6.0 DEVELOPMENTAL STATUS OF THE ASSAY AND RECOMMENDATIONS FOR PREVALIDATION STUDIES

6.1 Current Status

The endpoint included in the sectioned testes assay has been evaluated in other studies. However, the protocol itself has not been validated. Pending a final decision on the study design, the protocol would be ready to enter the prevalidation phase.

6.2 Recommendation for Optimization of the Sectioned Testis Assay Protocol

6.2.1 Testicular Preparation Issues

Optimization of the assay could be determined for the amount of testis actually needed to obtain a given level of sensitivity. For example, a single testis from an adult SD rat weighs approximately 1 g. If such a testis were quarter sectioned, then each section would weigh approximately 250 mg, which is the weight of the sample generally described by investigators who have used quartered sections of testis. However, no documentation was found that demonstrates whether smaller sections would give similar results. Thus, it would be advantageous to conduct a study that investigates whether the sensitivity of the preparation is affected by the amount of testicular tissue used and, if so, if there is an optimal and/or threshold amount to use. The weight of the sections to be tested could range from the customary amount used, i.e., 250 mg, down to an amount of tissue that represents a practical minimum, e.g., 5 to 10 mg.

It would also be useful to explore storage and viability of the testis and/or sections. One possible scenario to assist in meeting an ICCVAM and U.S. EPA objective to reduce, refine, and replace animal usage would be to use testes from animals in a separate study, that requires castration in the experimental design, e.g., Hershberger studies. It may be possible to store testes after removal in such a way that they remain viable, can be shipped to various locations, and are used at a later date for the in vitro sectioned testis steroidogenesis assay. A storage condition, stability, and viability study could be designed and tested.

6.2.2 Endpoint Issues

The importance of measuring progesterone could be evaluated. For example, it is possible that the stoichiometric molar relationship for progesterone and testosterone is not 1:1. If such a relationship exists and a substance inhibits progesterone production but the “pool” of progesterone is sufficiently large such that the production of testosterone is not affected, then the assay would not detect an effect on steroidogenesis if only testosterone were measured. However, this effect would be observed if progesterone were measured. This possibility could be determined during the initial experiments used to optimize the assay.
The stability of the media samples could be determined during these initial studies. Since the assay lends itself to multiple endpoints but the assay is most efficient by measuring a single important endpoint, i.e., testosterone, it would be useful to evaluate the length of time that the media could be stored and used at a later date to measure other endpoints, e.g., progesterone, estradiol. This information could easily be obtained by conducting storage stability studies of the media collected from studies used to optimize the experimental design.

Another experimental design factor that could be optimized by experimental determination is the number of collection time points. The current study design includes four time points. Media samples are collected at 1, 2, 3, and 4 hours after the incubation is initiated. The possibility exists that other time points are better suited to characterize the effect of a substance on steroid hormone production. Along those same lines, perhaps fewer time points are equally as useful to measure an effect. Statistical analysis could be used to determine whether concentrations measured at 1 and 4 hours provide no more or no less information than that obtained by measuring samples at four different time points. Such information could be used to reduce extraneous collection and analysis steps.

6.2.3 Stimulation Factor Issues

The initial studies need to optimize the concentration of stimulant added to the testicular preparation. The stimulant planned for use is hCG. The amount of stimulant used is important because it can affect whether a steady proportional increase in steroid hormone production occurs over the entire duration of the incubation period. Also, a measure of the variability of different lots of hCG could be determined during such experiments. This would serve to provide needed information about factors that affect the variability of the assay.

6.3 Recommendation for Sectioned Testis Assay Prevalidation Studies

Prevalidation studies following the ICCVAM validation process should be initiated. Prevalidation studies should include evaluation of six to eight substances to establish the database for the validation studies. It is recommended that the study be performed using test substances with different chemical classifications, as well as varying sites and/or mechanisms of action, which will aid in the development of the prevalidation database for the assay. The recommended positive and negative control test substances were selected based on their sites of action, i.e., aminoglutethimide inhibits P450<sub>SCC</sub> and finasteride inhibits 5α-hydroxylase, which is not found in the testes. Other test substances of interest that are recommended for testing in the prevalidation studies include:

- bisphenol A (inhibits steroidogenic signal transduction)
- lindane (inhibits signal transduction and the StAR protein)
- ketoconazole (a weak imidazole anti-fungal; inhibits P450<sub>SCC</sub> and aromatase)
- genistein (a weak phytoestrogen/flavonoid; inhibits 3β-HSD)
- flutamide (inhibits P450c17)
- econazole (a potent imidazole; inhibits aromatase).

6.4 **Recommendation for Further Development of Cell Line Methods**

In addition to the prevalidation studies for the *in vitro* sectioned testis assay, further characterization and development of the cell lines as screening tool assays is recommended. Based on the information summarized in Section 4.5 (Table 4-10), there are 2 to 3 cell lines that could be studied further for their possible use as assays for testing substances for steroidogenesis altering activity. The recommended cell lines are the MA-10, R2C, and H295R cells. These cell lines are recommended because they represent cell lines from three different species, i.e., mouse, rat, and human, respectively. The Leydig-like steroidogenic properties of the MA-10 cell line have been characterized to the greatest extent and exhibit many of the properties of Leydig cells up through the production of progesterone. In addition, the MA-10 cell line will provide a good standard for comparison as the properties of the other cell lines are more fully investigated, which is also recommended in the prevalidation studies.

Another reason that these cell lines are recommended for further study is that they are readily available; Dr. M Ascoli (University of Iowa, Ames, Iowa) holds the MA-10 cell line and the American Type Culture Collection (ATCC) stocks the R2C and H295R cell lines. As for the H295R cell line, it is unique in that it is an immortalized human cell line and, although it is derived from non-gonadal tissue, it appears to possess many of the properties that would make it a viable tool for testing substances for their effects on both the gonadal and adrenal steroidogenic pathways. Finally, the goal of the EPA to develop non-animal assays could be further attained if one or more of these cell lines were found to be useful investigative paradigms. For these reasons, it is recommended that consideration be given to further study of cell lines as screening tools for identifying substances with steroidogenic altering activity.
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7.0 REFERENCES


APPENDIX A:

LITERATURE SEARCH
APPENDIX A

Literature Search

A comprehensive literature search was conducted in support of this DRP. Appendix A describes the initial results from a Dialog search of electronic databases. In addition, as reference sources were obtained and reviewed following this initial screen, additional information was gathered, reviewed, and included in the DRP as appropriate. The Reference section (7.0) presents citations for all works discussed in the DRP.

Databases Searched

Steroidogenesis Search Strategy

Date of Search: 13 April 2001

Database Files

The following files from the database vendor, Dialog®, were searched:

File 155 MEDLINE—Covers virtually every area in the broad field of biomedicine. Coverage is from 1966-present.

File 156 TOXLINE—Information on the toxicological effects of chemicals, drugs, and physical agents on living systems. Coverage is from 1965-2000.

File 144 PASCAL—Provides access to the world's scientific and technical literature in the fundamental disciplines of physics and chemistry; life sciences (including biology, medicine, and psychology), applied sciences and technology, earth sciences, and information sciences. Includes about 450,000 new citations per year. Coverage is from 1973-present.

File 5 Biosis Previews—Comprehensive, worldwide coverage of research in the biological sciences and biomedical sciences. Coverage is from 1969-present from nearly 6,000 primary journals and monographic titles.

File 73 EMBASE—Comprehensive index of the world's literature on human medicine and related disciplines. EMBASE provides access to periodical articles from more than 3,300 primary journals from approximately 70 countries. Coverage is from 1973-present.

File 34 SciSearch®: A Cited Reference Science Database—An international, multidisciplinary index to the literature of science, technology, biomedicine, and related disciplines produced by the Institute for Scientific Information® (ISI®). SciSearch contains all of the records published in the Science Citation Index® (SCI®), plus additional records from the Current Contents® publications. Coverage is from 1990-present.
Database Search Strategies

English Language Articles
Foreign language articles with English abstracts

Keywords and phrases considered

- steroidogenesis
- minced testis
- minced ovary
- dihydrotestosterone
- P450 isoforms/isozymes
- testis
- Leydig cells
- ovary
- Luteinizing hormone (LH)
- aromatase
- adrenal cortex
- 5-alpha reductase
- fetal steroidogenesis
- cholesterol

Storage of electronic database information in Reference Manager format

Results of the online search:

- The search term "steroidogenesis" was used; the term was limited to appear in at least the titles or descriptors of relevant records; this search set was then combined with the search set on the term “vitro.” This resulted in 7008 records.

- This search then combined a search on the terms "method or methods or methodology or assay* or test or tests or testing or protocol* or guideline*.” This reduced the set to 887 records. [Note: The asterisk denotes the use of a truncation symbol to gather plurals or alternate endings of terms.]

- The term “testosterone” was added to this second set and limited the results to be in the English Language only. After removing duplicate records, 179 items remained.

- Next, I added the term “dihydrotestosterone” to the second set. After limiting by English Language only and removing duplicate records, 11 items remained.

- Going back to the second set (method or methods), duplicate records were removed from that set to see how many unique records were there. This was also limited to English Language only. This reduced the set from 887 to 607 records. From this set, a sample of 12 items was retrieved for initial review to determine appropriateness of journals retrieved. Based upon examination of the sample records, it was apparent that the terms “method or methods” were problematic. It was decided to limit those terms to appear in only the descriptors of relevant records. This resulted in 110 items from search Set 2 having the terms “method or methods” appearing in the descriptors.
C The second search was repeated by initially leaving out the terms “method or methods” and then searching them separately with Set 1. This search was then combined with our previous search, limited by English Language only and removed duplicate records, resulted in 530 records remaining, down from 607 records.

C A sample of records from this set of 530 were then reviewed. After review of this sample, a decision was made to include the concept “procedure*,” in doing so, this search was combined with Set 1, resulting in 111 records retrieved. The term “procedure*” was limited to appear in at least the titles or descriptors of relevant records. After removing duplicate records and limiting to English Language, only 6 records remained.

C Upon investigation of the titles of the 6 records, it was concluded that they were on target. However, during examination of other sample records, the terms, “test, tests, or testing” were also problematic. Articles where the terms “test, tests, or testing” appeared only in the abstract were removed, leaving a total of 420 articles remaining. This set was combined with the 6 from the “procedure” search, making a total of 426 records.

C From this set of 426 records, a online review of approximately 12 articles was conducted to determine whether identified articles met research expectations. It was concluded that this set of articles closely matched the research objectives of this task. However, to ensure adequate coverage of the published literature on steroidogenesis, a slightly different retrieval approach was used to find additional records.

