

# **Steroidogenesis: The Sliced Testis Protocol**

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ORD, U.S. EPA

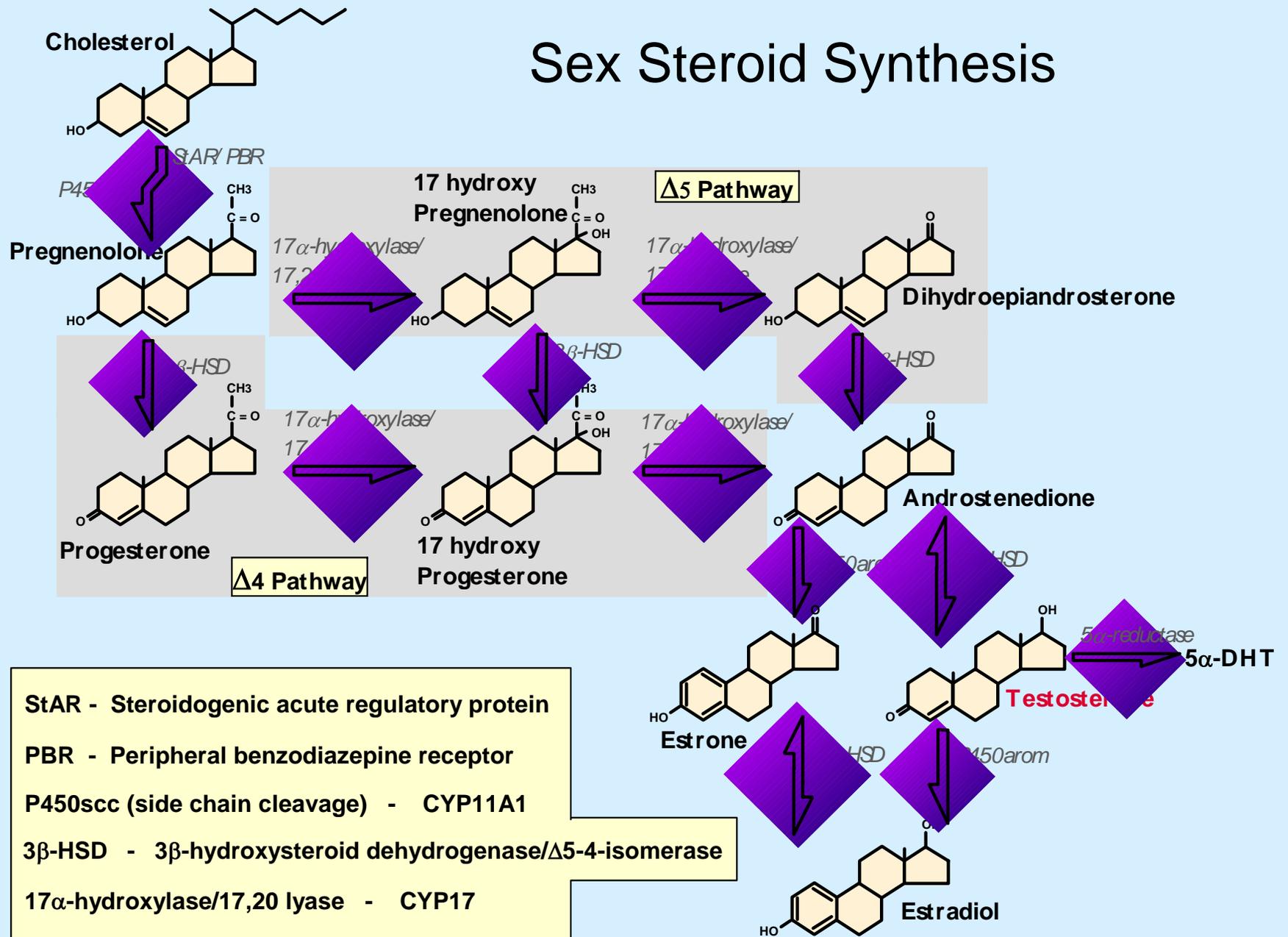
# **Steroidogenesis - Sliced Testis Protocol**

- **Selecting a Screen- Considerations**
- **The Protocol**
- **Protocol Evaluation**
  - I - Multichemical Evaluation**
  - II - Inter-lab Comparisons**
- **Cytotoxicity**
- **Strengths / Limitations of the Procedure**
- **Performance Criteria**
- **The Sliced Testis Protocol: Utility as a Steroidogenesis Screening Procedure**
  - Adjustments to the Protocol**

## Considerations in the Selection of a Screen to Evaluate Toxicant Effects on Steroidogenesis

- **Predictiveness** - Is the screen able to identify an effect?
- **Sensitivity** - Is the test sufficiently sensitive at lower concentrations of exposure?
- **Ease of Use** - Does the assay involve specialized training beyond general laboratory competence?
- **Variability** - Would variations (intra- & interlaboratory) within group data preclude detecting an effect?
- **Standardization** - Can the assay be sufficiently standardized with clear guidelines for its conduct?
- **Animal usage** - Can the use of animal subjects be refined, reduced, replaced, or even eliminated?
- **Costs** - Are the costs in equipment or personnel required to perform the assays at a manageable level?
- **Time requirements** - Is the time required to perform the assay reasonable to maintain the stability of the preparation?
- **Throughput** - Can sufficiently large numbers of samples be assayed concurrently?
- **Metabolic activation** - Does the assay permit any necessary metabolic activation?

# Sex Steroid Synthesis



StAR - Steroidogenic acute regulatory protein

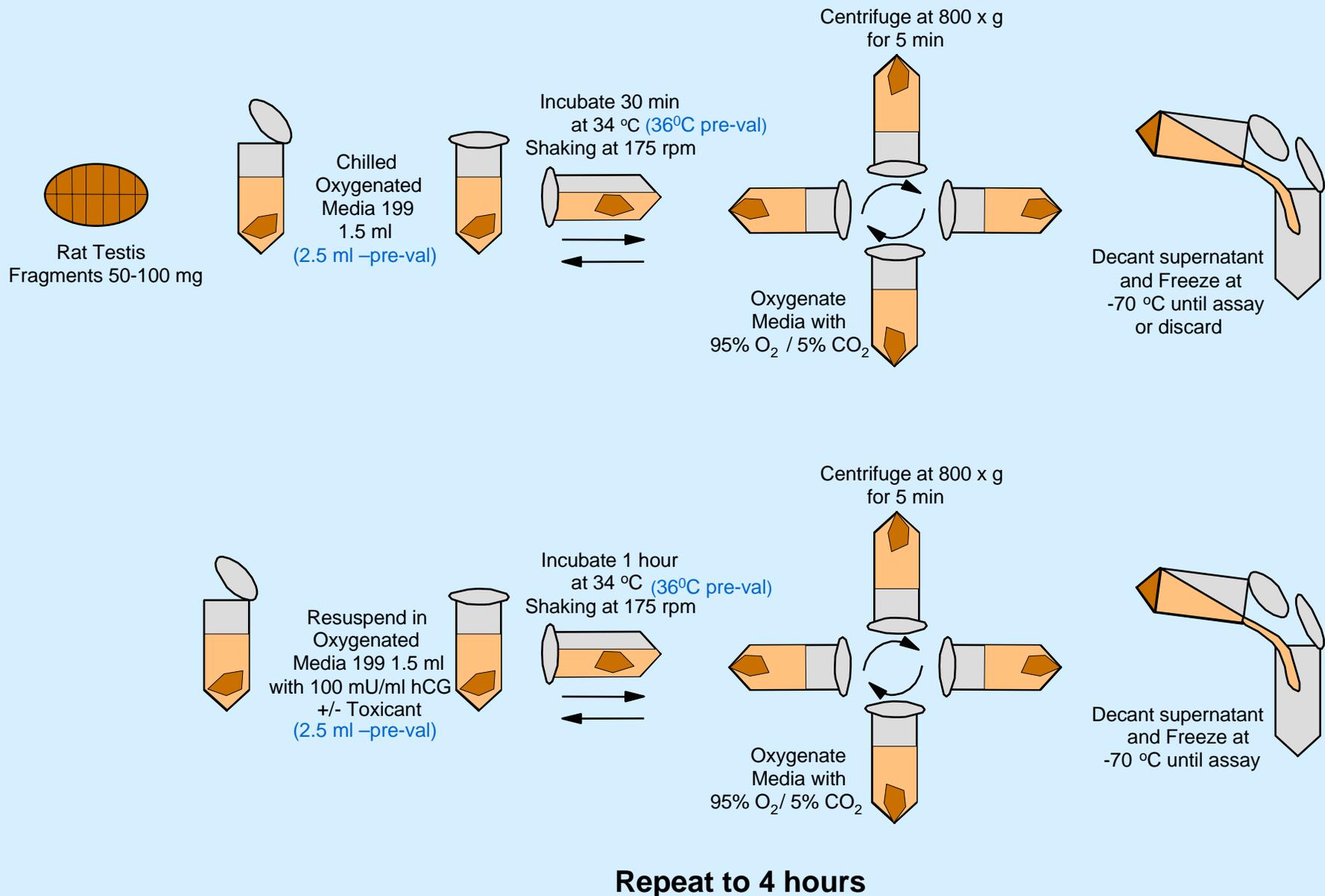
PBR - Peripheral benzodiazepine receptor

P450<sub>sc</sub> (side chain cleavage) - CYP11A1

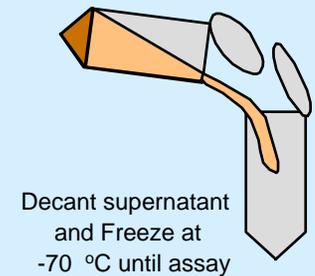
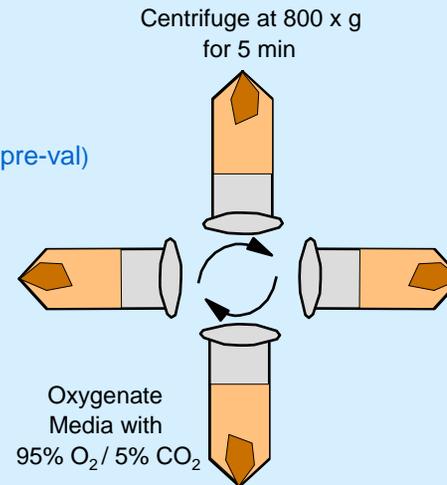
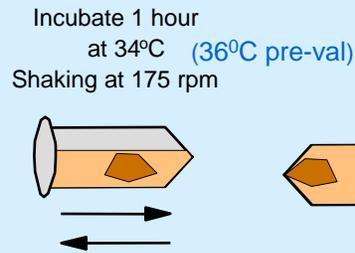
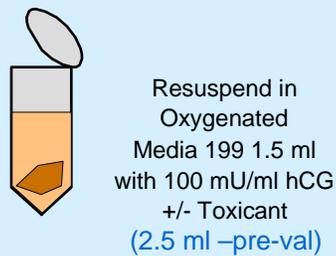
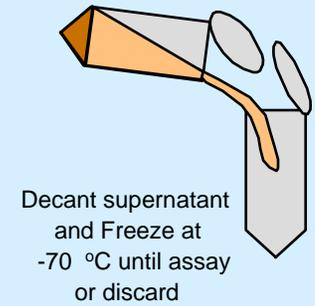
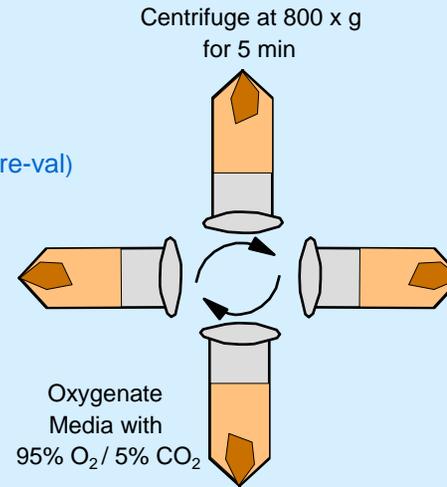
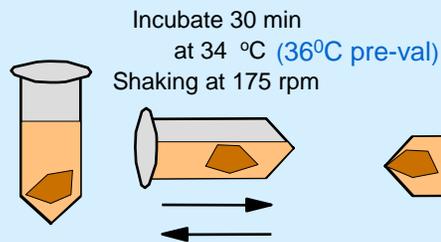
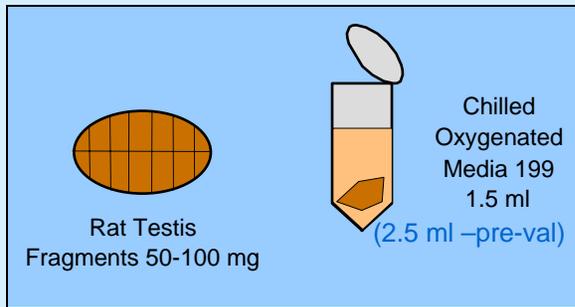
3 $\beta$ -HSD - 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ -4-isomerase

