

10.0 ANIMAL WELFARE CONSIDERATIONS

10.1 Refinement, Reduction, and Replacement Considerations

ICCVAM promotes the scientific validation and regulatory acceptance of new methods that refine, reduce, or replace animal use where scientifically feasible (ICCVAM, 1997; P.L. 106-545). Refinement, Reduction, and Replacement are known as the three Rs of animal protection. These principles of humane treatment of laboratory animals are described as:

- Refining experimental procedures such that animal suffering is minimized;
- Reducing animal use through improved science and experimental design; and
- Replacing animal models with nonanimal procedures (e.g., *in vitro* technologies), where possible.

Combes (2000) and Phillips (2000) recommended that adequate consideration be given to animal welfare concerns by careful development and validation of all proposed endocrine disruptor screening methods. With respect to the proposed use of *in vitro* ER binding assays as screening methods to detect substances that potentially exhibit estrogenic or anti-estrogenic activity, it is important to evaluate the current level of animal use in these assays and to consider what opportunities exist for refining, reducing, or replacing procedures that use animals.

10.2 Use of Animals in *In Vitro* ER Binding Assays

Of the 14 *in vitro* ER binding assays considered in this BRD, three assays (RUC, MUC, RBC) require the collection of uterine tissue from female rats, mice, or rabbits. Because the animals are not treated with a test substance, treatment-related pain and suffering are avoided. Some investigators that use the RUC and MUC assays obtain the uterus from ovariectomized mature female rats, while other investigators use nonovariectomized, sexually immature female rats. Some investigators prefer the former procedure because removal of the ovaries appears to increase uterine ER production in the rat for about 5 to 14 days after an ovariectomy. Thus, more ER can be obtained per gram of uterine tissue in comparison to the procedure using non-ovariectomized, sexually immature females. One investigator who uses uteri from overiectomized rats in the RUC assay estimates that one average-sized mature rat uterus (~200 mg) generates enough cytosol to test one substance at six concentrations in triplicate

(personal communication, Dr. Hong Fang, NCTR). Corresponding information on the amount of cytosol generated from sexually immature rats was not obtained.

With respect to refining the uterine cytosol assays, procedures that are the least invasive and distressful to the animals should be used. As for reducing the number of animals used in these assays, protocols should maximize the number of substances that can be tested per gram of tissue, for example, by optimizing the protocol to use the lowest possible concentration of ER per assay tube. In addition, the use of sexually mature versus immature animals should be carefully considered. While the use of immature animals only would reduce the need for ovariectomies, using sexually immature animals, which have substantially smaller uteri than mature ovariectomized animals (e.g., 30-50 mg versus 200 mg for the rat), would require that more animals be used.

The other 11 *in vitro* ER binding assays considered in this BRD do not use animals. Two of these assays -- the MCF-7 cell and MCF-7 cytosol assays -- use a human cell line, while the remaining assays use purified or semi-purified human or animal receptors derived from cDNA or from GST-ER fusion proteins. The experimental systems using purified receptors, semi-purified receptors, or fusion proteins can be carried out in multiwell plates, which permit smaller reaction volumes and allow data collection to be partially or fully automated. With the potential for automation, these systems would be more economical to perform than the uterine cytosol assays, which require animal care and surgical costs. Another advantage to using purified (cloned) ER is that ER α and ER β can be used selectively.

The assays using human ER or the ligand binding domain of the human ER are directly relevant to humans, as compared to ER derived from rodent or rabbit tissues. However, because of the relative newness of these assays, they have not been used as extensively as the uterine cytosol assays for the routine testing of substances; thus, their reliability and performance have not been demonstrated to the same extent. Despite the lack of a substantial database on assays using purified and semi-purified ERs, these assays, with further development and validation, could potentially replace the use of uterine cytosol to determine the ER binding of substances.