C Another search was conducted on the term “steroidogenesis,” limiting this term to appear in at least the titles or descriptors of relevant records, and combining that with the phrases “minced ovary or minced testes or minced testis.” After removing duplicate records from the above set of 426, 9 items remained. This set was then combined with the previous set for a grand total of 432 records. All 432 records were downloaded in a tagged format.

Literature Evaluation Process

Criteria for Literature Selection

C Appropriateness of methods for measuring endpoints of interest,
C method clearly described
C appropriate use of controls
C data adequately reported
C appropriate statistical analysis

Literature Review
Summary of the Review Process

Literature describing *in vitro* studies of mammalian reproduction and/or endocrine disruption, and/or applicable steroidogenesis test protocol evaluation was retrieved. Approximately 232 of the 432 references were reviewed for relevancy. The references were given a number from the reference list. These references were divided into ovarian, testicular, and adrenal steroidogenesis. Twenty-eight papers regarded adrenal steroidogenesis and were not used in this paper. Twenty-two were not in mammals and were not included in the ovarian steroidogenesis descriptions of methods. The ovarian references were divided into categories such as human IVF granulosa cell cultures (32), by species and compounds, and by methods used to determine steroidogenesis. Approximately 11 papers were purely mechanistic in nature. The references were then read by the authors and placed in the following categories:

C 5—Excellent methods with technical details, highly relevant
C 4—Good methods, relevant
C 3—Good for other areas such as background, introduction, references, some relevance
C 2—Poor for the purpose of this report, little interest
C 1—Not useful for this report, no interest
C 0—Not relevant to this report

Each article in each group was then evaluated according to the criteria described. Summaries of the best articles that illustrated the usefulness of an *in vitro* exposure protocol for detection of endocrine-disrupting activity of the test compound are presented below.

Hazard- and risk-based study designs may examine different endpoints, but if these endpoints will be used for risk assessment, they must first be shown to be robust, reproducible, appropriately sensitive, biologically plausible, and relevant to the adverse outcomes of concern. Definitions of the attributes of such endpoints are as follows:

**Reproducible:** These endpoints must be reliable; the same findings occur under the same conditions within the initial reporting laboratory (intra-laboratory) and among other laboratories (inter-laboratory). If the results from endpoints are not reproducible, they cannot form the basis for future research and are most likely not useful for risk assessment.

**Robust:** These endpoints must be present after comparable routes of exposure (e.g., whole organism as well as cell or organ culture). Different effects may be observed by different exposure routes, different species, or different neurological controls. The findings from routes unrelated to human or environmental exposures may not be useful for risk assessment. These findings must also be present at the same routes and doses over time.
**Sensitive:** These endpoints should not be dependent on unique conditions, especially those which are not relevant to the species at risk. These endpoints should not exhibit high variability (insensitive) or be greatly affected by confounders (too sensitive).

**Relevant:** These endpoints must be biologically plausible and related to adverse effects of interest/concern. If there are no adverse effects at the dose/duration/route evaluated, these endpoints should be predictive of other adverse effects at higher doses, after longer exposure duration, and/or by different routes, etc.

**Consistent:** These endpoints must occur in the presence of effects in other related, relevant endpoints, if possible, at the same dose, timing, duration, routes of exposure, etc.

The literature was evaluated in terms of how well the study design was described. Studies that were described in such a way that they could be repeated in the authors’ laboratory only on the basis of information contained in the manuscript were further evaluated for scientific soundness, and for the likelihood that the results could be reproduced by repeating the experiment.

The literature was evaluated for the use of appropriate controls. In *in vitro* studies, the use of a concurrent control sample (media) that is under the same culture conditions as the treated samples is essential for valid statistical analysis.

Data and results were considered to be adequately reported if the data followed the study design in a logical manner, and all of the animals or samples could be tracked through the description of the results and placed appropriately in their data groups. In addition, it was necessary that the data appear to be consistent and realistic within groups and compared to the concurrent control.

Data analysis was evaluated with regard to the use of appropriate statistical methods, appropriate comparisons with the control group, tightness of data, and appropriate interpretation of the statistical results.

This appendix contains the entire “online” search results in chronological fashion such that one can discern the number of articles indexed for given key word combinations and phrases that were encountered during the online search conducted in April 2001. This section also includes the title and abstract for all articles retrieved.

**Summary of Literature Retrieved**

The purpose of this section is to numerically categorize and organize the articles (hard copy retrieved articles) for the steroidogenesis reference manager database. The criteria used are species, test species sex, tissue type, chemicals or steroids used, and various components of experimental design. The experimental design is further categorized into those studies involving the investigation of a certain chemical on the
steroidogenesis process, along with those studies that simply investigate the general biological function of the steroidogenesis process. Many of the papers in this database explore several different species, tissues, chemicals, and methods. Therefore, the numbers displayed in the following tables do not add up to the total number of papers retrieved.

Of the 432 titles identified during the initial search, approximately 264 were identified for full text retrieval; of those, 232 were able to be obtained and consequentially reviewed.

**Table A-1. Summary of Database Count**

<table>
<thead>
<tr>
<th>Total Number of Papers in Database</th>
<th>264</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Papers Retrieved</td>
<td>232</td>
</tr>
</tbody>
</table>

The papers in this database that were retrieved were categorized into two different types. The first type included all papers that investigated the basic biological function of a system. This included papers describing alterations in hormone levels and the biological activity of naturally produced hormones and steroids etc. The second type included those papers that investigated the effects of an outside substance on the steroidogenic process or system.

**Table A-2. Breakdown of Paper Objectives**

<table>
<thead>
<tr>
<th>Papers Describing Biological Function of a System</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papers Describing Chemical Influence on a System</td>
<td>32</td>
</tr>
<tr>
<td>Papers Not Yet Retrieved</td>
<td>32</td>
</tr>
</tbody>
</table>

This database contained papers studying multiple species of organisms. As stated previously, many papers included more than one species of organism in their study, so the values given will not add up to the total number of papers in the database.
Table A-3. Breakdown of Species Types Used in Database Research Papers

<table>
<thead>
<tr>
<th>Species Type</th>
<th>Number of Papers Concerning Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>92</td>
</tr>
<tr>
<td>Humans/Primates</td>
<td>62</td>
</tr>
<tr>
<td>Mice</td>
<td>18</td>
</tr>
<tr>
<td>Fish</td>
<td>14</td>
</tr>
<tr>
<td>Cows</td>
<td>13</td>
</tr>
<tr>
<td>Pigs</td>
<td>10</td>
</tr>
<tr>
<td>Rabbits</td>
<td>8</td>
</tr>
<tr>
<td>Horses</td>
<td>5</td>
</tr>
<tr>
<td>Hamster</td>
<td>4</td>
</tr>
<tr>
<td>Sheep</td>
<td>4</td>
</tr>
<tr>
<td>Birds</td>
<td>4</td>
</tr>
<tr>
<td>Reptile</td>
<td>3</td>
</tr>
<tr>
<td>Snakes</td>
<td>2</td>
</tr>
<tr>
<td>Lizards</td>
<td>2</td>
</tr>
<tr>
<td>Frogs</td>
<td>1</td>
</tr>
<tr>
<td>Gerbil</td>
<td>1</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>1</td>
</tr>
<tr>
<td>Goats</td>
<td>1</td>
</tr>
<tr>
<td>Shellfish</td>
<td>1</td>
</tr>
<tr>
<td>Crustacean</td>
<td>1</td>
</tr>
<tr>
<td>Insects</td>
<td>1</td>
</tr>
</tbody>
</table>

Several papers in the database only reviewed the methods and studies of others. These papers included no new experimental research, therefore not involving any specific species or chemicals.

Table A-4. Listing of Review Papers

| Number of Review Papers (no experimental research conducted) | 3 |

Many of the papers in the database were sex-specific studies on certain cells and systems. However, there were several papers that included both sexes. Table A-5 shows the breakdown of studies concerning the male systems, and Table A-6 those concerning female systems.
Table A-5. Papers Investigating the Male Reproductive and Hormonal System

<table>
<thead>
<tr>
<th>Tissues/Systems Studied Concerning Male Endocrine System</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes/Testosterone</td>
<td>112</td>
</tr>
<tr>
<td>Leydig Cells</td>
<td>47</td>
</tr>
<tr>
<td>Male Infertility</td>
<td>1</td>
</tr>
</tbody>
</table>

Table A-6. Papers Investigating the Female Reproductive and Hormonal System

<table>
<thead>
<tr>
<th>Tissues/Systems Studied Concerning Female Endocrine System</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovaries/Hormones</td>
<td>170</td>
</tr>
<tr>
<td>Granulosa Cells</td>
<td>80</td>
</tr>
<tr>
<td>Follicle</td>
<td>88</td>
</tr>
<tr>
<td>Placenta</td>
<td>26</td>
</tr>
<tr>
<td>Mammary</td>
<td>1</td>
</tr>
<tr>
<td>Uterine</td>
<td>3</td>
</tr>
</tbody>
</table>

The database included 59 non-sex-specific studies. These included studies on the adrenal, pituitary, neurological, and urinary systems. Although these papers study the effects of non-sex-specific organs, many of them did study the effects of these organs and their products on sex-specific systems.

Table A-7. Papers Investigating the Adrenal, Pituitary, Neurological, and Urinary Systems

<table>
<thead>
<tr>
<th>Adrenal/Pituitary/Neurological/Urinary Papers</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal</td>
<td>39</td>
</tr>
<tr>
<td>Pituitary</td>
<td>7</td>
</tr>
<tr>
<td>Neurological</td>
<td>13</td>
</tr>
<tr>
<td>Urinary</td>
<td>3</td>
</tr>
</tbody>
</table>

Many different assay methods were used in the papers included in this database. Radioimmunoassay (RIA) was the most frequently used, but again, many of the papers used more than one method of bioassay.
Table A-8. Methods of Gathering Data

<table>
<thead>
<tr>
<th>Method</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioimmunoassay Procedure (RIA)</td>
<td>138</td>
</tr>
<tr>
<td>Scintillation</td>
<td>15</td>
</tr>
<tr>
<td>Fluorometry</td>
<td>2</td>
</tr>
<tr>
<td>Chromatography</td>
<td>49</td>
</tr>
</tbody>
</table>

Most of the research conducted in the database papers was done \textit{in vitro}, although 44 studies used \textit{in vivo}. Some studies included both methods.

Table A-9. \textit{In Vitro} vs. \textit{In Vivo}

<table>
<thead>
<tr>
<th>Method</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro studies</td>
<td>249</td>
</tr>
<tr>
<td>In vivo studies</td>
<td>53</td>
</tr>
</tbody>
</table>

Multiple hormones, steroids, enzymes, and proteins were studied in these papers. Numbers given in Table A-10 only indicate the presence of a certain chemical in a specific study. It does not indicate that it was the \textit{only} chemical studied, however.
### Table A-10. Breakdown of Steroids, Chemicals, and Hormones Examined

**Note:** Indication of use does not imply that the study only examined one chemical, but rather that it was involved somewhere in the process.