17 $\alpha$ -hydroxylase/17,20 lyase - CYP17

P450<sub>arom</sub> (aromatase) - CYP19



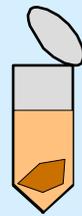
# Flow Diagram for In Vitro Sliced Testis Incubation



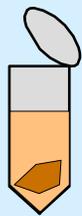
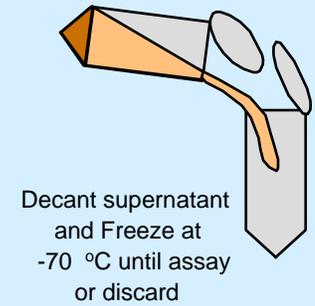
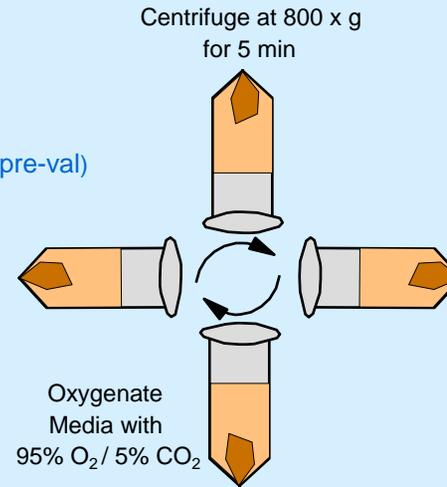
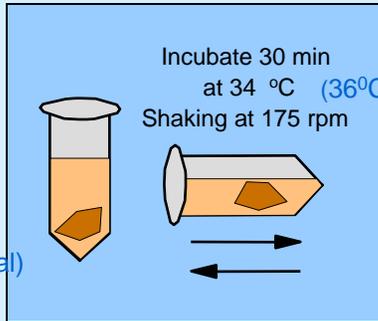
Repeat to 4 hours

## Flow Diagram for In Vitro Sliced Testis Incubation

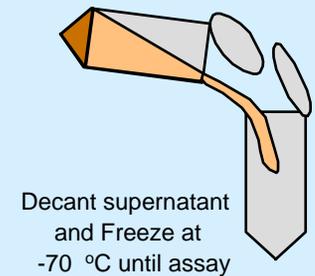
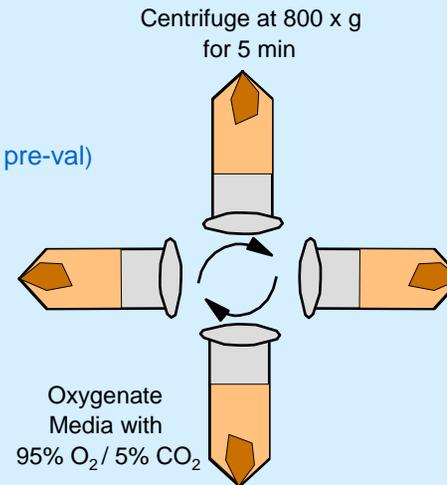
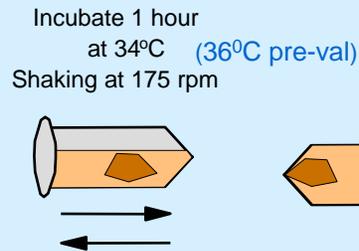
Rat Testis  
Fragments 50-100 mg



Chilled  
Oxygenated  
Media 199  
1.5 ml  
(2.5 ml -pre-val)



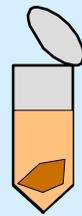
Resuspend in  
Oxygenated  
Media 199 1.5 ml  
with 100 mU/ml hCG  
+/- Toxicant  
(2.5 ml -pre-val)



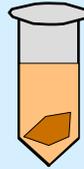
Repeat to 4 hours

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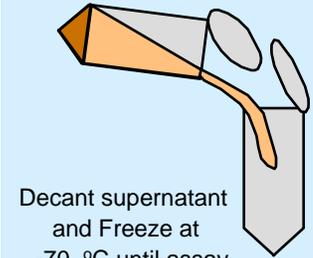
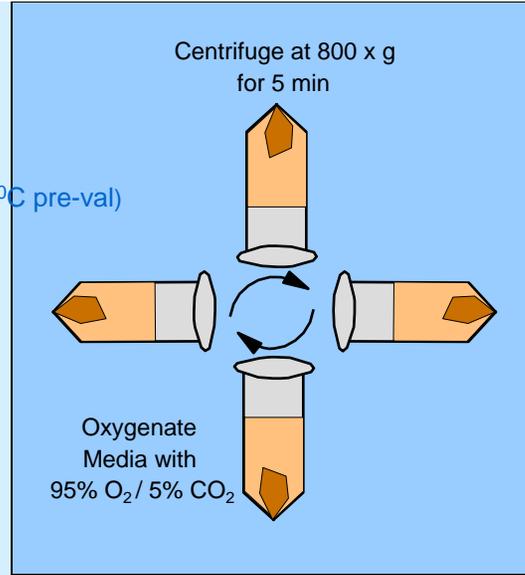
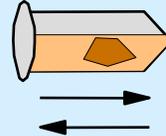
Rat Testis  
Fragments 50-100 mg



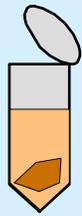
Chilled  
Oxygenated  
Media 199  
1.5 ml  
(2.5 ml -pre-val)



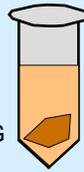
Incubate 30 min  
at 34°C (36°C pre-val)  
Shaking at 175 rpm



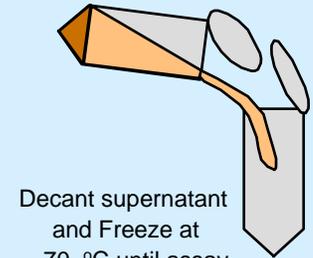
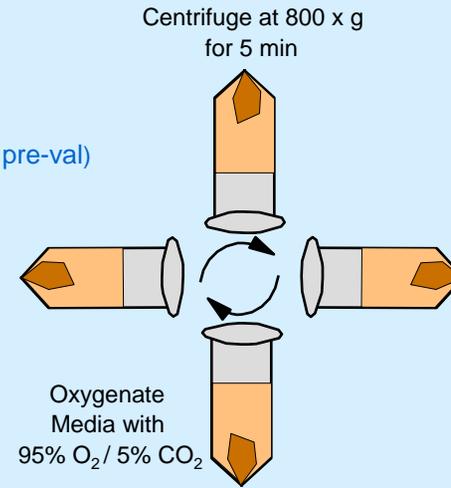
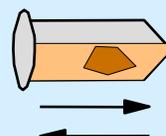
Decant supernatant  
and Freeze at  
-70 °C until assay  
or discard



Resuspend in  
Oxygenated  
Media 199 1.5 ml  
with 100 mU/ml hCG  
+/- Toxicant  
(2.5 ml -pre-val)



Incubate 1 hour  
at 34°C (36°C pre-val)  
Shaking at 175 rpm

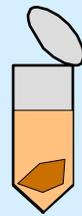


Decant supernatant  
and Freeze at  
-70 °C until assay

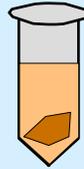
Repeat to 4 hours

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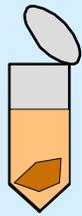
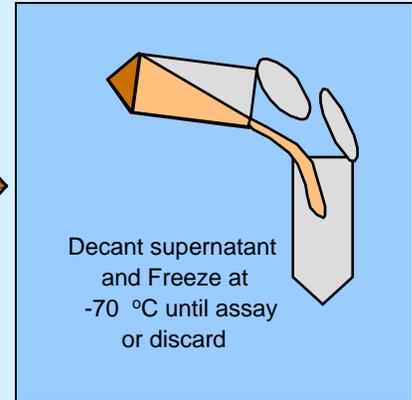
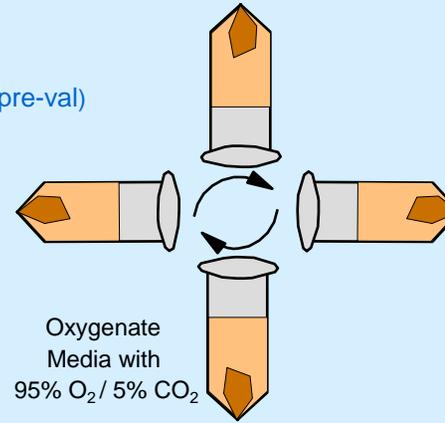
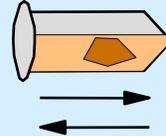
Rat Testis  
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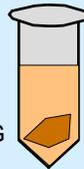
Chilled  
Oxygenated  
Media 199  
1.5 ml  
(2.5 ml -pre-val)



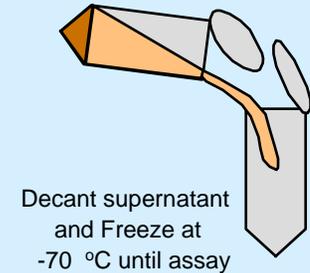
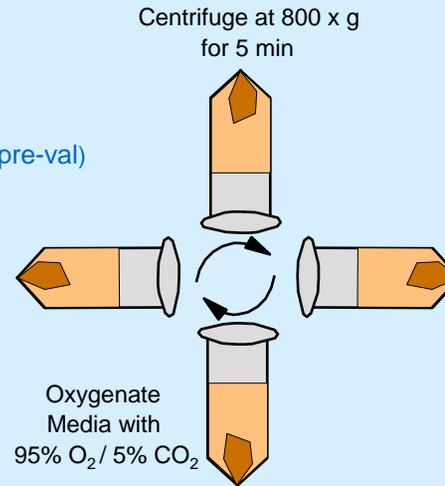
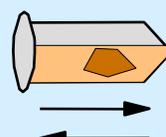
Incubate 30 min  
at 34°C (36°C pre-val)  
Shaking at 175 rpm



Resuspend in  
Oxygenated  
Media 199 1.5 ml  
with 100 mU/ml hCG  
+/- Toxicant  
(2.5 ml -pre-val)



Incubate 1 hour  
at 34°C (36°C pre-val)  
Shaking at 175 rpm



Repeat to 4 hours

## Flow Diagram for In Vitro Sliced Testis Incubation

# Assay Parameters – Pre-validation

**Assay Parameters for sliced testis incubation previously determined by RTI International as lead laboratory (EDMVS presentation Aug. 2003)**

- Age of rats - 11-15 weeks
- Fragments from single testis distributed across all treatment conditions < 1hr from euthanasia to incubation.
- Fragment weight ~100 mg
- Incubations in oxygenated M-199 (w/o phenol red), Earles salts, 0.1% BSA, HEPES buffer, pH 7.4, 36°C
- 30 minute wash followed by hourly samplings (1 – 4 h)
- Testosterone - radioimmunoassay  
Aliquots from hourly samplings pooled
- Cytotoxicity – Lactic dehydrogenase leakage

