 2005), and chromosome number / aneuploidy (Hou *et al.*, 2008; Li *et al.*, 2009; Pacchierotti and Ranaldi, 2006) can be observed as well. In

these types of investigations non-mammalian donors such as the Zebrafish (Seki *et al.*, 2008) and Xenopus (LaChapelle *et al.*, 2007; Zhang *et al.*, 2009) were also used.

Discussion

The process of oocyte IVM is highly sensitive to toxic effects, even more than the IVF (Lazzari *et al.*, 2008). The meiotic process (formation of gametes with half the original number of chromosomes) can reveal toxicity of chemicals *in vitro* at lower concentrations than the mitotic process (duplication of cells) in somatic cells (Lazzari *et al.*, 2008; Luciano *et al.*, 2010). Rossi *et al*. (2006) found accordingly that the oocyte IVM test can be used as a sensitive test for assessing pesticide impact, even at low concentrations.

The response range of the IVM covers several orders of magnitude, allowing a distinction between highly toxic and less toxic chemicals (Lazzari *et al.*, 2008).

Lazzari *et al.* (2008) have tested 15 chemicals (each 3 times) with the oocyte IVM test. The good concordance of results shows a high within-laboratory reproducibility of this assay, according to the authors (Lazzari *et al.*, 2008).

Luciano *et al.* (2010) tested 8 of the 15 chemicals mentioned above in two laboratories. They concluded a high between-laboratory reproducibility and good concordance of IVM test results from a maximum ratio of the obtained EC50 means of 1.35 (for one of the tested compounds) (Luciano *et al.*, 2010). Moreover, Luciano stated that IVM procedures are routinely and successfully applied for animal breeding purposes indicating that this procedure can closely mimic the corresponding *in vivo* process (Luciano *et al.*, 2010). As far as bovine oocytes are used for this test, these are isolated from ovaries of slaughtered animals; thus no additional sacrifice of animals is required (Luciano *et al.*, 2010). Mouse oocytes are used as well, for their ease of harvest and shorter culture period (Hou *et al.*, 2008). A method for collection of mouse oocytes without euthanasia has been published (Byers *et al.*, 2009).

Schenk *et al.* (2010) tested 10 chemicals with a test battery consisting of 14 *in vitro* tests resulting from the ReProTect project, including the bovine oocyte IVM assay. Due to the lack of methodological details, results of this ReProTect-feasibility study for individual chemical compounds were not included into DB-ALM as test result data sheets. The results from the IVM tests led to a correct prediction (an observed dose-response relationship was taken as evidence of a positive result in the *in vitro* assay) of the occurrence of *in vivo* effects (the ability to impair female fertility) for 6 chemicals (2 correctly positive, 4 correctly negative) and to a false prediction for 3 chemicals (1 false positive, 2 false negative). One chemical gave ambiguous results in vivo and in vitro (Schenk *et al.*, 2010). Therefore, it is concluded by the authors that the IVM assay could advance towards validation and might become part of an integrated testing strategy with other *in vitro* assays to predict chemical hazards to mammalian fertility in future (Lazzari *et al.*, 2008; Luciano *et al.*, 2010).

The higher sensitivity (i.e., observable effects at lower test compound concentrations) of the IVM assay compared to the IVF assay might not directly apply to the *in vivo* situation, as oocyte maturation takes place in the ovary and fertilisation in the oviduct. Therefore, the type of substance (test substance or metabolite) and the amount of the chemical reaching the target process might differ between the two locations (Lazzari *et al.*, 2008).

The effects of chemicals on oocyte IVM can differ between species. This fact suggests including several model species in alternative test strategies for reproductive toxicity (Lazzari *et al.*, 2008).

The outcome of the IVM assay might also be influenced by the presence of cumulus cells; chemicals may act different on oocytes surrounded by cumulus cells than on denuded oocytes (Lazzari *et al.*, 2008).

Until now, no attempt was made to address the issue of possible test substance metabolisation; further studies are planned (Lazzari *et al.*, 2008).

Status

Participation in evaluation study:
The herewith reported experimental procedure of the Oocyte *In Vitro* Maturation Assay was developed in the ReProTect project (Lazzari *et al.*, 2008; Luciano *et al.*, 2010). Within the frame of this project, Lazzari *et al.* (2008) and Luciano *et al.* (2010) tested 15 and 8 chemicals with the IVM assay, respectively, for within- and between laboratory reproducibility and transferability. Luciano *et al.* (2010) concluded that the IVM test could advance towards validation as an alternative *in vitro* method as part of an integrated testing strategy with other *in vitro* assays to predict chemical hazards to mammalian fertility. This assay was also tested in the ReProTect feasibility study as part of an integrated *in vitro* testing strategy for detecting potential chemical hazards to female fertility (Schenk *et al.*, 2010). Therein, Schenk *et al.* (2010)

tested 10 chemicals with a test battery of 14 *in vitro* assays, including the bovine oocyte IVM assay, and found that the majority of the predictions based on the obtained *in vitro* results turned out to be correct compared to *in vivo* data.

So far, no information on formal validation studies and no prediction model are available in the international literature. This method is not mentioned in any official regulations, guidelines or recommendations on reproduction toxicity testing.

Known Laboratory Use:
A questionnaire survey was performed among members of EPAA (The European Partnership for Alternative Approaches to Animal Testing, a collaboration between the European Commission, European trade associations, and companies from seven industry sectors) in November 2010 to investigate the real use of each method among the industries.

The occasional, project-dependent use of this method was reported by one company (a service provider; one out of 11 questionnaire responses) for the assessment of chemical effects on the female reproductive system for substance-related mechanistic studies. It is used as part of a test battery or as part of an Integrated Testing Strategy (ITS). More than 20 substances have been tested with this method since its introduction in this laboratory in 2003. Predictivity, evaluated against results from other tests, is described as good by this company.

Abbreviations & Definitions

COC: Cumulus-Oocyte Complexes
DB-ALM: DataBase on Alternative Methods to animal experimentation
EC: European Commission
EC50: half maximal Effective Concentration
EPAA: The European Partnership for Alternative Approaches to animal testing
EU: European Union
FSH: Follicle-Stimulating Hormone
ICH: International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use.
ITS: Integrated Testing Strategy
IVF: *In Vitro* Fertilisation
IVM: *In Vitro* Maturation
LH: Luteinising Hormone
OECD: Organization for Economic Cooperation and Development
SOP: Standard Operating Procedure
TCM: Tissue Culture Medium

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