<table>
<thead>
<tr>
<th>Hormone/Steroid</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>140</td>
</tr>
<tr>
<td>Testosterone</td>
<td>111</td>
</tr>
<tr>
<td>Estradiol</td>
<td>108</td>
</tr>
<tr>
<td>Luteinizing Hormone (LH)</td>
<td>93</td>
</tr>
<tr>
<td>Human Chorionic Gonadotropin (hCG)</td>
<td>82</td>
</tr>
<tr>
<td>Follicle Stimulating Hormone (FSH)</td>
<td>69</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>45</td>
</tr>
<tr>
<td>Estrogen</td>
<td>37</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>31</td>
</tr>
<tr>
<td>Insulin</td>
<td>26</td>
</tr>
<tr>
<td>ACTH</td>
<td>24</td>
</tr>
<tr>
<td>Aromatase</td>
<td>21</td>
</tr>
<tr>
<td>Corticotropin</td>
<td>15</td>
</tr>
<tr>
<td>Prolactin</td>
<td>12</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>9</td>
</tr>
<tr>
<td>Serotonin</td>
<td>9</td>
</tr>
<tr>
<td>Forskolin</td>
<td>8</td>
</tr>
<tr>
<td>Growth Hormone (GH)</td>
<td>7</td>
</tr>
<tr>
<td>Lutropin</td>
<td>5</td>
</tr>
<tr>
<td>Melatonin</td>
<td>3</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>3</td>
</tr>
<tr>
<td>Gonadotropin Releasing Hormone</td>
<td>2</td>
</tr>
<tr>
<td>Moult Inhibiting Hormone</td>
<td>1</td>
</tr>
</tbody>
</table>

Table A-11 lists the specific chemicals studied in the papers describing the effects of an outside chemical on a specific system.
### Table A-11. List of Chemicals Examined for Their Possible Role in the Disruption of Steroidogenesis

<table>
<thead>
<tr>
<th>Chemical</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)</td>
<td>4</td>
</tr>
<tr>
<td>Aspirin</td>
<td>1</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>2</td>
</tr>
<tr>
<td>Cadmium or Cadmium Salts</td>
<td>4</td>
</tr>
<tr>
<td>Colchicine</td>
<td>1</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>1</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>1</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>1</td>
</tr>
<tr>
<td>Taxol</td>
<td>1</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>1</td>
</tr>
<tr>
<td>Danzol</td>
<td>3</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>1</td>
</tr>
<tr>
<td>Etomidate</td>
<td>1</td>
</tr>
<tr>
<td>Epostane</td>
<td>1</td>
</tr>
<tr>
<td>Trilostane</td>
<td>2</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>1</td>
</tr>
<tr>
<td>Megestrol acetate</td>
<td>1</td>
</tr>
<tr>
<td>Aminoglutethimide</td>
<td>1</td>
</tr>
<tr>
<td>Stanozolol</td>
<td>1</td>
</tr>
<tr>
<td>Gossypol</td>
<td>1</td>
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<td>Ketoconazole</td>
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<td>Lead</td>
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<td>Methoxychlor</td>
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<td>MGK repellent-11</td>
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<tr>
<td>Mitomycin C</td>
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<td>Nicotine</td>
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<td>Anabasine</td>
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<td>Cotinine</td>
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<td>Nitric Oxide</td>
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### Chemicals

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<td>Omeprazole</td>
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</tr>
<tr>
<td>Polycyclic Aromatic Hydrocarbons (PAH's)</td>
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</table>

### Summary

The following generalities can be made from this database:

- Rat and human steroidogenic systems were studied most frequently when investigating the steroidogenic process.
- The majority of the papers in this database investigate the basic biological function of the system. This includes procedural papers simply describing how to assay and perform experiments that would describe broad functions in the steroidogenic process.
- Radioimmunassay was by far the most commonly used method of ascertaining the presence and amounts of specific hormones.
APPENDIX B:
INTERVIEWS
APPENDIX B: INTERVIEWS

Interviews with Principal Investigators—Published and Unpublished Studies
(See Appendix 1)

Researchers contacted:

Gary R. Klinefelter, Ph.D.
Reproductive Biologist and Toxicologist
Reproductive Toxicology Division, MD#72
National Health and Environmental Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711
Phone: (919) 541-5779
Fax: (919) 541-4017
E-mail: klinefelter.gary@epa.gov

Jerome M. Goldman, Ph.D.
Endocrinology Br. MD-72, Reproductive Toxicology Div.
National Health & Environmental Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711
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Patricia A. Fail
Manager, Laboratory of Reproductive and Endocrine Toxicology
Research Triangle Institute
P.O. Box 12194, 3040 Cornwallis Road
Research Triangle Park, NC 27709-2194
Phone: (919) 541-6079
Fax: (919) 541-5956
E-mail: patf@rti.org
Reproductive and endocrine toxicologist

David T. Armstrong
E-mail: david.armstrong@adelaide.edu.au

John W. Laskey
7017 Branton Drive
Apex, NC 27502
Phone: (919) 362-3945
Fax: (919)362-3946
E-mail: JohnL45198@aol.com
Reproductive and endocrine toxicologist
National Health & Environmental Effects Research Laboratory
U.S. Environmental Protection Agency
Consultant

Clarification of Study Design, Interpretation, and Data Analysis of Studies Through Interviews with These Scientists

The interviewer identified him/herself, identified the contract, and indicated the title and objectives/goals of this particular work assignment. He/she briefly described the study designs under consideration (in vitro culture of ovarian or testicular cells). The questions were designed to be open-ended and to encourage discussion; and follow-up questions were asked, as appropriate. The questions were modified to suit each interviewee’s experience, as appropriate.

The written text will become part of the permanent record.

At a minimum, the questions provided in the following template should be answered. Other ideas, opinions, thoughts, suggestions, tangents, or anecdotal type of information will be welcomed and will become part of the permanent record. Other information should be recorded on this form under the "Open Dialogue" heading.
Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up-regulation or down-regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases, it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name of Interviewer: Patricia A. Fail, PhD

Name of Interviewee: Dr ________________________________

Date: __________________________________

Have the questions been responded to in writing by interviewee (circle or omit one)? Yes/No

If so was a follow up phone call made to discuss any of the major points (circle one)? Yes/No

Note to reviewer: Please feel free to add more space or modify question as needed. Please put your modifications in italics.

--Thanks, Pat Fail
QUESTIONS

Q1. Do you know of any references that you would recommend that investigate the status of various \textit{in vitro} methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis?

Specific comment(s) to individual "expert" from me, such as, "Jerry, I have the early ones for ovarian steroidogenesis from John Laskey's work. Are there any of yours we can include or have you published the follicle method yet?"

In our experiments (for the EPA CA with Earl Gray) we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays or your own, please respond to the following question to the best of your ability.

Each form was customized for the specific scientific "expert."

Ovary

Q2. What are the limitations of this method?

- Whole follicle
- Minced ovary
- Other

Q3. What are the strengths?

- Whole follicle
- Minced ovary
- Other

Q4. What would you recommend to further enhance this method or what changes would you recommend (if any)?

- Whole follicle
- Minced ovary
- Other

Q5. Do you know of any other published literature that corroborates or refutes the findings for these methods?

Q6. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share...or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?
- Whole follicle
- Minced ovary
- Other

Q7. **Quality Control:** What are the quality control measures to be included in these assays that you favor?

a. Are there specific or special circumstances when additional quality control measures need to be added?

Q8. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?

Q9. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

a. What specific step of steroidogenesis or organ of steroidogenesis does each access?

Q10. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?

Q11. Are there variations of the assay that should be considered that you did not have time to validate or access? Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

Q12. Is there anyone else you can think of that we should contact? Yes/No

a. If so, whom?
b. Can we mention your name when we contact him/her? Yes/No

Q13. Open Dialogue

Testes

Q14. What are the limitations of this method?

Q15. What are the strengths?

Q16. What would you recommend to further enhance this method or what changes would you recommend (if any)?

Q17. Do you know of any other published literature that corroborates or refutes the findings in this paper?
Q18. In running this method/procedure are their any steps that are especially difficult that require special attention (i.e., lessons learned that come after numerous runs that you would like to share) or is there any special set-up strategy you would recommend that would save time or resources that come from experience in running the assay?

Q19. Quality Control: What are the quality control measures to be included in these assays that you favor?

   a. Are there specific or special circumstances when additional quality control measures need to be added?

Q20. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?

Q21. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

   a. What specific step of steroidogenesis or organ of steroidogenesis does each access?

Q22. Do you have any unpublished data relevant to these assays that you would be willing to share?

   a. If so, are there any restrictions?

Q23. Are their variations of the assay that should be considered that you did not have time to validate or access?

   Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

Q24. Is there anyone else you can think of that we should contact? Yes/No

   a. If so, whom?
   b. Can we mention your name when we contact him/her? Yes/No

Q25. Open Dialogue
Detailed Review Paper on Steroidogenesis
EPA Contract Number 68-W-01-023
Work Assignment # 1-6

Interviewee

Patricia A. Fail, PhD
Manager
Laboratory of Reproductive and Endocrine Toxicology
Research Triangle Institute
P.O. Box 12194 (RTI)
Durham, NC 27709
Phone: (919) 541-6079; 1 800 334-8571 ext. 6079
Fax: 919 541-7208

Instruction for interviewing researchers for input regarding procedures, methods, and
lessons learned regarding Steroidogenesis (minced testes, ovary, adrenal leydig cells,
or granulosa cells etc). Use a separate template to capture information for each person
interviewed. This information can be scribed by hand during the interview or you may
ask the person being interviewed if he or she would like to provide his or her own written
response to the questions to be followed up with a phone call.

The written text will become part of the permanent record.

At a minimum the questions provided in the template below will be asked. All
impromptu questions, thoughts, suggestions, tangents or anecdotal type of information
will be captured and become part of the permanent record. This information will be
recorded on this form under the "Open Dialogue" heading.
Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name of Interviewer: Carol S. Sloan, MS

Name of Interviewee: Patricia A. Fail, PhD

Date: August 2 and 10, 2001

Have the questions been responded to in writing by interviewee? Yes

If so was a follow-up phone call made to discuss any of the major points? Yes

Q1. Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis?

A1. Laskey et al., 1994 and many others. See Literature Search and Methods in Toxicology Vol. 3a and 3b for technical methods. Also see attached list of abstracts and reports from my own CV.

In your papers and reports you describe methods (or assays)—the testicular steroidogenesis and ovarian steroidogenesis assessments in vitro—in regards to these papers please respond to the following question to the best of your ability.