Data subjected to log transformations for ANOVA

Heterogeneity of variance

Treatment *standard deviation*. proportional to treatment *mean*



# Phase 1

## Multichemical Study – Lead Laboratory

**Media Control**

**Media-Vehicle (0.1% DMSO) Control**

**- hCG / + hCG (0.1 IU/ml)**

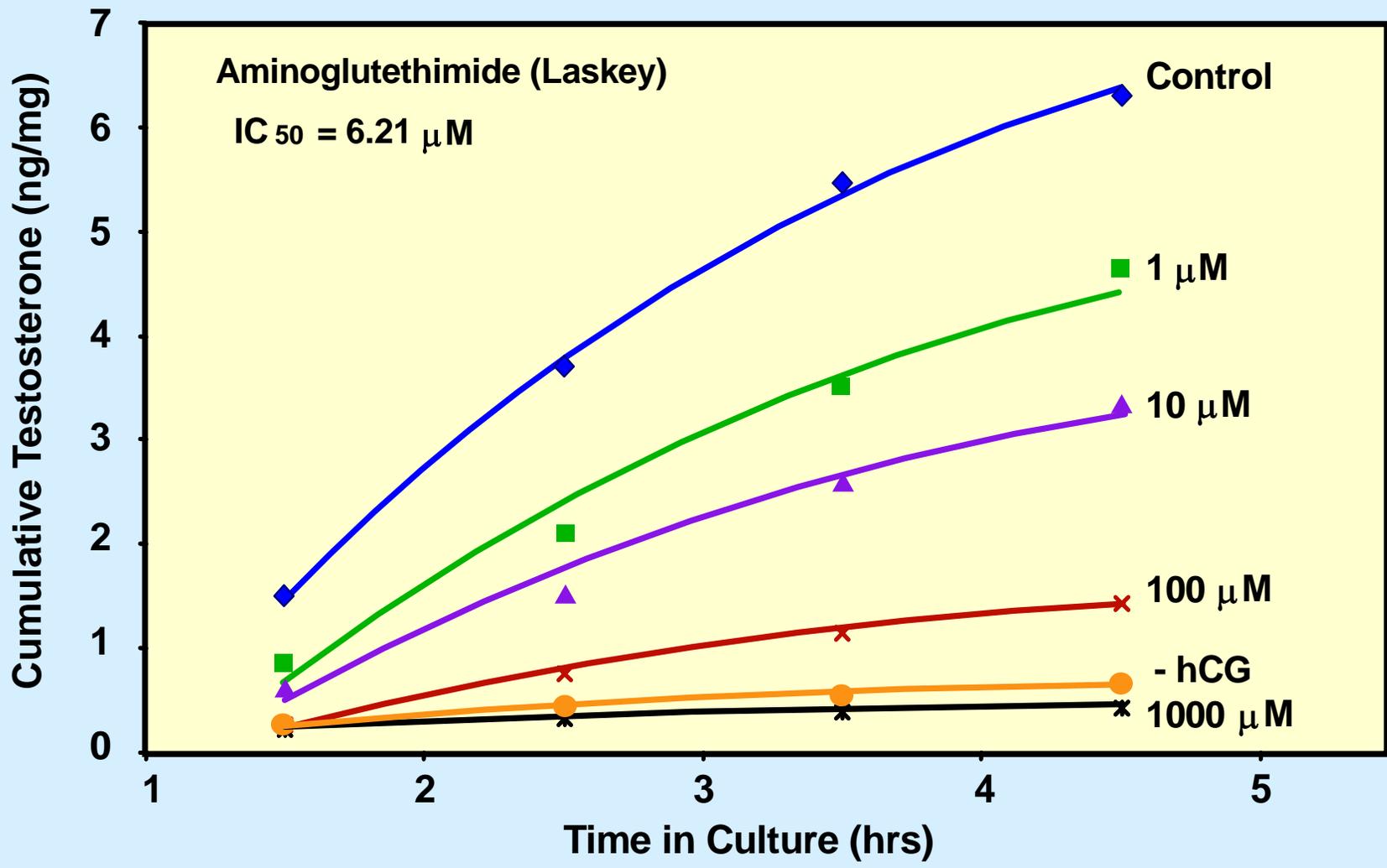
- **9 Chemicals - hCG stimulation**
- **Positive Control (aminoglutethimide)**
- **Cytotoxicant (2,4-dinitrophenol)**

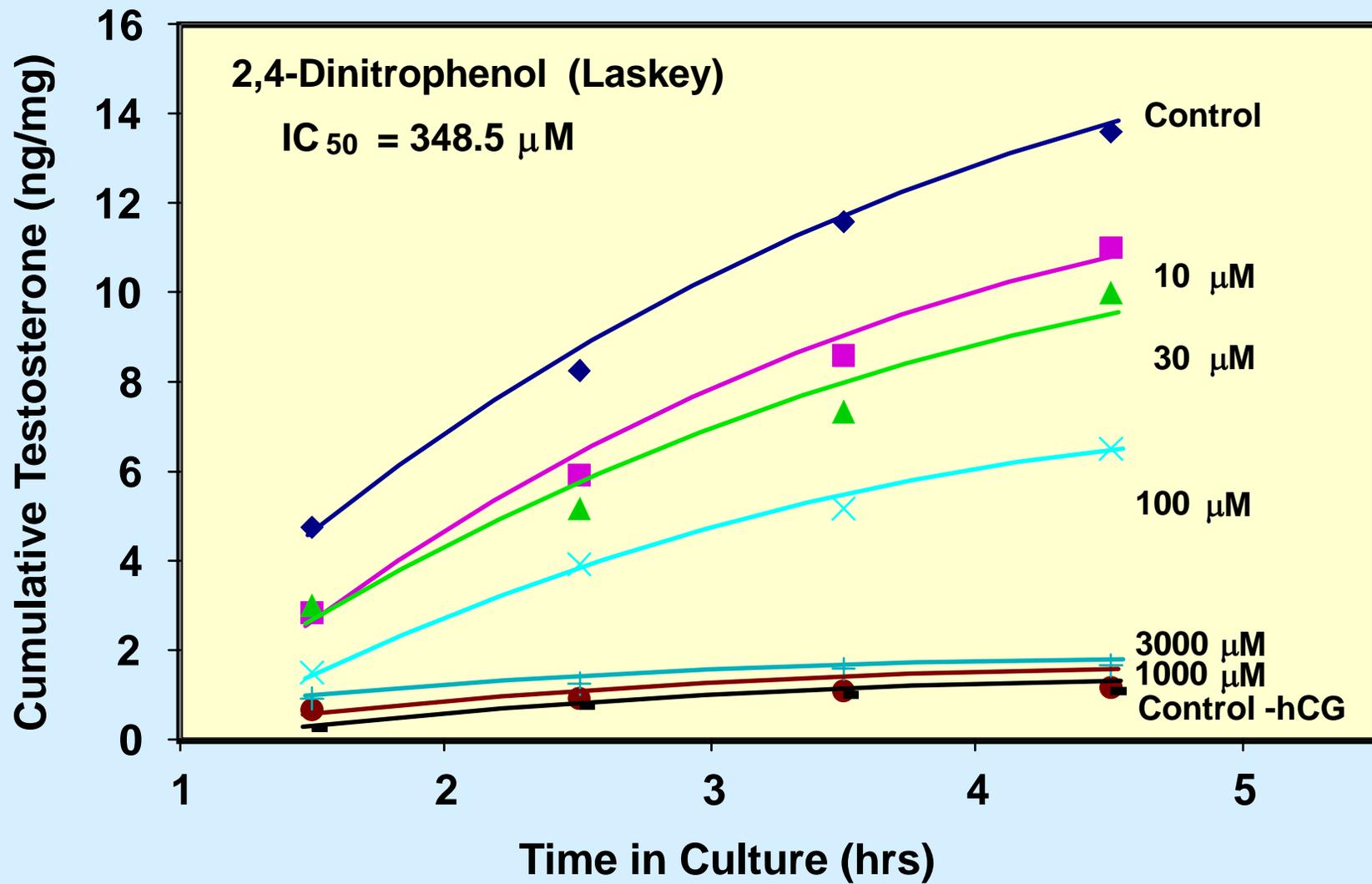
- **2 replications**
- **Each treatment condition within the replicate- n=3**

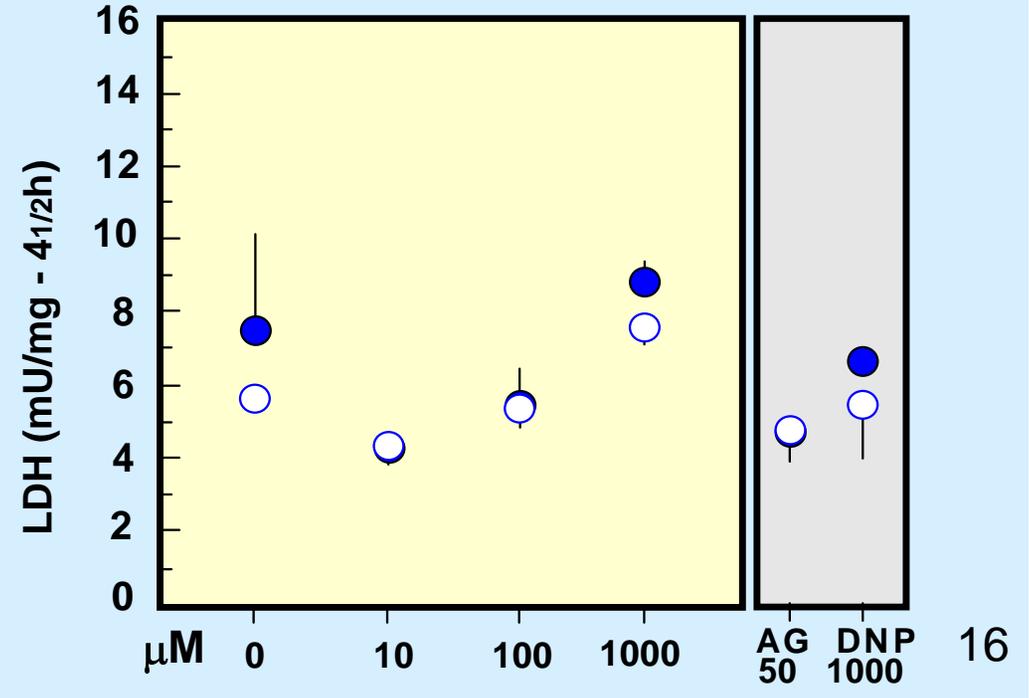
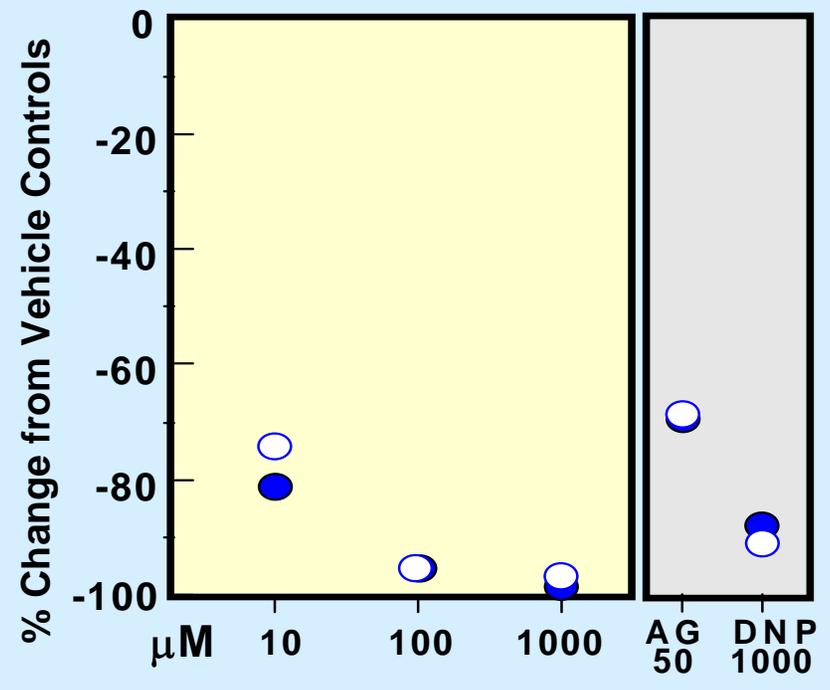
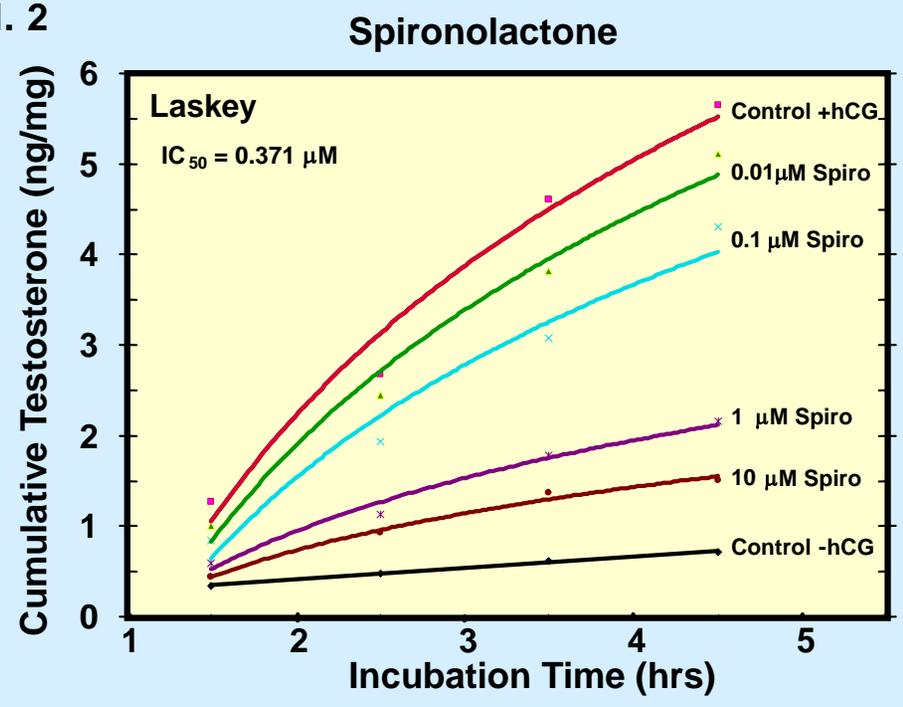
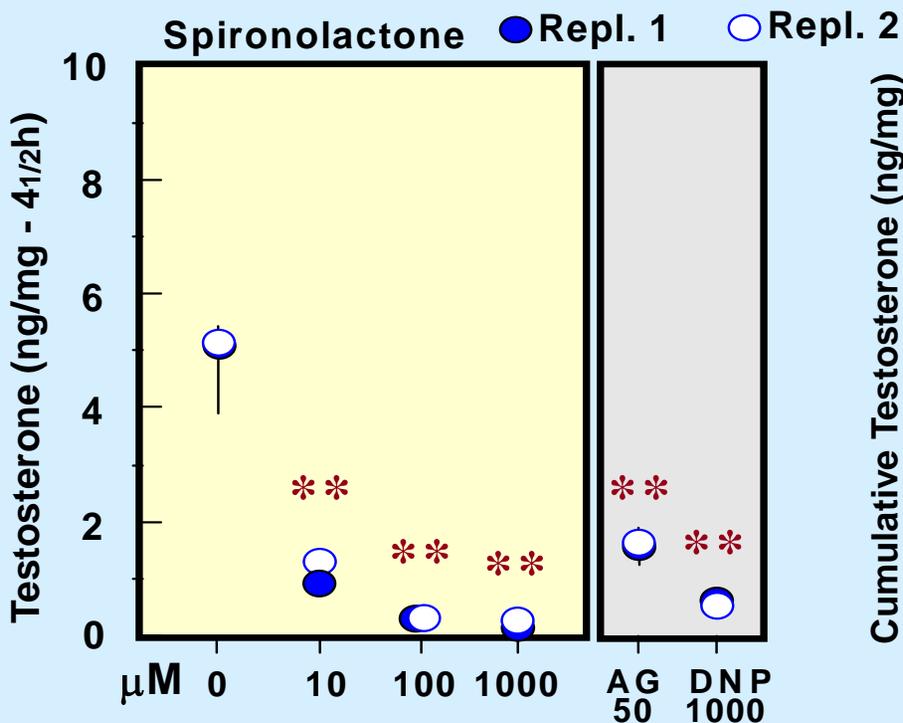
**Initial 1/2h wash. Samples taken at 4 hourly intervals and media changed. Aliquots from each sampling time were pooled.**

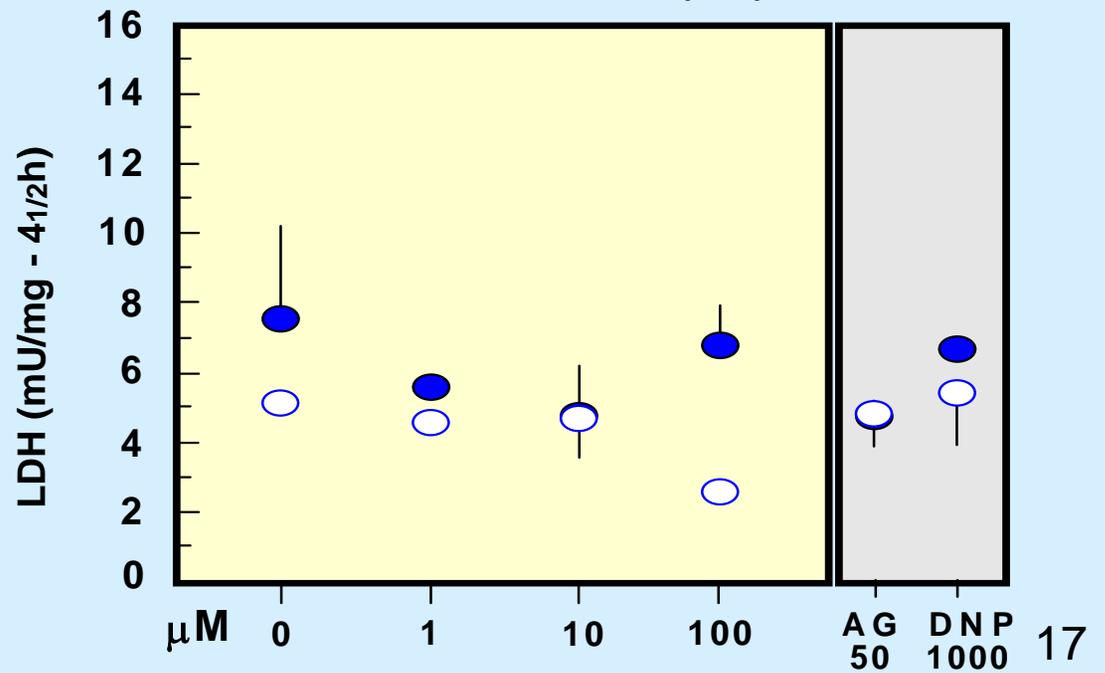
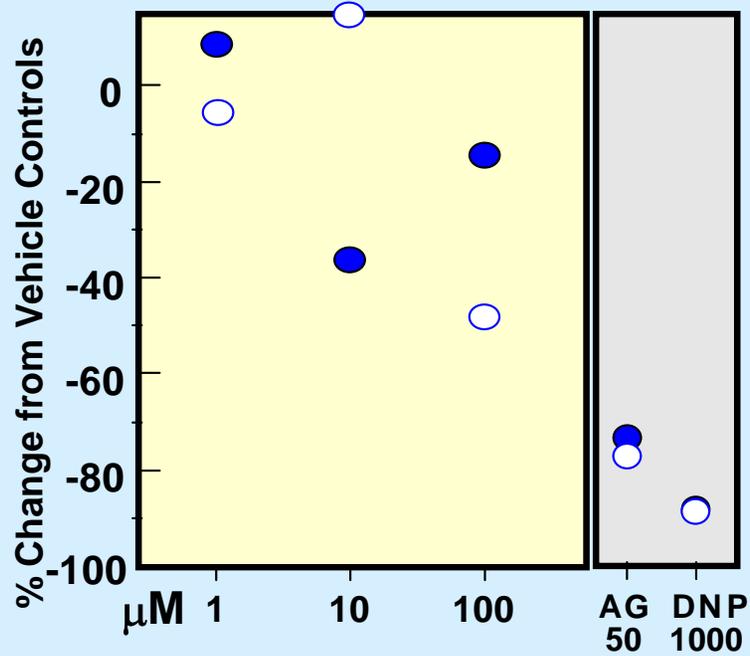
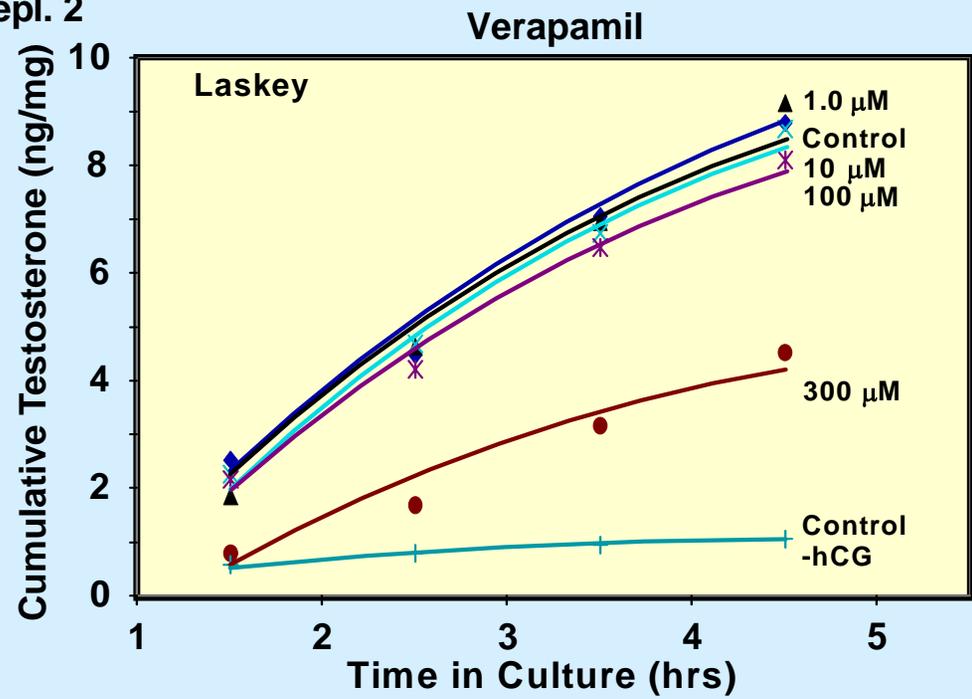
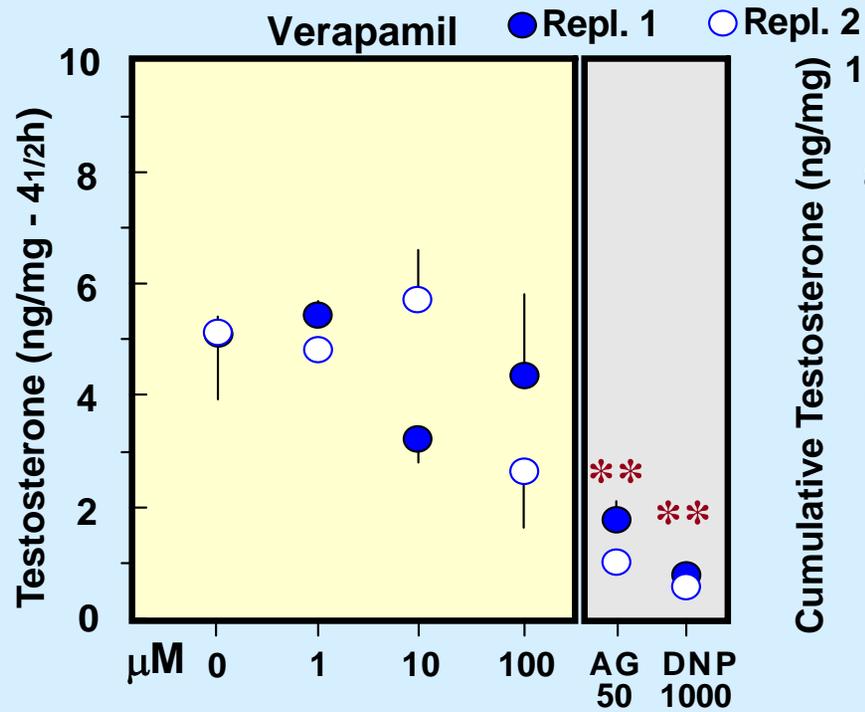
# Prevalidation - Chemicals