Q2. What are the limitations of these methods? [All answers apply to both assays unless otherwise stated]

A2. High variability when assessing individual animal's testis. See Table M-5 of document for a measure of variation. A general test.

Q3. What are the strengths?

A3. Ease, rapidness, fairly sensitive, relatively low cost. For our lab, fewer technician hours dramatically lowers the total cost. This assay can be made more specific by supplying precursors. For example, if a potential toxicant is shown to disrupt testosterone basal release or stimulated release, specific intermediates can be used to test for the "health" of specific enzyme substrates.
This assay can represent one animal's response to *in vivo* or *in vitro* exposure. Requires less time, technical skill or major equipment than cellular isolations such as Leydig cell purification, Granulosa or Theca cell isolation, and/or isolation of whole follicles.

A nice compromise might be a crude Leydig Cell isolation.

**Q4.** What would you recommend to further enhance this method or what changes would you recommend (if any)?

**A4.** Use of ED 50 or ED75 (of hCG) but no more than ED100, for the challenge test.

Variability must be addressed by using triplicate determinations (or more) per test point. I also recommend that the variation be summarized for these two specific methods within and between labs and compared with variation in other methods (in literature).

I believe that further reduction in costs are possible by downsizing it—using a smaller volume of media and a decreased amount of testis used per evaluation replicate. Klinefelter et al are now using 50 mg testis/ml media. This can be accomplished by using 1.5 ml vials rather than the 20 ml vials or by using a 24-well plate.

**Q5.** Do you know of any other published literature that corroborates or refutes the findings in this paper?

**A5.** All HEERL EPA literature from Laskey et al., Klinefelter et al., and Goldman et al. J. W. Laskey and others taught us to use these assays in a cooperative research program.

**Q6.** In running this method/procedure are there any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share...or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?

**A6.** I believe that further reduction in costs are possible by using 96 well plate assay and decrease amount of testis used per evaluation replicate.

**Q7.** Quality Control: What are the quality control measures to be included in these assays that you favor?

**A7.** Always include negative and positive controls (two compounds) at one or more doses. Doses for the positive control should be on the straight part of the dose response curve. Both the positive and a negative controls should be easily obtainable and reasonable inexpensive. Always include procedural controls (e.g., blank media that receives all treatments).
Q7a. Are there specific or special circumstances when additional quality control measures need to be added?

A7a. For the RIA being used—testosterone, estradiol, and/or progesterone—the test chemicals should be tested for cross reactivity. That is, do these chemicals themselves bind to the antibody used in the RIA?

All labs doing interlab validation should use same RIA kit source or have RIAs done at a central facility. Storage time and temperature should be controlled.

Q8. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?

A8. First and foremost, the endproducts of steroidogenesis in the system being tested. For the whole testis—testosterone; for the whole or minced ovary—estradiol. Androstenedione and progesterone are also easily assayed and could be included in the first analyses or as a secondary triggered measure if the primary product is affected.

Q9. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

A9. See Table in Goldman interview, incorporated into the text.

Q9a. What specific step of steroidogenesis or organ of steroidogenesis does each access?

A9a. Each targets one or more aspects of gonadal steroidogenesis or the effect of gonadal steroid on the target organ (pituitary and accessory sex organ’s receptors). If I understand the major recommendation of EDSTAC, it is to rapidly access the overall synthesis of steroidogenesis. Thus the overall process in ovary (estradiol and or progesterone) or testes (testosterone) should be tested initially. Then if desirable the specific parts or place [of steroidogenesis affected] can be evaluated. The overall processes include the integrity of the LH receptors, P450 enzyme systems, cholesterol uptake (and possible biosynthesis), labile protein synthesis (e.g., STAR), bioactivity of specific steroidogenic enzymes, and precursor uptake. These steps can be tested only if the "pure" mechanism of action(s) are known. I doubt that they do for many toxicants. Do not use cytotoxicants such as EDS.

Q10. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?
A10. The effective doses of hCG in 1/6 testes slabs. See Table M-2. At this point, please do not cite, quote or publish. This information will help us establish the appropriate doses to use for challenge assays. Similar data must be established for ovarian cultures or smaller pieces of testes (i.e., to use in 96 well plates). For all tables, the sponsor will be contacted and the conditions for use established. Also the Methoxychlor data are from an EPA cooperative agreement and have not yet been published in other that abstract form (See reference list).

LIST OF TABLES ATTACHED TO DRP

Table M-1: Effects of daily administration of methoxychlor on testicular testosterone (ng/g testis) In f0 male Long-Evans rats: in vitro incubation

Table M-2: Preliminary studies to define the dose and time response in testicular cultures: in vitro testosterone at 0, 1, 2, and 3 hours after hCG challenge

This is an example of an in vitro study for a dose response to hCG. Any (or several) toxicant(s) would be substituted in appropriate doses

Table M-3: Experimental design: Endocrine toxicity of a toxicant (xxx) on rat testes after in vivo exposure for 2, 7, and 14 days. An example of a definitive study design with in vitro testing after in vivo exposure

Table M-4: Preliminary Studies to Define Dose and Time Response in Adult Male Sprague Dawley Rats: Plasma and Testicular Testosterone at 1, 3, or 6 Hours Post-hCG Challenge

Table M-5: Characteristics of Radioimmunoassays Validated for Determination of Testosterone in Adult Male Sprague Dawley Rats

Q11. Are there variations of the assay that should be considered that you did not have time to validate or access. Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

A11. Differences in aged or prepubertal males; differences in females in different stages of cycle; use of the prepubertal PMSG primed females (also see Laskey's interview); use of ovarian whole follicles (see goldman interview).

Q12. Is there anyone else you can think of that we should contact? Yes
   a. If so, whom?
   b. Can we mention your name when we contact him/her?
John Laskey, Jerome Goldman, Gary Klinefelter, David Armstrong.
Also Anita Payne (Leydig cells)

Q13. Open Dialogue
REFERENCES

Publications


Abstracts (Poster or Platform Presentations)


Study Reports - Commercial Client

Toxicity Testing of a Fungicide, XXXX, in Adult Male CD® Sprague Dawley Rats. P.A. Fail, S.A. Anderson, and S.W. Pearce, Project Number 65C-5703, October 20, 1994, Confidential Client.

Toxicity Testing of a Fungicide, XXXX: Endocrine Toxicology Studies of Testes From Adult Male CD® Sprague Dawley Rats Exposed to XXXX In Vivo. P.A. Fail, S.A. Anderson, and S.W. Pearce. Project No. 65C-6169, September 16, 1996, Confidential Client.
Toxicity Testing of a Fungicide, XXXX: Endocrine Toxicology Studies of Testes From Adult Male CD® Sprague Dawley Rats Exposed to XXXX In Vitro. P.A. Fail, S.A. Anderson, and S.W. Pearce. Project No. 65C-6169, September 16, 1996, Confidential Client.

Study Reports - Government Client


INTERVIEWEE: Testicular steroidogenesis

Gary R. Klinefelter, Ph.D.
Reproductive Biologist and Toxicologist
Reproductive Toxicology Division, MD#72
National Health and Environmental Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711
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The written text will become part of the permanent record.

At a minimum the questions provided in the following template should be answered. Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of information will be welcomed and will become part of the record. Other information should be recorded on this form under the "Open Dialogue" heading.
Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name Interviewer: Patricia A. Fail, PhD

Name of Interviewee: Dr. Gary Klinefelter

Date of Response: August 1, 2001; follow-up interview on October 11, 2001

Have the questions been responded to in writing by interviewee (circle or omit one) Yes

If so was a follow up phone call made to discuss any of the major points (circle one) Yes/No

NOTE to reviewer: Please feel free to add more space or modify question as needed. Please put your modifications in italics. Thanks, Pat Fail
QUESTIONS

1) Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis?


2) In our experiments [for the EPA CA with Earl] we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays, please respond to the following question to the best of your ability.

Testis

a. What are the limitations of this method?

Sensitivity and linearity appear to be issues associated with incubations of minced ovarian tissue

b. What are the strengths?

Incubations of minced testis (or ovarian) tissue permits acquisition of data on individual animals and is relatively easy
c. What would you recommend to further enhance this method or what changes would you recommend (if any)?

For testicular assessments, consider going away from minced testis parenchyma incubations and moving forward with modifications of Leydig cell purification that permit a yield of enriched Leydig cells from an individual animals that is sufficient for steroidogenic profile assessment (see Salva et al., J. Androl., In Press).

For ovarian assessments, consider use of preovulatory follicles and in vivo challenges for experimental correlate. Regardless of organ/cell type ensure that linear steroid production is achieved [over time], and that assessments are made within the linear response range.

d. Do you know of any other published literature that corroborates or refutes the findings in this paper?

e. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay.

f. Quality control

1. what are the quality control measures to be included in these assays that you favor?

Tests for linearity of steroid production and cell viability. For testis, include 3B-HSD histochemistry at end of assessment period.

Incubations with intermediate substrates as well as hCG.

An in vivo experimental correlate. For example challenge in vivo with hCG in control and exposed animals to determine changes in hCG responsivity, examining steroid in serum as well as in intersititial fluid (testis).

2. Are there specific or special circumstances when additional quality control measures need to be added?

3) Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?

Degree of LH/hCG responsivity, i.e. to what degree does stimulation with hCG enhance steroid production. If only 2 fold over baseline, cells aren't happy.
Ability to respond to a greater degree to intermediate substrates of steroid production. Does stimulation with Cholesterol increase steroid production over that achieved with hCG stimulation?

4) Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

Chemicals such as aminoglithemide, and other suicide substrates for P450 enzyme activity. Chemicals that inhibit membrane signal transduction and cholesterol trafficking (i.e. StAR) should also be evaluated.

Duration [of incubation] should be within the linear range of steroid production.

What specific step of steroidogenesis or organ of steroidogenesis does each access.

5) Do you have any unpublished data relevant to these assays that you would be willing to share? If so, are there any restrictions?

6) a. Are there variations of the assay that should be considered that you did not have time to validate or access?
   b. Anything you would change about the assay to increase it’s sensitivity, efficiency, relevancy, or robustness?

7) Is there anyone else you can think of that we should contact?
   a. If so, whom?
   b. Can we mention your name when we contact him/her?

8) Open Dialogue
DETAILED REVIEW PAPER ON STEROIDOGENESIS
EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT #1-6

INTERVIEWEE:  Ovarian Steroidogenesis

Jerome M. Goldman, Ph.D.
Endocrinology Br. MD-72, Reproductive Toxicology Div.
National Health & Environmental Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711
(919) 541-2320  FAX: (919) 541-4017
email: goldman.jerome@epa.gov

The written text will become part of the permanent record.