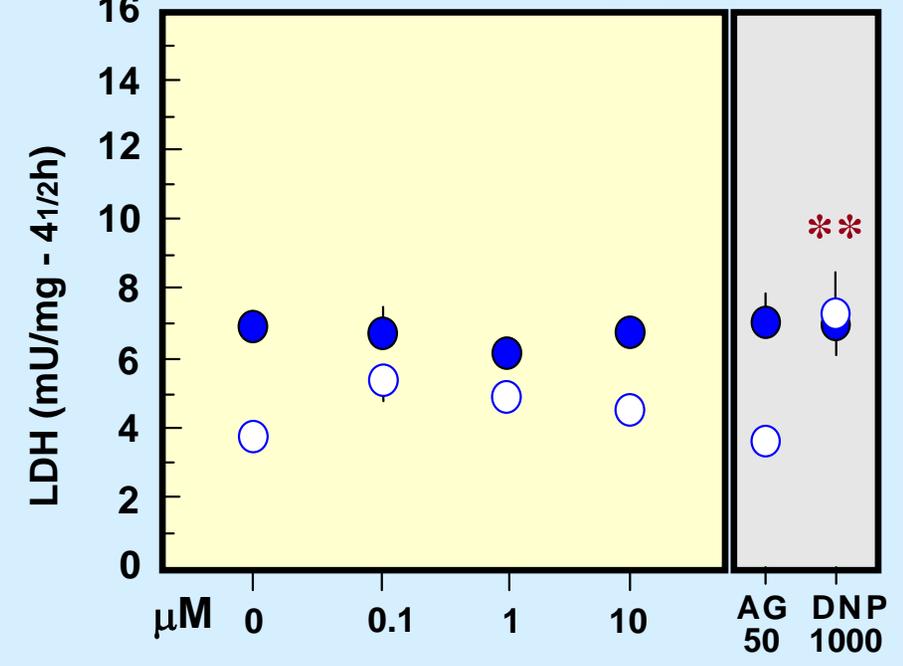
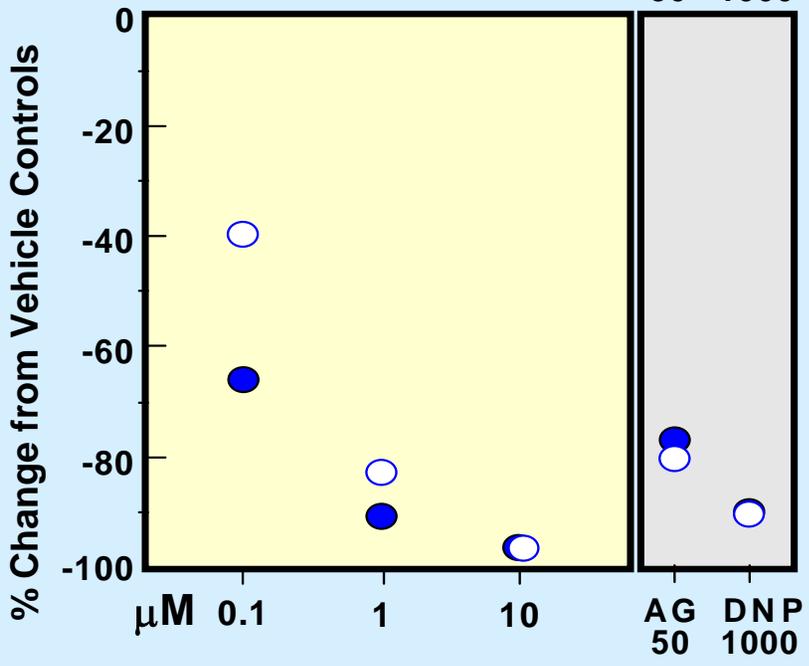
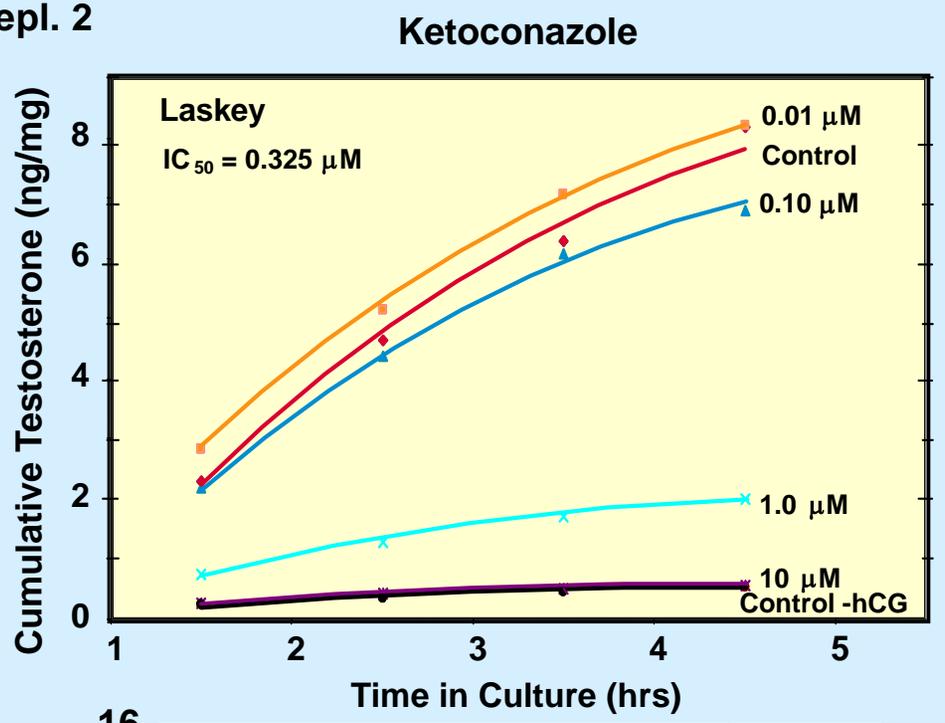
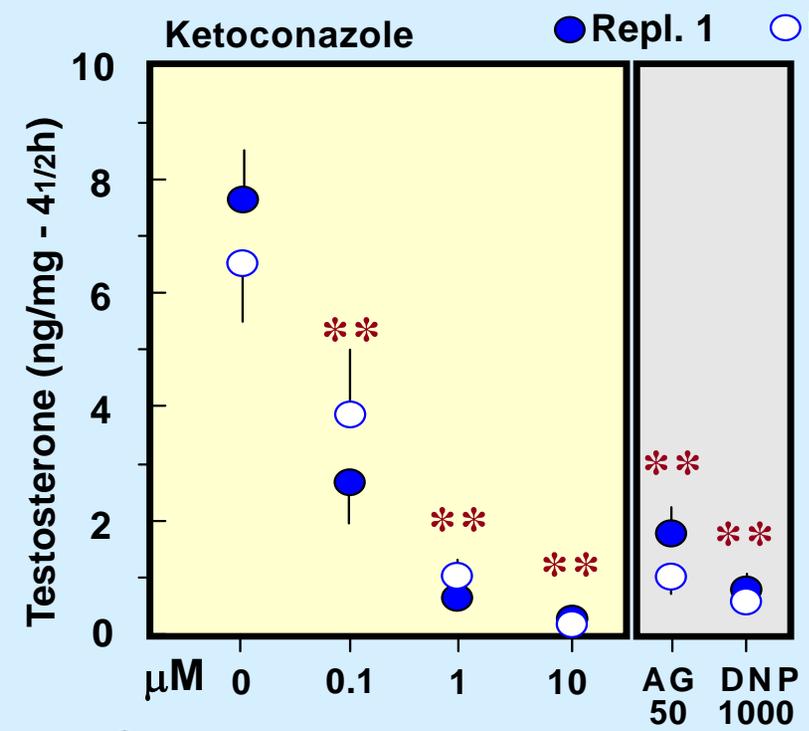
<b>Ketoconazole</b>	—	P450scc (CYP11A) inhibition
<b>Spirolactone</b>	—	17 $\alpha$ -hydroxylase / 17,20-lyase (CYP17) inhibition
<b>Dimethoate</b>	—	Some evidence of StAR inhibition
<b>Prochloraz</b>	—	Aromatase inhibition, AR block, some evidence for inhibition of T production
<b>Flutamide</b>	—	Androgen receptor antagonist
<b>Finasteride</b>	—	5 $\alpha$ -reductase inhibition
<b>Verapamil</b>	—	Calcium channel blocker
<b>Vinclozolin</b>	—	Anti-androgen / AR-antagonist (metabolites) Some evidence for aromatase induction
<b>Atrazine</b>	—	Evidence for inhibition of phosphodiesterase activity / induction of aromatase activity.
<b>Aminoglutethimide</b>	—	Inhibition of CYP11A & P450arom (CYP19)
<b>2,4-Dinitrophenol</b>	—	Inhibition of oxidative metabolism; uncouples mitochondrial respiration from ATP synthesis

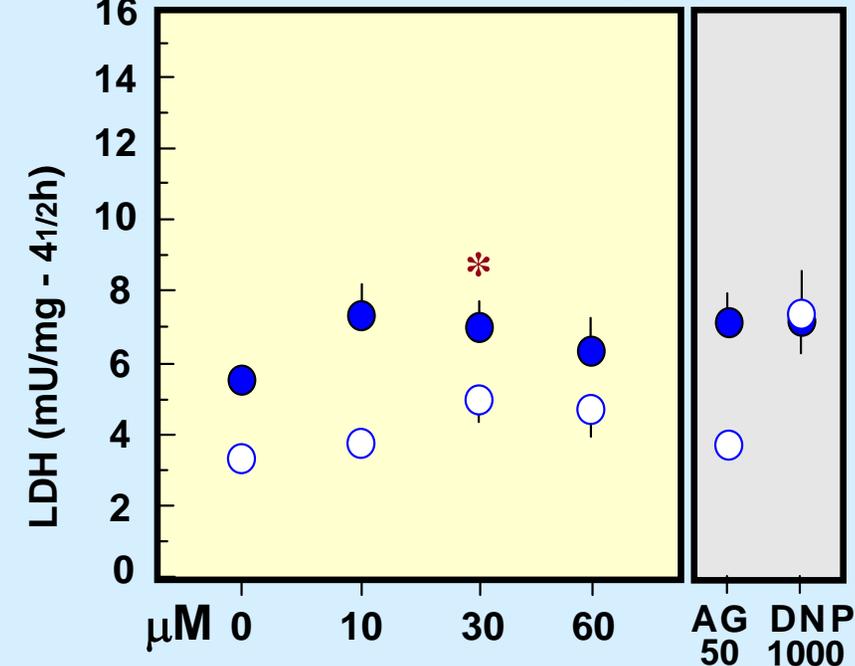
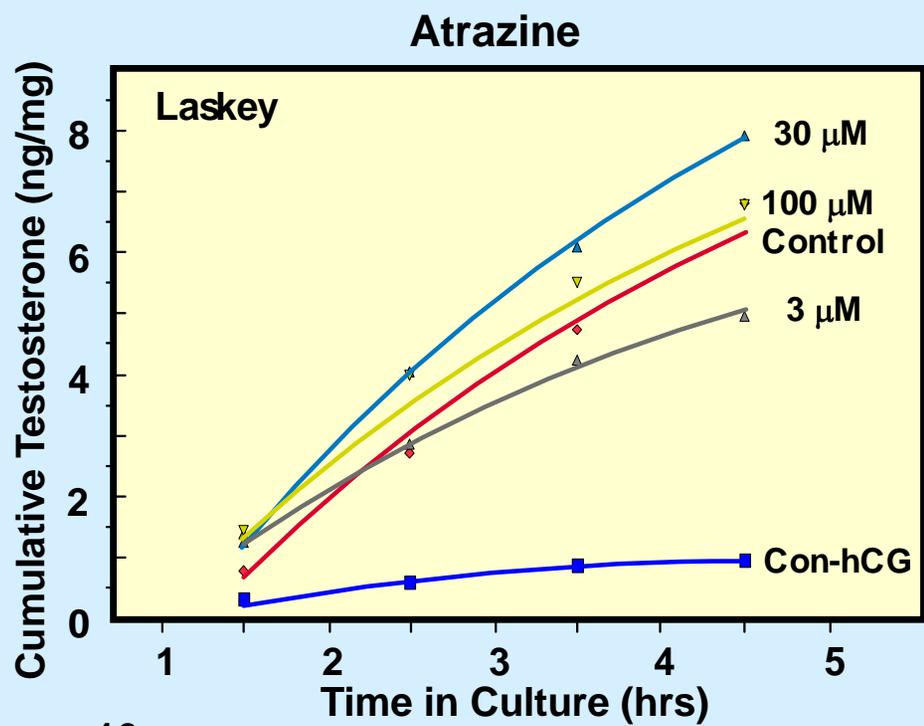
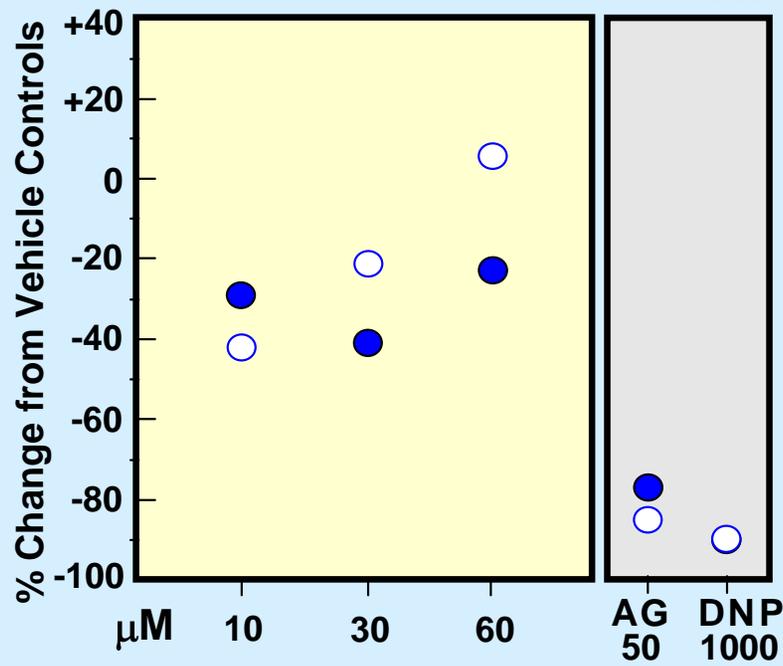
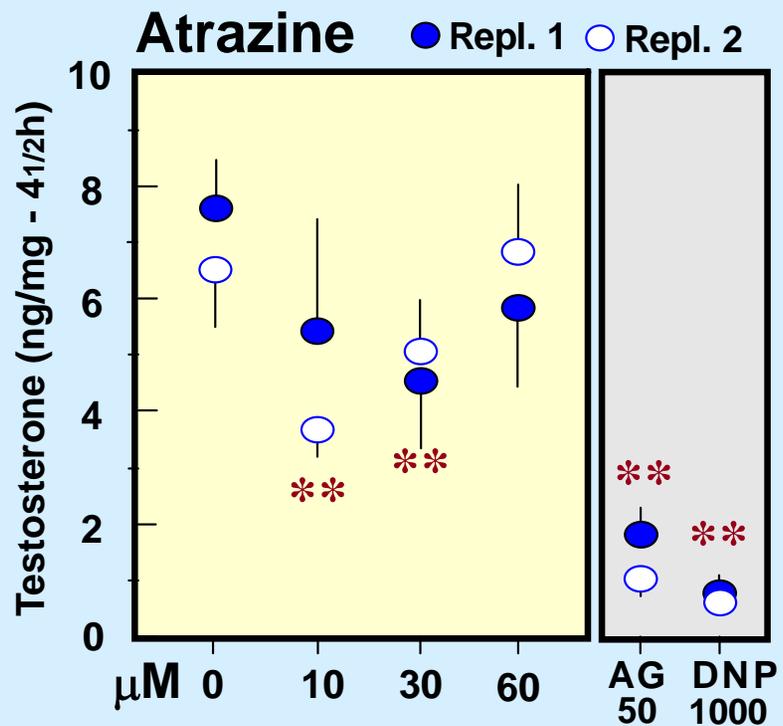


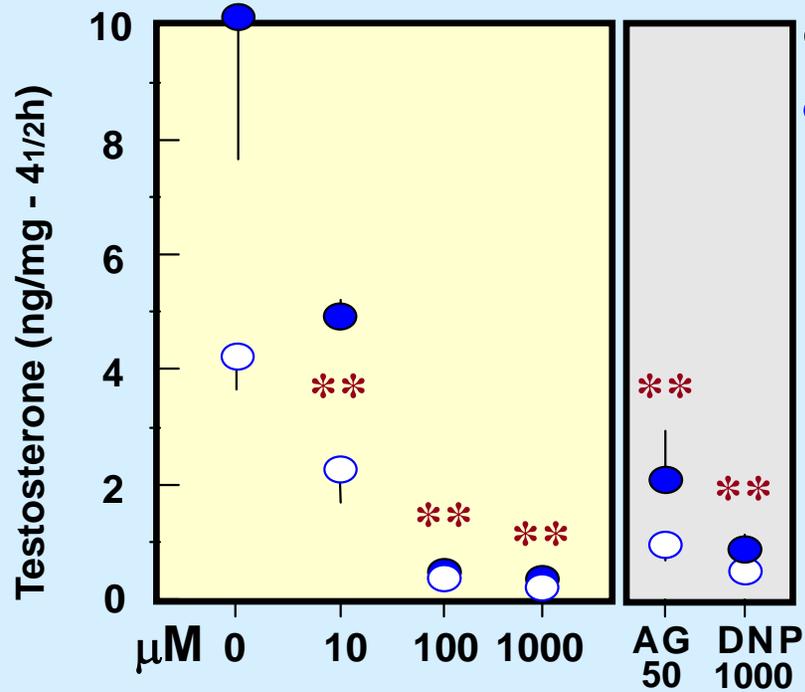




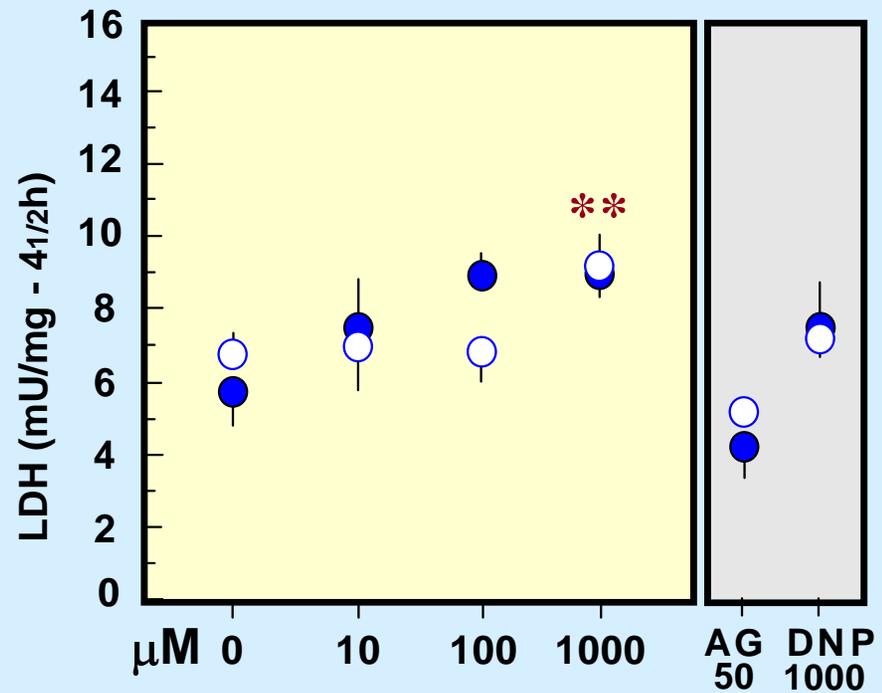
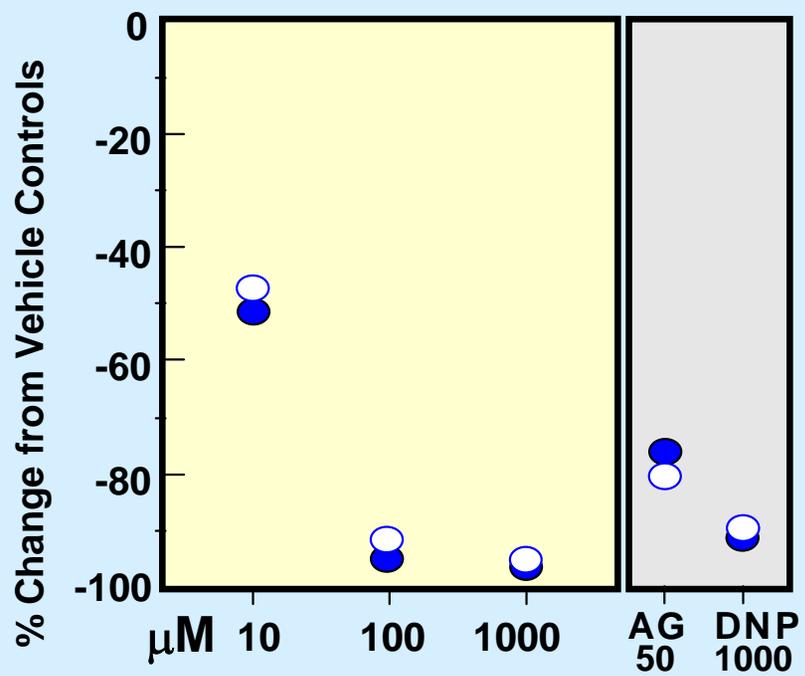


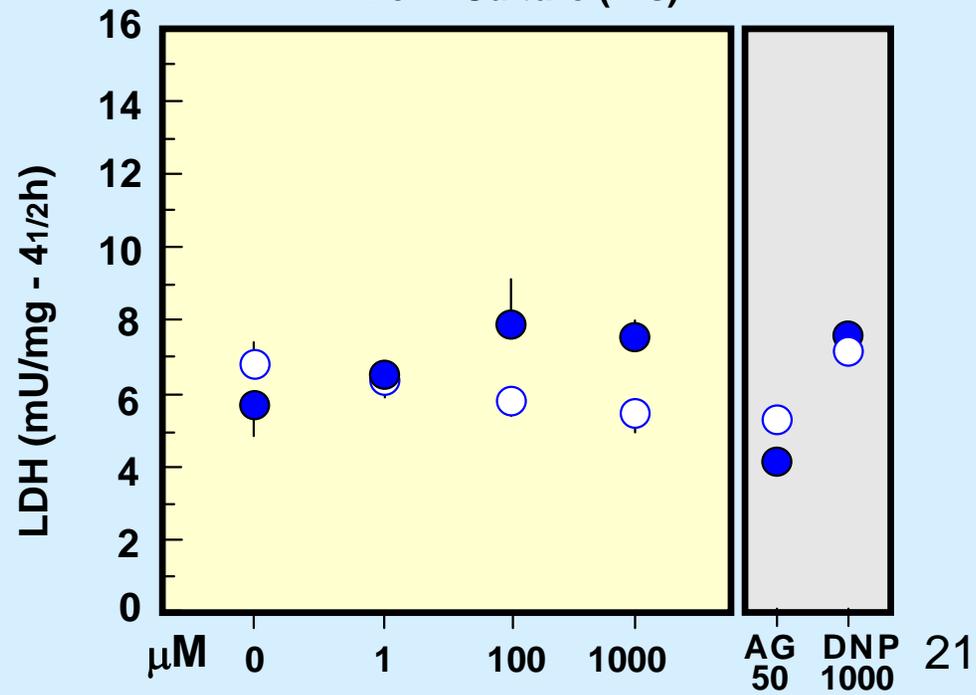
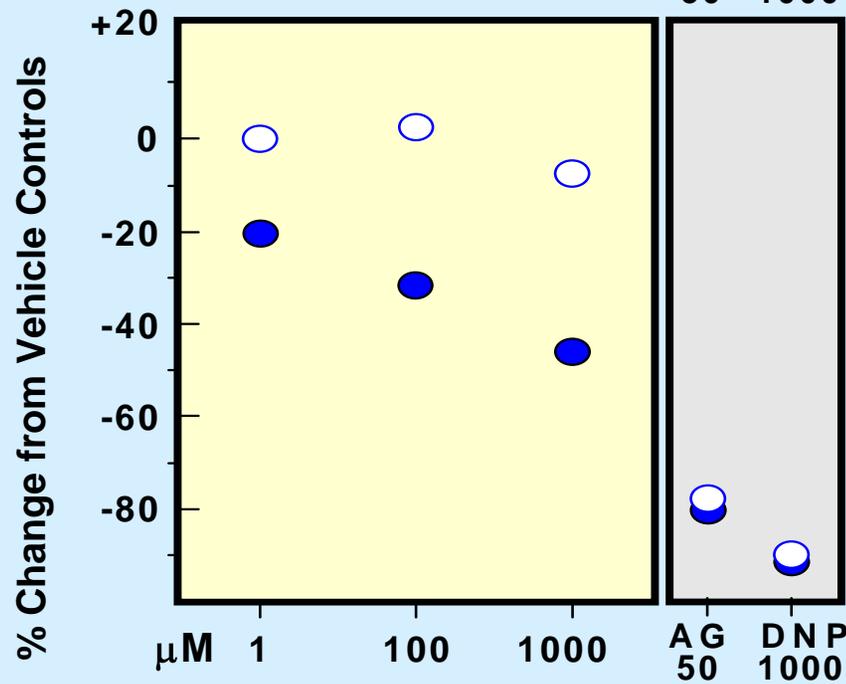
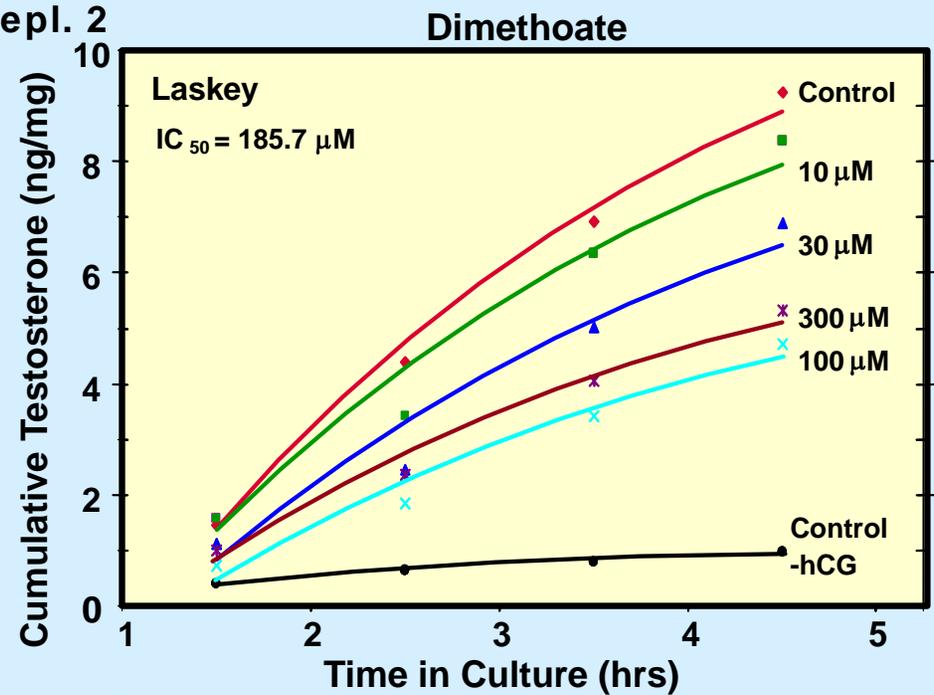
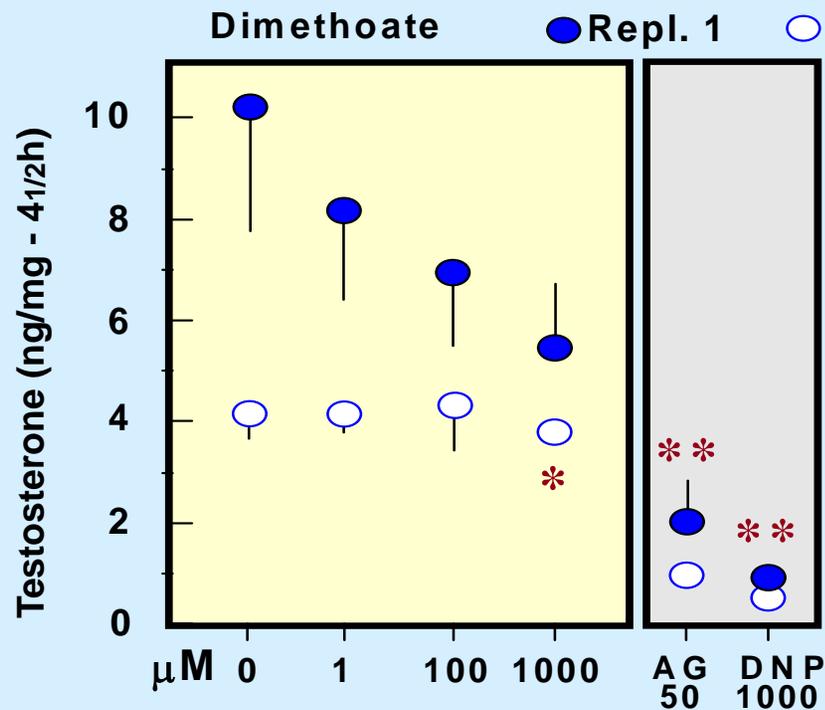