At a minimum the questions provided in the following template should be answered. Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of information will be welcomed and will become part of the record. Other information should be recorded on this form under the "Open Dialogue" heading.
Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name Interviewer: Patricia A. Fail, PhD
Name of Interviewee: Dr Jerome Goldman
Date: August 1, 2001

Have the questions been responded to in writing by interviewee (circle or omit one) Yes
If so was a follow up phone call made to discuss any of the major points (circle one) Yes

NOTE to reviewer: Please feel free to add more space or modify question as needed. Please put your modifications in italics. Thanks, Pat Fail

QUESTIONS

1. Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Off-hand, I don't know of an article that compares the different ovarian approaches for use in a Tox Study (if that is what you are asking).


Our lab has used in vitro exposures of pre-ovulatory follicles under hCG-stimulated and non-stimulated conditions (Balchak et al., Repro. Toxicol.14: 533, 2000). A second manuscript has been submitted for publication using our improved approach, but exploring sites of toxic impact along the early portions of the steroidogenic pathway.

In our experiments (for the EPA CA with Earl Gray) we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays or you own, please respond to the following questions to the best of your ability.

OVARY

2. What are the limitations of this method?

   - Whole follicle

     This is not a pure cell population. Isolations (of follicles) are more technically demanding than a Laskey-type minced ovary approach.

   - Minced ovary

     Minced ovaries are much more heterogeneous than either isolated cell preps or follicular preps. Ovarian perifusions, as described by Peluso/Pappalarda and Brannstrom are reasonably time-consuming and more technically demanding, but would generate more reliable data than simply placing minced ovaries in a tube without controlling the oxygen content of the medium.

   - Other?

     Isolated cell preparations (i.e., granulosa cells or theca cells). Loss of architectural structure and interactions among different cell types that would normally occur.

     Likely require longer baselines to characterize "normal" cells. Viability should be tested.

3. What are the strengths?

   - Whole follicle

     Maintains follicular structure and intercellular communication (theca-granulosa).
By using immature rats (26d) primed with PMSG for 48h, can obtain first generation pre-ovulatory follicles at a comparable stage of maturation (can select similar-sized follicles).

Better tissue penetration of test compound than with minced ovaries.

Can obtain multiple follicles per rat and randomly assign follicular pairs among treatment conditions to reduce any variability between animals/individual ovaries. Also allows a reduction in the number of animals used per study.

**- Minced ovary:**

Laskey type incubations- Minimum of preparation time; easy to obtain.

Ovarian perfusions or perifusions- obtain a picture of the dynamics of steroid secretion under different experimental conditions.

**- Other?**

Isolated cell preparations (i.e., granulosa cells or theca cells)

Homogeneous cell types may have advantage in mechanistic studies and improve consistency of hormonal data.

Better penetration of test compound for in vitro exposures.

4. **What would you recommend to further enhance this method or what changes would you recommend (if any)?**

**- Whole follicle**

Besides whole follicles, isolated corpora lutea (from rats made pseudo pregnant) can be used as well. Choice would depend on the nature of the questions asked (pregnancy maintenance/focus on progesterone production, maintenance of cyclicity. Also both can be used in a perifusion system (We've done some unpublished work with CLs. Came out pretty well, but we never took the approach further).
- Minced ovary

This really depends on which approach to ovarian incubations you mean.

- Other?

5. Do you know of any other published literature that corroborates or refutes the findings for these methods?

I don't understand. The utility of the approaches?

6. In running this method/procedure are there any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share...or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?

For any of the in vitro procedures using steroid secretory endpoints, use of teflon vials/tubes/tubing is the ideal, since the sex steroids (particularly progesterone) adhere quite readily to plastic, less so to glass (see Bruning et al. J. Steroid Biochem. 14:553, 1981; Higuchi and Espey. J. Reprod. Fertil. 87:821, 1989).

Use well-oxygenated media for incubations.

There are always concerns about maintenance of viability over time. Steroid secretion will eventually fall off. For most experiments, 24 hr should be sufficient to determine any toxicant effects on steroidogenesis.

- Whole follicle:

Practice- either for a chemical isolation or a surgical one.

- Minced ovary:

For the perfusion or perifusion approaches, practice and confirmation of a marked increase in steroid secretion under stimulation (e.g., cAMP). This would also be true for a simple minced ovarian prep (a general check on viability). In fact, under experimental conditions, it is always informative to include comparisons of baseline release under control and treatment levels in addition to a response to stimulation (hCG, cAMP, etc.) to evaluate hormonal release when the tissue is "pushed".

- Other?

7. Quality Control: What are the quality control measures to be included in these assays that you favor?
pH checks, maintain sterility as much as possible (even though antibiotics are
generally included in incubation media).

Discussion with Fail: Likely should characterize how often pH should be
measured, or how long it will hold in validation experiments. With oxygenated
follicle sealed in flasks, it was stable up to 4 hours.

For most cultures, an atmosphere of 95% Oxygen 5% CO2 works nicely.

**Are there specific or special circumstances when additional quality control
measures need to be added?**

Although it is not generally done, it would be helpful to be able to sample the
dissolved oxygen content of the incubation media. However, this can be tricky to
do under most circumstances without a more elaborate setup. For some data on
the effects of oxygenation on follicular steroid secretion, see Roby KF, Terranova
PF. (1990). Effects of tumor necrosis factor-alpha in vitro on steroidogenesis of
healthy and atretic follicles of the rat: theca as a target. Endocrinology 126:
2711-2718.

8. **Based upon your expertise and experience, what endpoints would be most
appropriate for elucidating the effects of chemicals on testicular/ovarian/
adrenal steroidogenesis when investigating an in vitro study design?**

Depends on the general focus. For toxicant effects on pregnancy maintenance,
it would be more appropriate to focus on P4, using corpora lutea. For general
effects on ovarian steroidogenesis, the most obvious endpoints for whichever
cell/tissue approach employed are E2 and P4 release. Supplementation of the
media with known concentrations of hormones such as pregnenolone or
testosterone may provide some information about the synthetic capacity of the
system under toxic insult and indications of effects on the particular enzymes
(3b-HSD, P450arom, or others) involved.

9. **Based on your experience, what chemicals, routes, duration, and doses
would you recommend to be used to validate an in vitro steroidogenesis
assay/protocol?**

Give me a call to discuss. August 3, 2001 Discussion

Table x.x. Chemical Candidates for Controls

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<td>Tamoxifen</td>
<td>??receptor</td>
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<td>Compound</td>
<td>Effect</td>
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<td>receptor</td>
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<td>Vinclozolin</td>
<td>receptor</td>
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<tr>
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<td>Dibutylphthalate</td>
<td>Sertoli cell?</td>
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<td>Solubility problems</td>
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<td>DDE</td>
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<tr>
<td>Finasteride</td>
<td>enzyme</td>
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<tr>
<td>Dibromo acetate</td>
<td>TBD</td>
</tr>
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</table>

**What specific step of steroidogenesis or organ of steroidogenesis does each access?**

See above, authors, this aspect needs additional attention. Up to 40 hours could be easily used selecting appropriate controls, but after the assays are selected.

PAF August 3, 2001

**10. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?**

Maybe. Contact me to discuss circumstances.

**11. Are there variations of the assay that should be considered that you did not have time to validate or access? Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?**

There are a variety of things to try when issues of sensitivity are considered. Robustness of the assays is always affected by cell/tissue viability.
If the minced ovarian tissue is to be used one should consider using PMSG primed immature female rat to get similar follicles (size and status). The pseudopregnant females could be used to donate ovaries with coprae lutea dominate status.

12. Is there anyone else you can think of that we should contact?
   a. If so, whom?
   b. Can we mention your name when we contact him/her?

13. Open Dialogue

The Effective dose issue is an important one. When stimulating tissues, it is important not to overwhelm the system, but the challenge dose must be high enough to evoke a response. In our follicle cultures we add the hCG and the toxicant at the same time and incubate for two hours. That avoids opening the sealed vials to take a sample.

14. Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Specific comment(s) to individual "expert" from me, such as, "Jerry, I have the early ones for ovarian steroidogenesis from John Laskey’s work. Are there any of yours we can include or have you published the follicle method yet?"

In our experiments (for the EPA CA with Earl Gray) we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays or you own, please respond to the following question to the best of your ability.
OVARY

15. What are the limitations of this method?
   - Whole follicle:
   - Minced ovary
   - Other?

16. What are the strengths?
   - Whole follicle:
   - Minced ovary
   - Other?

17. What would you recommend to further enhance this method or what changes would you recommend (if any)?
   - Whole follicle:
   - Minced ovary
   - Other?

18. Do you know of any other published literature that corroborates or refutes the findings for these methods?

19. In running this method/procedure are there any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?
   - Whole follicle:
   - Minced ovary
   - Other?

20. Quality Control: What are the quality control measures to be included in these assays that you favor?

   Are there specific or special circumstances when additional quality control measures need to be added?

21. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?

22. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?
What specific step of steroidogenesis or organ of steroidogenesis does each access?

23. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?

24. Are their variations of the assay that should be considered that you did not have time to validate or access? Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

25. Is there anyone else you can think of that we should contact?
   a. If so, whom?
   b. Can we mention your name when we contact him/her?

26. Open Dialogue
The written text will become part of the permanent record.

At a minimum the questions provided in the following template should be answered. Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of information will be welcomed and will become part of the record. Other information should be recorded on this form under the "Open Dialogue" heading.
Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name Interviewer Patricia A. Fail, Ph.D.
Name of Interviewee: Dr John W. Laskey
Date of Response 08/12/2001

Have the questions been responded to in writing by interviewee (circle or omit one) Yes or NO?
If so was a follow up phone call made to discuss any of the major points (circle one) Yes or NO?

QUESTIONS

1. Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Check the References included in my CV – sent separately

John, I am especially interested in the reference(s) for the testicular assay. I have the ovarian references. Pat - in the listed references there should be several references for the testicular assay G. Klinfelter and I have considered the possible problems with this assay and in consultation with Dr. L. Ewing have concluded that this minced testicular assay is consistent with the isolated Leydig cell assay.

In our experiments (for the EPA CA with Earl Gray) we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays or you own, please respond to the following question to the best of your ability.
OVARY

2. What are the limitations of this method?
   
i. For all these responses check the attached freelance file Pathway.jpg or Pathway.pre file.

   ii. The steroidogenic pathway in humans is somewhat different than in laboratory species probably due to the order of the order of the enzymatic reactions.

   iii. The stage of the ovarian cycle (estrous, diestrus, ... pregnancy) makes a great deal of difference in the response to stimulation and/or inhibition.

   iv. Blocking of the study to insure that day to day changes in assay conditions (technical differences, media prep, stimulant prep, inhibitor prep, sample origin, etc.) can be statistically corrected.