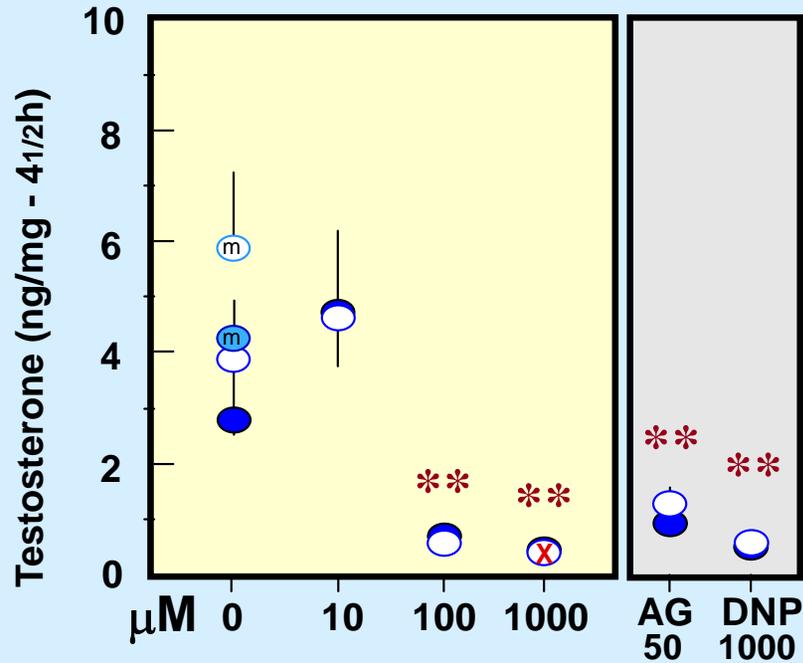


## Finasteride

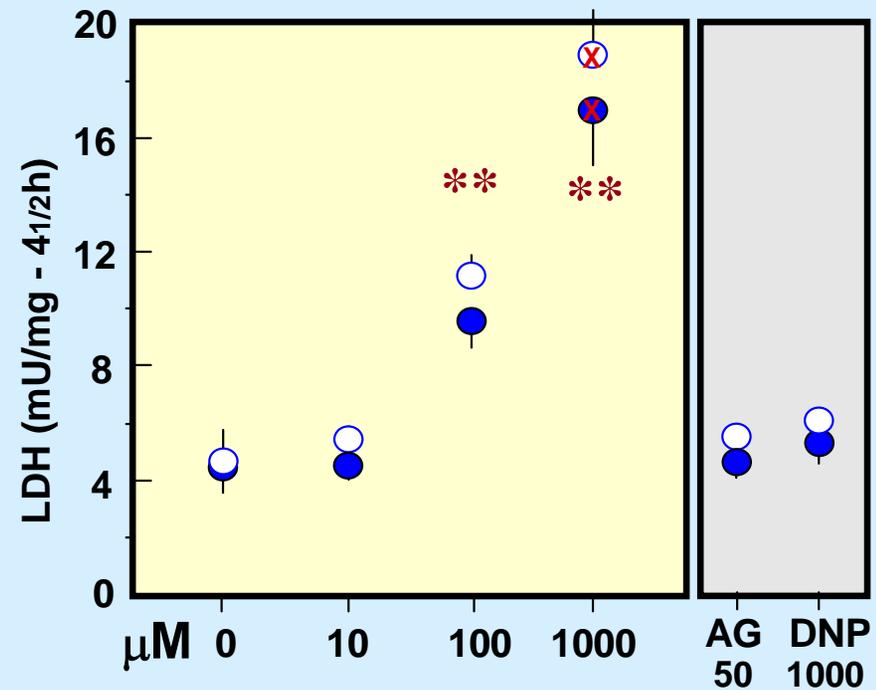
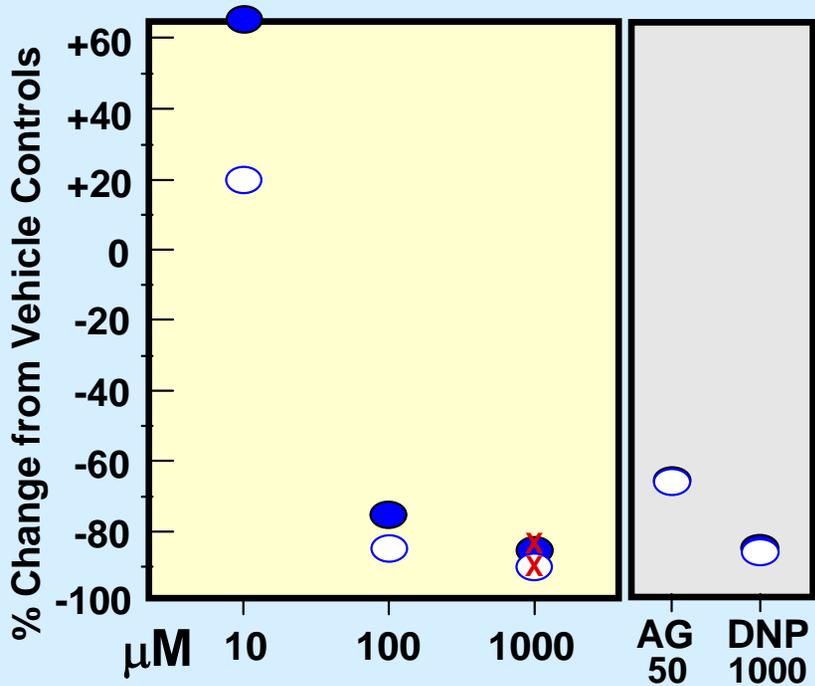




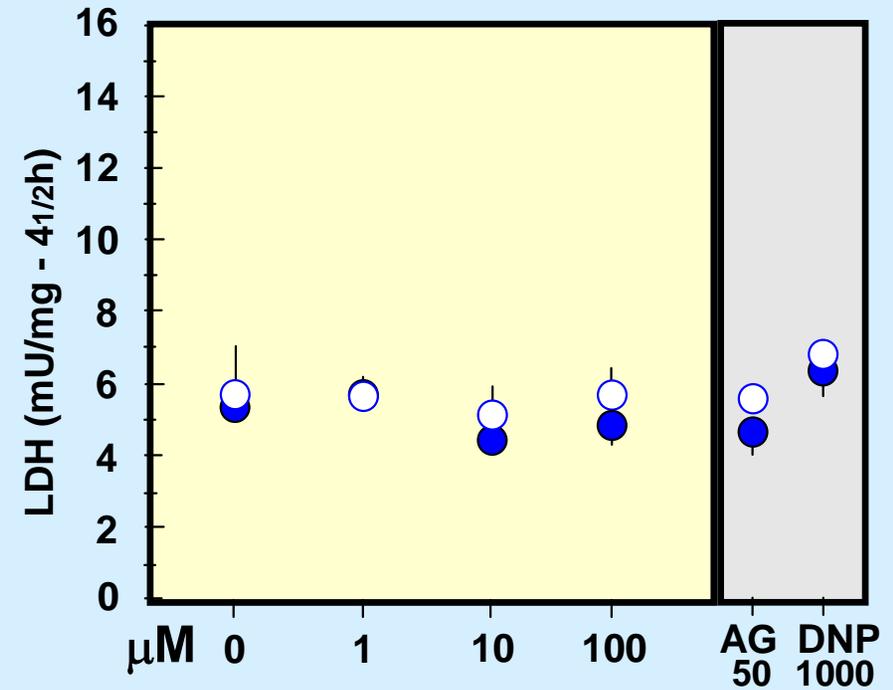
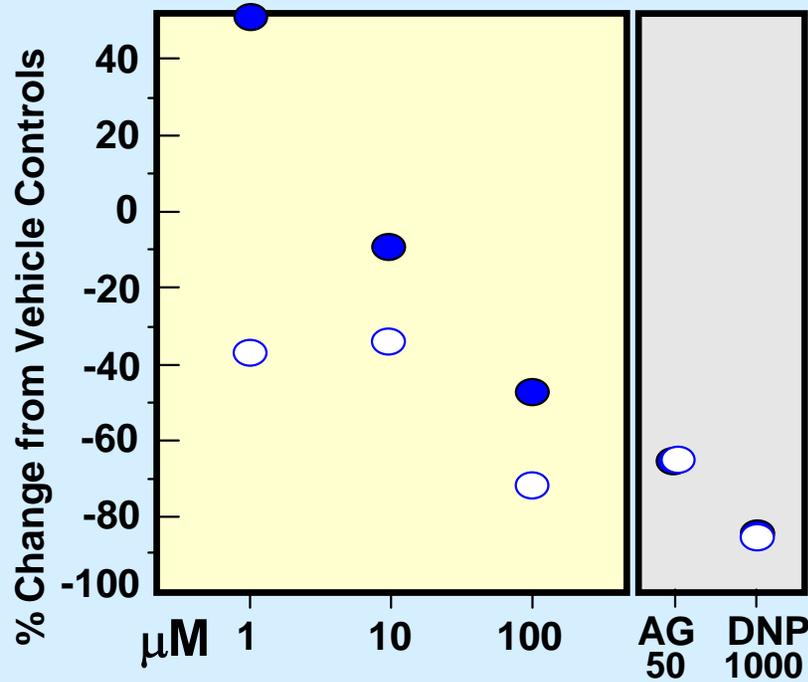
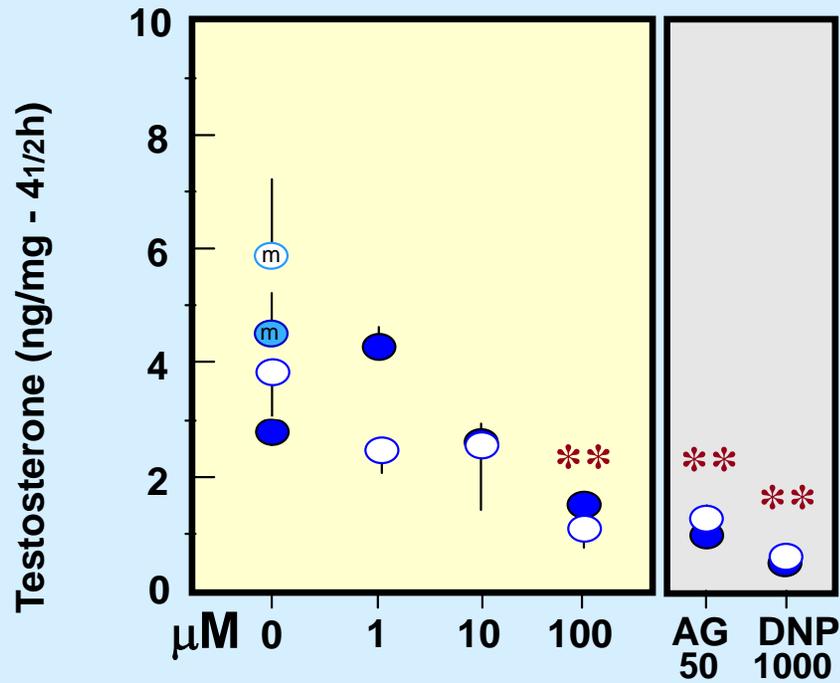
# Flutamide