3. What are the strengths?
   
i. Ease of sample preparation.
   
   ii. With good quality/technical control there is excellent reproducibility.

4. What would you recommend to further enhance this method or what changes would you recommend (if any)?
   
i. With normal laboratory precautions this method doesn't require any changes.

5. Do you know of any other published literature that corroborates or refutes the findings for these methods?
   
i. I haven't been following the literature for the past five years.

6. In running this method/procedure are there any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share...or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?
   
i. See above
7. Quality Control: What are the quality control measures to be included in these assays that you favor?
   
i. See above

   Are there specific or special circumstances when additional quality control measures need to be added?
   
i. See above

8. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?
   
a. Responses to appropriate hormone/stimulation.

9. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?
   
What specific step of steroidogenesis or organ of steroidogenesis does each access?

10. Do you have any unpublished data relevant to these assays that you would be willing to share? If so, are there any restrictions?

11. Are there variations of the assay that should be considered that you did not have time to validate or access? Anything you would change about the assay to increase its sensitivity, efficiency, relevancy, or robustness?

12. Is there anyone else you can think of that we should contact?
   
a. If so, whom?
   b. Can we mention your name when we contact him/her?

13. Open Dialogue
The written text will become part of the permanent record.

At a minimum the questions provided in the following template should be answered. Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of information will be welcomed and will become part of the record. Other information should be recorded on this form under the "Open Dialogue" heading.
Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name Interviewer: Patricia A. Fail, PhD
Name of Interviewee: Dr. D. T. Armstrong
Date: Sept 7, 2001

Have the questions been responded to in writing by interviewee (circle or omit one)

NO  Not all of them,

If so was a follow up phone call made to discuss any of the major points (circle one)

NO

QUESTIONS:

1. Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

There are a lot of papers that use in vitro methods to study regulation of various aspects of steroidogenesis. Many of them could be potentially useful in identification of chemicals that affect the specific steroidogenic outcomes being investigated. I don't know of a specific critical review of the status of such methods for your purposes. It would be a good topic for someone to write; perhaps an honours student with interests along these lines.

I wrote a review which touched on some of these ideas some years ago.
2. In your experiments did you use both the ovarian steroidogenesis and Testicular steroidogenesis methods?

With regards to these assays, please respond to the following question to the best of your ability.

OVARY

a. What are the limitations of this method?
b. What are the strengths?
c. What would you recommend to further enhance this method or what changes would you recommend (if any)?

Our research deals primarily with regulation of ovarian steroidogenesis and its relationship to oocyte competence and ovulation. We have used a variety of ovarian cell systems and end points. Studies with isolated systems of single cell types have the limitation that they are able only to identify actions that involve the cell type under study. Since a lot of the more subtle effects of compounds that influence ovarian regulation involve cellular interactions, e.g. between oocytes and granulosa cells, or thecal cells and granulosa cells, their effects may be missed by restricting study of a single cell type. In addition, many agents that alter steroidogenesis do so through extra-ovarian actions such as the hypothalamus or pituitary (or even the liver, as in agents that affect steroid clearance and hence alter feedback regulation of the ovary).

The strength of in vitro methods is their sensitivity, as well as the ability to identify sites and mechanisms of action. In addition to the limitations alluded to above, in vitro methods usually have to make assumptions as to the probable site of action of a given compound. Therefore they may be inappropriate for screening of compounds suspected to affect reproductive functions but for which the site or mechanism of action has not yet been identified. This limitation may be overcome by a sequential strategy that includes a series of tests such as:

- whole animal approaches (e.g. ovarian and uterine weights, ovulation, pregnancy) followed by
- whole ovary or isolated whole follicle culture in which steroids are measured in culture media;
- strategic cell combinations, such as thecal and granulosa cell co-culture; oocyte-granulosa cell co-cultures; macrophage-luteal cell co-culture; isolated single cell types;
- subcellular components that enable study of a single component, e.g hormone receptor, signal transduction molecule, specific intracellular reaction (enzymatic, transport mechanism)
d. Do you know of any other published literature that corroborates or refutes the findings in this paper?

e. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share...or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay.

f. Quality control

1. What are the quality control measures to be included in these assays that you favor?
2. Are there specific or special circumstances when additional quality control measures need to be added?
3. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?

There are important linkages and interactions between germ cell and somatic cell compartments of the ovary that have not been explored as much as warranted, and that could undoubtedly be exploited for design of specific test systems that could be of interest to your group. Thus, in addition to steroidogenesis end points, oocyte end points could also be used to assess effects of chemicals. Specific oocyte end points that have proven useful in our studies include effects (of regulatory agents) on meiotic maturation in vitro (both spontaneous and gonadotropin-induced), ability to undergo normal fertilization in vitro, and developmental competence after fertilization or artificial activation. All of these end points depend on or are influenced by input from follicular somatic cells, and hence would have potential for development of assay systems that could prove useful for your purposes.

4. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

What specific step of steroidogenesis or organ of steroidogenesis does each access?

5. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?

6. Are there variations of the assay that should be considered that you did not have time to validate or access. Anything you would change about
the assay to increase its sensitivity, efficiency, relevancy, or robustness?

7. Is there anyone else you can think of that we should contact?
   a. If so, whom?
   b. Can we mention your name when we contact him/her?

Professor Fulvio Gandolfi
Istituto di Anatomia degli Animali Domestici
via Trentacoste 2
I - 20134 Milano. Italy
Tel: (+39) 02-2154-036
Fax: (+39) 02-2140-745
Email: gandolfi@imiucca.csi.unimi.it

8. Open Dialogue

Pat, I'm not sure whether I've addressed this in a way that is useful. It will be evident that our research over the years has not been directly aimed at ovarian regulation from the standpoint of chemicals that disrupt/modify ovarian function. Rather it has attempted to better understand normal (physiological) ovarian regulatory processes. New concepts and details are being discovered in this field at an unprecedented rate, as increasingly powerful methodology is focussed on the topic. As our understanding of normal regulatory processes in the ovary increases in both depth and breadth, it should be possible to use this information to design approaches to study specific actions of chemicals such as those that your institute wishes to investigate.

TESTES:

a. What are the limitations of this method?
b. What are the strengths?
c. What would you recommend to further enhance this method or what changes would you recommend (if any)?

d. Do you know of any other published literature that corroborates or refutes the findings in this paper?

e. In running this method/procedure are there any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share...or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay.

f. Quality control
1. what are the quality control measures to be included in these assays that you favor?

2. Are there specific or special circumstances when additional quality control measures need to be added?

9. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?
APPENDIX C:

PARAMETERS FOR COMPARISON SUMMARY OF
IN VITRO METHODS IN TABLE 4-11
Appendix C: Parameters for Comparison Summary of
in Vitro Methods in Table 4-11

Cost – The overall cost of doing the screen including labor, cost of animals, and supplies. The
relative scale used in the table is defined as: ($) - inexpensive; ($$) - moderately expensive;
($$$) - very expensive. The cost of conducting a steroidogenic assay using sectioned testes was
estimated to be $11,600 per chemical (EDSTAC). This was considered an ($) inexpensive assay.

Time - Under this parameter, the time necessary for the initial set-up and time to conduct an
assay were evaluated and compared. As for the initial set-up, the evaluation was based on the
laboratory equipment and apparatus needed for the assay. Also, the time needed to set-up the
apparatus and verify that it was functioning properly. In regard to the time needed to conduct the
assay, information from the literature was reported (when ever possible) using the number of
preparations that could be used for testing in a day. It does not include the time needed to
process samples since that would vary based on the selected endpoints, as well as would not be a
distinguishing factor between the assays.

Laboratory - Under this parameter, the level of training and type of equipment were compared
for each in vitro assay. In regard to the level of training and type of equipment, the in vitro
methods were designated as either general or specialized. General was used to describe those
assays that can be conducted with standard laboratory training and equipment. Specialized
describes those in vitro methods that require unique equipment, as well as additional and
advanced laboratory training in order to be able to perform the assay.

Sensitivity--The ability of the assay to detect an effect. The more sensitive the screening
bioassay, the less likely it will be that it fails to detect the positive action of a compound. Failure
to detect a positive effect (whether that effect increases or decreases some component of
steroidogenesis) is a false negative (a type II error). In the radioimmunoassays (RIAs) or other
determination of the hormone concentration, sensitivity is defined as the minimum detectable
amount of that hormone for a given set of assay conditions.

Specificity--Specificity refers to the ability to detect very well defined or specific activities. In
the case of the steroidogenesis screen, it is more desirable to rank any chemical that interferes
with any step of steroidogenesis as having positive activity. It is desirable then for the bioassay
to be less specific. For example, any substance that affects the P450 enzymes should cause an
altered hormone secretion in the assay. In RIAs, the antibody (detector) used must be
categorized to define which if any related substances it is detecting. For example, in a
testosterone RIA it is important to know if only testosterone is being detected or if the antibody
also recognizes dihydrotestosterone, androstenedione, or other androgens.

Multiple endpoints – The assays were evaluated for their capacity to foster multiple endpoints.
The steroidogenic pathway’s hormones are the most frequently measured endpoints. The
number of different steroid hormones measured are included in the table (in parenthesis) along
with the reference. In addition, enzyme activities and architectural changes observed by
microscopic examination are also used as endpoints, but less frequently.
**Metabolic Activation** - An evaluation of whether the assay has an endogenous capacity to metabolize pro-xenobiotics into active toxicants. Also, whether the assay lends itself to exogenous addition of a metabolic fraction, i.e. S9.

**Stability** - A measure of the time that the preparation has been used by investigators to characterize a steroidogenic response in the presence of a substance being tested. In addition, the times listed in the table were based on papers in the literature that reported some measure of viability. Viability was assessed using data about linear steroid hormone production in the presence of a stimulating agent, e.g. hCG, as well as other viability type tests, e.g. Trypan Blue.

**Cytoarchitecture** - Describes the degree that the organ remains intact once removed from the animal and is prepared for testing. Preparation of the organ for treatment, e.g. treatment with collagenase in order to isolate cells, then the cytoarchitecture is changed, which may affect how closely the preparation mimics the *in situ* environment.

**Repeatability (Inter-assay variability)** – Chemicals that test positive (or negative) in the subject bioassay will do so repeatedly and with the same degree of effect. That is, the initial assay is predictive of the same or similar results when repeated in another assay of the same type but set up on different dates. In this case the variation associated with the repeated evaluation is actually characterized (e.g. 10+ 0.5 %; Mean and SEM, n=y dates). For example, Chemical X results in depressed testosterone secretion in a bioassay using adult male rat testes each time it is tested in sequential evaluations and the amount (quantitative) of that suppression is similar. That amount is calculated and reported as a characteristic of the bioassay.
APPENDIX D:

RECOMMENDED PROTOCOL
TITLE: Testicular Steroidogenesis Bioassay Screening Protocol

SPONSOR:

TESTING FACILITY:

PROPOSED STUDY IN-LIFE DATES: _______________________

AMENDMENTS:

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1.0 OBJECTIVE AND BACKGROUND

Steroid hormones produced by the gonads affect most of the organs in the body including bone, muscle, brain, and reproductive organs. It is for this reason that the EDSTAC recommended the *in vitro* steroidogenesis assay in testicular tissues as a component of the Tier 1 Screening (T1S) battery. The objective of this assay is to detect disruption of the steroidogenic pathway in vitro. It may: (1) be used as one of the protocols recommended by EDSTAC for the Tier 1 screening battery, (2) serve as a follow-up test for certain substances for which additional data are required or desired, and/or (3) predict the likelihood that steroidogenesis and downstream biologically dependent processes would be affected by the same or similar substances *in vivo*. The endpoints were selected for their potential to detect toxicant-induced alterations of steroidogenesis in gonadal tissue.

The Food Quality Protection Act of 1996 and the Safe Drinking Water Act of 1996 required the EPA to develop and implement a screening program for determining the potential in humans for estrogenic (and anti-estrogenic) effects from pesticides. This program has been expanded on the advice of the EDSTAC to include androgenic (and anti-androgenic) effects and effects from thyroid-hormone (TH)-like (or anti-TH) substances. The EDSTAC, assembled by the EPA in 1996, believed, to the best of its knowledge, that the recommended Tier 1 screening battery, if validated, would have the necessary breadth and depth to detect any currently known disruptors of estrogen, androgen, and thyroid (EAT) hormones.

The suggested T1S protocols are being tested within the Endocrine Disruptor Screening Program (EDSP) “to characterize the nature [and] likelihood of a dose-response relationship of endocrine disruption in humans and wildlife” (EDSTAC, 1998). To this end, the EPA has requested the development of a screening protocol that identifies compounds having the potential to affect steroidogenesis.

The testis steroidogenesis bioassay was selected as a component of the Tier 1 screening (T1S) by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to evaluate the potential toxicity of substances on the endocrine system (EDSTAC, 1998). The T1S tests were selected to obtain minimum yet sufficient estimates of potential endocrine disrupting activity. The Committee stated that these tests should be relatively inexpensive, quick, and technically easy to perform. Furthermore, they should be sensitive and specific, capture multiple endpoints, and be predictive across species, gender, and age. Finally, they should be validated and standardized as soon as possible (EDSTAC, 1998).

The *in vitro* testis steroidogenesis bioassay assesses non-receptor mediated effects on P450 steroidogenic enzymes. This assay has been used with fetal, neonatal, and adult testis, and is not limited to mammalian species, having been used to assess steroidogenesis in fish, reptile, avian, and amphibian systems as well. Thus, the steroidogenesis bioassay as a component in the T1S phase should be broadly understood to screen for any disruption of the overall steroid biosynthetic pathway. Both synthesis and release can be tested in gonads from normal animals.
The goal of the in vitro steroidogenesis Tier 1 screen is to evaluate simultaneously all of the processes involved with gonadal synthesis of steroid hormones (receptor binding, signal transduction, transcription, translation, and cellular secretion of the steroids). A number of compounds can inhibit the synthesis of various steroid hormones. These compounds inhibit one or more enzymatic steps in the biosynthetic pathway of steroidogenesis (e.g., aminoglutethimide, cyanoketone, finersteide, ketoconazole). Estrogen biosynthesis can be inhibited by exposure to aromatase inhibitors such as the fungicide fenarimol (Hirsch et al., 1987). In addition, a number of other steps in gonadal steroidogenesis might be disrupted, such as binding of LH to the receptor on the cell membrane, cholesterol synthesis, and cholesterol intracellular transfer.

Antiandrogens and antiestrogens act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors (on the target organs). One prominent mechanism of antihormonal activity is inhibition of hormone synthesis by inhibiting the activity of P450 enzymes in the steroid pathway. Such activity will be detected with this in vitro procedure using testicular tissue obtained from adult male rats. It will, however, detect only pesticides and other substances that are active without metabolism, that is, the parent material is active. The assay will also detect other mechanisms that alter gonadal steroid synthesis via the LH receptor, cholesterol biosynthesis, and intracellular cholesterol transfer.

Substances that interfere with steroidogenesis primarily by inhibiting cytochrome P450 enzymes in the steroid pathway include two major classes of herbicides, the imidazoles and the triazoles (Taton et al., 1988). They inhibit P450 enzymes in the sterol synthesis pathway for lanosterol, a vital precursor of cholesterol (required for steroidogenesis and a component of fungal membranes). Cytochrome P450 inhibitors tend to be nonspecific, and these fungicides can also inhibit other P450 enzymes such as those required for mammalian steroid hormone synthesis (Murray and Reidy, 1990). Inhibition of mammalian steroid synthesis can potentially result in a broad spectrum of adverse reproductive effects in vivo, including abnormal serum hormone levels, pregnancy loss, delayed parturition, demasculinization of male pups, lack of normal male and female mating behavior, altered estrous cyclicity, and altered reproductive organ weights.

Measures of not only testosterone, but also other intermediate hormones of the pathway can be determined from media collected during the incubation period. Aromatase, another P450 enzyme, is also present, albeit at low concentrations, in the testes. Thus, it may also be possible to utilize this assay for determination of aromatase disruption by measuring estradiol concentrations. In support of this notion, Ammonium Perfluorooctanoate increased estradiol production is isolated Leydig cell cultures (Biegel et al., 1995). Thus, while testosterone is the ultimate endpoint as it is the end-product hormone, the assay has the advantage of having multiple endpoints that can be used to assess effects at various sites of the pathway.

The purpose of this protocol is to outline a procedure for the quantitation of steroidogenic hormone production from rat testicular tissue. The hormone measured – testosterone – is, if altered, indicative of altered gonadal enzyme activity. This generic protocol is written for 250 mg testicular sections with a focus upon the components that are to be included in a validation.
2.0 MATERIALS AND METHODS

2.1 Test Substances

2.1.1 Test Substance—A Negative Control

Common Name:
Chemical Name:
Synonyms:
CAS No.:
Molecular Formula:
Molecular Weight:
Appearance:
Odor:
Melting Point:
Density/Specific Gravity:
Solubility:
Vehicle:
Supplier:
Batch/Lot Number:
Purity:
Storage Conditions:

2.1.2 Test Substance—A Positive Control

Common Name:
Chemical Name:
Synonyms:
CAS No.:
Molecular Formula:
Molecular Weight:
Appearance:
Odor:
Melting Point:
Density/Specific Gravity:
Solubility:
Vehicle:
Supplier:
Batch/Lot Number:
Purity:
Storage Conditions:
2.1.3 Test Substance—Each Tested Unknown and Each Stimulant

Unknown test substances will be selected by the client. All information as listed above will be inserted.

2.1.4 Standard Substance—Each Radioimmunoassay Standard

Radioimmunoassay Standards of the highest purity will be obtained and prepared. The following standard curves can be used: Testosterone 0.07 to 500 ng/mL.

2.2 Chemical Safety and Handling

An MSDS for each substance used will be attached.

2.3 Dose Formulation and Analysis

Each test substance, as well as the positive and negative controls, should have known purity, stability in bulk, as well as stability and homogeneity in solution (in the range to be used). The solubility of the test substance in the media to be used must be known. It has been standard procedure to mix the substance in the incubation media. If it is necessary to use a special solvent such as DMSO as a vehicle to enhance solubility in media, then that solvent should be tested alone (as a vehicle control) for its effect in the assay system.

The formulation will be prepared at a frequency determined by stability tests performed prior to the start of the study. Suspensions will be prepared and stored in wide-mouth, amber bottles. The test materials will be suspended in appropriate vehicles or media, with the concentration determined by the experimental design. At least three concentrations of the unknowns should be tested.

An aliquot of each concentration per formulation will be analyzed. The formulation bottles will be identified by a five-digit, random number Rx code and a color code. Personnel, other than the Laboratory Supervisor, Project Toxicologist, and Study Director, will not be informed of the test substances or formulation concentrations until all laboratory work is completed (i.e., the study technicians will be “blind” for substance and dose). Aliquots from the formulations will be collected on the day of treatment of the tissues and will be analyzed.

2.4 Animals

2.4.1 Species and Supplier

The proposed test animals will be the Sprague Dawley Derived Outbred Albino Rat Crl:CD®(SD) IGS BR supplied by Charles River Laboratories, Inc., Raleigh, NC.
2.4.2 Live Animals and Species Justification

The use of tissues from live animals has been requested by the Sponsor. Alternative test systems are not available for the assessment of effects of substances on reproduction and development in intact mammals for determining the potential risk for humans from endocrine-mediated effects of pesticides and other substances. The Charles River CD® rat has been the subject of choice on reproductive and developmental toxicology contracts, and has been used for other reproductive toxicology studies with this test material. Large historical data bases for reproductive performance and prevalence of spontaneous malformations in control rats are available from the supplier (Charles River, 1988). This strain of rat has been proven to have robust fertility and fecundity, and does not present any unusual endocrine patterns. This study does not unnecessarily duplicate any previous study.

2.4.3 Total Number, Age, and Weight

- Number of Males: 10
- Age on Receipt: ~10-12 weeks
  (no less than 8 weeks of age to ensure maturity)
- Animal Wt. Range: 250-275 g

NOTE: The number of animals needed will depend upon the size of the bioassay. Ten male rats are sufficient to provide tissue for 80 incubations. Minimum sample size requirements should be verified (based on assay variation). Estimates may be made from existing data. This is essential to providing adequate power for statistical comparison of data among treatment groups and will provide information needed to determine the number of testicular sections assigned to each dose group.

2.4.4 Quality Control (tests of animals to verify antibody free status)

Serological evaluation of animals for fecal or blood viral, bacterial, or protozoan antibodies is not deemed necessary for this protocol as they will not be housed at the laboratory for more than 1 or 2 weeks.

2.4.5 Quarantine

Upon receipt, animals will be quarantined for 7 days. They will be observed daily for general health status and ability to adapt to husbandry conditions. They will be released from quarantine by the attending veterinarian or his/her designate.
2.5 **Animal Husbandry**

2.5.1 **Housing, Feed, and Water**

During the quarantine period, animals will be randomly assigned to cages. Animals will be singly housed in solid-bottom polycarbonate cages (8"x19"x10.5") fitted with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ). Sani-Chip® cage bedding (P.J. Murphy, Forest Products, Inc., Montville, NJ) will be used in all solid-bottom cages. Pelleted feed (No. 5002 Purina Certified Rodent Chow®) will be available *ad libitum* for the rats and tap water from the water system will be filtered and available *ad libitum* to all animals via an automatic water delivery system (Edstrom Industries Inc., Waterford, WI). Water is also available in plastic bottles with stainless steel sipper tubes *ad libitum*. The analysis of the rodent chow for chemical composition and possible chemical contamination and analysis of city water will be provided by the suppliers and maintained in the study records if deemed necessary. It is anticipated that contaminant levels will be below certified levels for both feed and water and will not affect the design, conduct, or conclusions of this study. Rat chow will be stored at approximately 60-70°F, and the period of use will not exceed 6 months from the milling date. At all times, animals will be housed, handled, and used according to the NRC Guide (NRC, 1996).

2.5.2 **Environmental Conditions**

Environmental conditions in the laboratory facility will be continuously monitored, recorded, and controlled during the course of the study by an automated system. Animal rooms used for this study will be maintained on a 12:12 hour light:dark cycle. Target conditions for temperature and relative humidity in the animal rooms will be between 64 and 79°F (18 and 26°C) and 30 and 70%, respectively, with 10 to 15 air changes per hour (NRC, 1996). Temperature and/or relative humidity excursions will be documented in the study records and the final report.

2.5.3 **Animal Identification**

During quarantine, animals will be individually identified by a cage card. They will not be tattooed or given ear tags. Data generated during the course of this study will not be tracked by these numbers.

2.5.4 **Limitation of Discomfort**

Discomfort or injury to animals will be limited, in that any animal will be humanely terminated by CO₂ inhalation.
3.0 EXPERIMENTAL DESIGN

3.1 Study Design

The study will consist of 24 treatment combinations (concentration by challenge groups), including at least one vehicle control group. Each group is comprised of at least 3 sections of gonadal tissue (incubated separately) selected at random from a pool of the tissue. Table 1 presents the study design and target doses of the test substance. A graphical representation of the study design is also presented in Figure 1 below.

Figure 1. Technical Flow Illustration of the Testicular Steroidogenesis Assay
### Table 1. Experimental Design

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Endocrine Challenge Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hCG Stimulation</td>
</tr>
<tr>
<td><strong>Vehicle controls</strong></td>
<td></td>
</tr>
<tr>
<td>media alone</td>
<td>3</td>
</tr>
<tr>
<td>others for solvents (each)</td>
<td>3</td>
</tr>
<tr>
<td><strong>Negative Control Test Substances</strong></td>
<td></td>
</tr>
<tr>
<td>one dose used in all assays</td>
<td>3</td>
</tr>
<tr>
<td><strong>Positive Control</strong></td>
<td></td>
</tr>
<tr>
<td>one dose used in all assays</td>
<td>3</td>
</tr>
<tr>
<td><strong>Test Substances</strong></td>
<td></td>
</tr>
<tr>
<td>Unknown 1, dose x</td>
<td>3</td>
</tr>
<tr>
<td>Unknown 1, dose y</td>
<td>3</td>
</tr>
<tr>
<td>Unknown 1, dose z</td>
<td>3</td>
</tr>
<tr>
<td>Unknown 2, dose x</td>
<td>3</td>
</tr>
<tr>
<td>Unknown 2, dose y</td>
<td>3</td>
</tr>
<tr>
<td>Unknown 2, dose z</td>
<td>3</td>
</tr>
<tr>
<td>Unknown N, dose x</td>
<td>3</td>
</tr>
<tr>
<td>Unknown N, dose y</td>
<td>3</td>
</tr>
<tr>
<td>Unknown N, dose z</td>
<td>3</td>
</tr>
</tbody>
</table>
3.1 **The Testicular Steroidogenesis Bioassay**

Figure 1 represents the technical flow of the testicular steroidogenesis assay. Briefly, the animals are killed and the testes removed, sectioned, weighed, and placed in media in scintillation vials. Vials are kept cold (4°C) until assayed. One testis of each male is separated into four longitudinal sections for an *in vitro* incubation to estimate testosterone synthesis and response to gonadotropin (hCG endocrine challenge test; hCG ECT). Vials contain media (see below) or media plus test substance (1 or more doses). Each condition is represented by three replicates. The vials are placed in the incubator and after the first period of incubation (e.g., 1 or more hours); an aliquot of media (0.5 mL) is collected. The sample is added to a small tube, the tube centrifuged, the sample removed and frozen. (This is the baseline sample or secretion sample.) One half of the replicates then are challenged with appropriate substance (i.e., hCG ECT), at one or more concentrations. Additional samples are removed after various incubation periods (1, 2, and 3 hours), frozen, and later quantified for hormone content. The percentage change in hormone concentration represents the response to hCG ETC. This tests the integrity and function of LH receptors and immediately associated functions (at least through pregnenolone synthesis).

For the testicular *in vitro* incubation, modified medium 199 (Medium 199 [GIBCO BRL, Life Technologies, Inc., Grand Island, NY] with 0.1% bovine serum albumin [BSA], 8.5 mM sodium bicarbonate, 8.8 mM HEPES and 0.0025% soybean trypsin inhibitor, pH 7.4) is used. No phenol red indicator will be used. The vials are incubated at approximately 34°C on a shaker in 5% CO₂/95% air. The hCG obtained from Sigma (St Louis, MO) or Calbiochem (San Diego, CA), is added in 0.5 mL media. To the other sections, media without hCG is added. Aliquots (0.5 mL) for testosterone RIA are collected 1, 2, and 3 hours after challenge. Typically, only 10 to 50 microliters are required and duplicate determinations are done. The *in vitro* synthesis and release of testosterone is compared with that released after modified Media 199 alone (media control), both within and between treatment groups.

3.2 **Radioimmunoassay of Samples**

Media samples from the cultured testicular preparations are assayed for the steroid hormone: testosterone using radioimmunoassays (RIA). Whatever assays are used, the same ones should be used in various laboratories or at the very least the antibody specificity and other assay characteristics should be reported.

Radioimmunoassay standards of the highest purity will be obtained (sigma) and prepared. The following standard curve will be used: Testosterone 0.07 to 500 ng/mL.

4.0 **STATISTICAL ANALYSES**

The unit of comparison will be hormone concentration (ng/mg testes/hour) for each replicate in the incubation vial (or tubes or wells). (Each replicate determination will be the mean of two replicates in each RIA.) Treatment groups will be compared to the concurrent
control group (within time) using either parametric ANOVA under the standard assumptions or robust regression method (Royall, 1986; Huber, 1967), which does not assume homogeneity of variance or normality. The homogeneity of variance assumption will be examined via Levene's test (Levene, 1960), which is more robust to the underlying distribution of the data than the traditional Bartlett's test. If Levene's test indicates lack of homogeneity of variance (p<0.05), then a log 10 conversion of the data will be made. These values will be tested for normalcy and an ANOVA applied to test all treatment effects. If the assumptions of ANOVA are not met, then robust regression methods will be applied. The robust regression methods use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They will be used to test for overall treatment group differences, followed by individual tests for exposed vs. control group comparisons (via Wald Chi-square tests), if the overall treatment effect is significant. The presence of linear trends (over the time points) will be analyzed by GLM procedures for homogenous data or by robust regression methods for nonhomogenous data (SAS Institute Inc., 1999a,b,c,d,e; 2000). Standard ANOVA methods, as well as Levene's test, are available in the GLM procedure of SAS® Release 8 (SAS Institute Inc., 1999a,b,c,d,e; 2000), and the robust regression methods are available in the REGRESS procedure of SUDAAN® Release 7.5.3 (Shah et al., 1997).

If Levene's test does not reject the hypothesis of homogeneous variances, standard ANOVA techniques will be applied for comparing the treatment groups. The GLM procedure in SAS® 6.12 will be used to evaluate the overall effect of treatment and, when a significant treatment effect is present, to compare each exposed group to control via Dunnett's Test (Dunnett, 1955, 1964). A two-tailed test (i.e., Dunnett's test) will be used for all pairwise comparisons to the vehicle control group.

A test for statistical outliers (SAS Institute, Inc., 1990b) will be performed on suspected outliers. If examination of pertinent study data do not provide a plausible technologically sound reason for inclusion of the data flagged as "outlier," the data will be excluded from summarization and analysis and will be designated as outliers. For all statistical tests, p \#0.05 (one- or two-tailed) will be used as the criterion for significance.

5.0 RETENTION OF SPECIMENS AND RECORDS

All specimens and records will be retained in archives for two years at the performing laboratory's expense. Beyond two years, continued retention will be at additional cost to the Sponsor.

6.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) prepared for this study.

A list of all laboratory-specific SOPs will be maintained with the study records and available for inspection by the Sponsor’s representative.
7.0 REPORTING

An executive summary will be prepared describing the number and strain of rats used in the study, the doses and substances tested, and the effects with levels of statistical significance for all endpoints. Electronic and hard copies of spreadsheets containing the raw data from all animals will be provided for each endpoint. In addition, the spreadsheet should include treatment means, standard deviation, standard error, coefficient of variation, and sample number below each endpoint. Data presented should include sample identification and treatment, and media progesterone, testosterone, and estradiol concentrations. A data summary table containing the mean, standard deviation, standard error, coefficient of variation, and sample size for each treatment group should be provided for all endpoints.

Tentative Study Dates\textsuperscript{a} (to be added to the protocol by amendment)

\begin{itemize}
  \item Male Rats arrive:
  \item Quarantine period (7 days):
  \item Preparation of chemical solutions (test toxicants):
  \item Sacrifice of animals:
  \item Preparation of tissues and incubation with test toxicants:
  \item Radioimmunoassay of media for hormones:
  \item Statistical analysis:
  \item Submission of nonaudited draft final report:
  \item Submission of audited draft final report:
\end{itemize}

\textsuperscript{a} The end dates are tentative and will depend on the dates of radioimmunoassay completion.

8.0 STUDY RECORDS TO BE MAINTAINED

\begin{itemize}
  \item Protocol and any Amendments
  \item List of any Protocol Deviations
  \item List of Standard Operating Procedures
  \item Animal Requisition and Receipt Records
  \item Quarantine Records
  \item Temperature and Humidity Records for the Animal Room(s)
  \item Animal Research Facility Room Log(s)
  \item Durham City Water Analysis (analyzed monthly, reported annually)
  \item Feed Type, Source, Lot Number, Dates Used, Certification, Analytical Results
  \item Dosage Code Records Containing Five-Digit Rx Code, Color Code, and Concentration
  \item Dose Formulation Receipt and Use Records
  \item Statistical Analysis Records
  \item Media Estradiol Analysis (E2)
  \item Media Testosterone (T4)
  \item Media Progesterone Analysis (P4)
\end{itemize}
Correspondence

9.0 REFERENCES


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