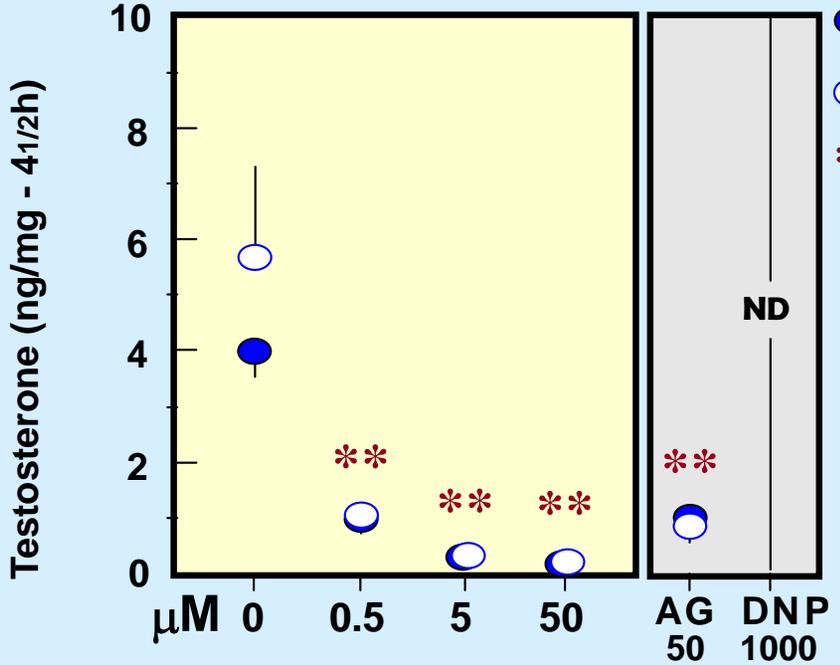


- Repl. 1
- Repl. 2
- Ⓜ Media Con-rep 1
- Ⓜ Media Con-rep 2
- ✕ Precipitate
- \*\* -  $p < 0.01$  for combined replicates 1 & 2 vs. combined replicates for M-V control



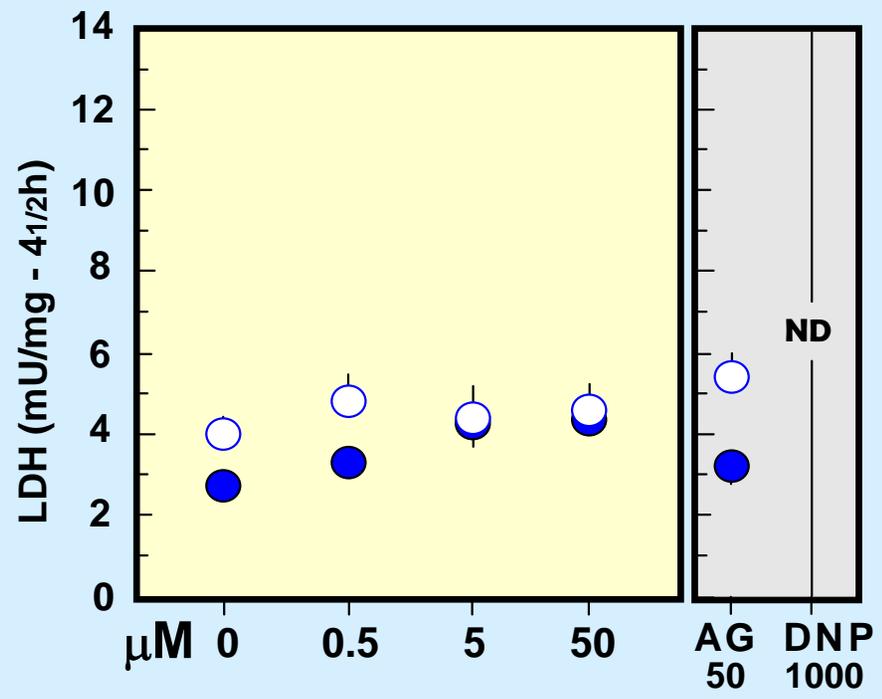
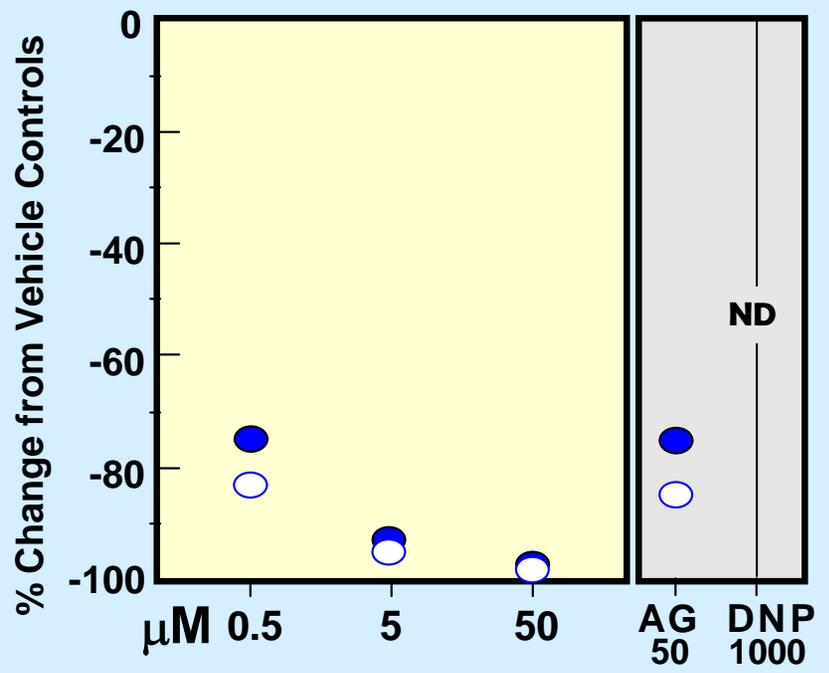
# Vinclozolin





● Repl. 1  
○ Repl. 2  
\*\* -  $p < 0.01$  for combined replicates 1 & 2 vs. combined replicates for M-V control

**Prochloraz**



# Prevalidation - Chemicals

<u>Compound</u>	<u>Effect Observed</u>	<u>Possible Explanation</u>
<b>Ketoconazole</b>	- Dose-related suppression	- P450scc (CYP11A) inhibition.
<b>Spiroinolactone</b>	- Dose-related suppression	- 17 $\alpha$ -hydroxylase/17,20-lyase inhibition (Ruiz de Galarreta et al. JBC 258:10988, 1983)
<b>Dimethoate</b>	- Dose-related suppression, modest effect at 1000 $\mu$ M	- Some evidence of StAR inhibition (Walsh et al. J. Endocrinol. 167:253, 2000)
<b>Prochloraz</b>	- Dose-related suppression	- Inhibition of fetal testicular T production Increase in P4- 17 $\alpha$ -hydroxylase inhibition? [Wilson et al. Tox. Lett. 146:207,2004; Vinggaard et al. Toxicol Sci. (in press)].
<b>Flutamide</b>	- Suppression at 100 / 1000 $\mu$ M	- Cytotoxic (LDH) at these doses
<b>Finasteride</b>	- Dose-related suppression 10 – 1000 $\mu$ M	- Cytotoxic (LDH) at high dose (1000 $\mu$ M). Also, P4 decreased at 1.34 & 2.68 $\mu$ M in R2C rat Leydig cells. Possible P450scc effect (Freeman et al. Endocrinology 133:1915, 1993).
<b>Verapamil</b>	- No effect up to 100 $\mu$ M (Laskey)- Suppression at 300 $\mu$ M	- Decreased stimulatory effect of LH on testosterone in primary cultures of mouse Leydig cells (Meikle et al. J. Androl. 12:148, 1991).
<b>Vinclozolin</b>	- Decrease at high dose (100 $\mu$ M)	- Equivocal / cytotoxicity?
<b>Atrazine</b>	- U-shaped dose response 10 – 60 $\mu$ M	- Equivocal. Laskey data indicates no effect (3, 30, or 100 $\mu$ M).

## Phase 2

### Multi-lab Comparisons: Selection of Laboratories

- **Selected by Contractor by open solicitation**
- **Selection criteria**
  - **Independent Laboratories**
  - **Experienced in:**
    - **In vitro test methods**
    - **Cell & tissue cultures**
    - **Test chemical administration**
    - **Enzyme kinetics & inhibition studies**
  - **Knowledge of steroidogenesis**
  - **Capable of compliance with GLP**

## 5 Labs

**Battelle**

**WIL Laboratories**

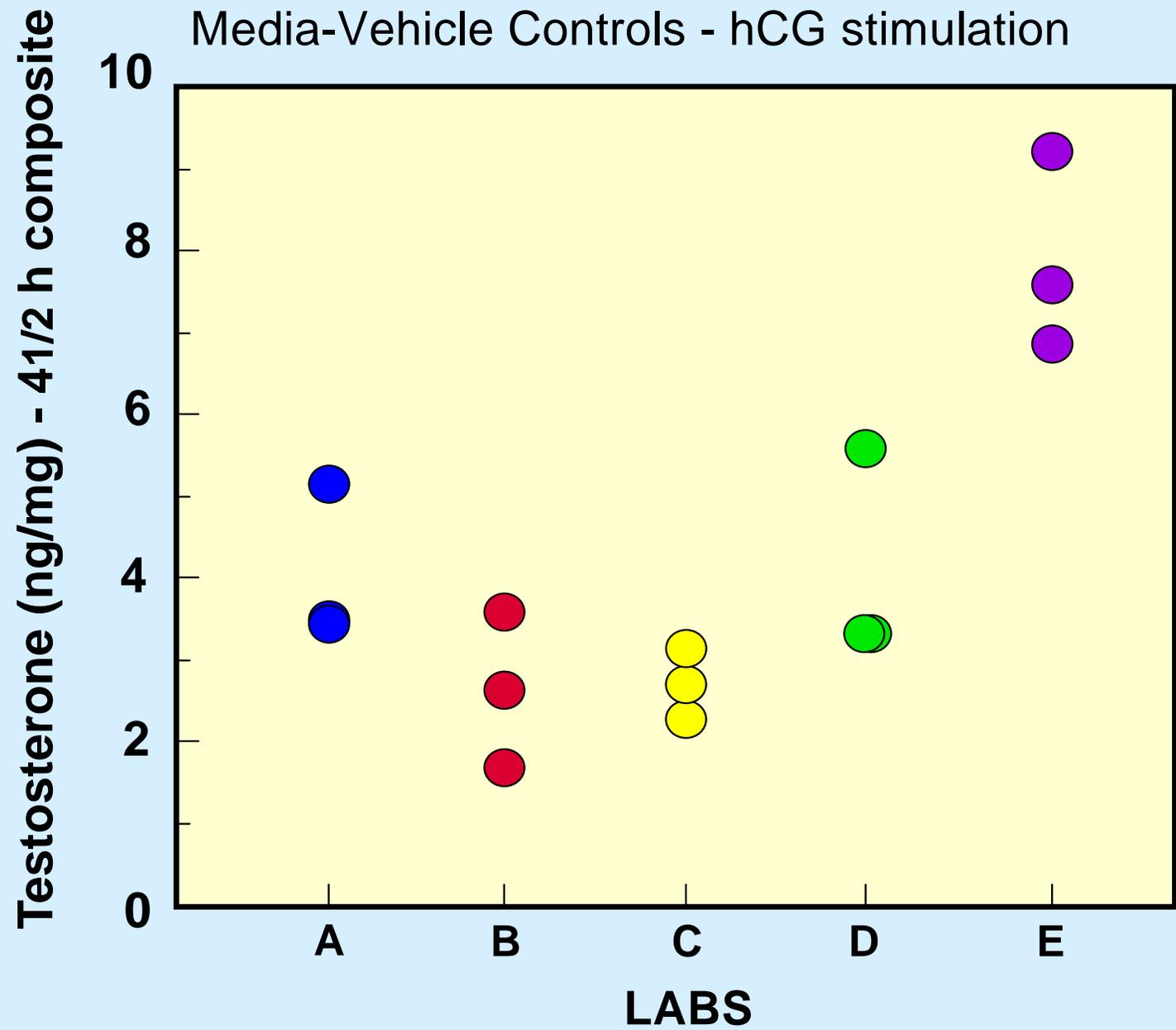
**RTI, International**

**Toxikon**

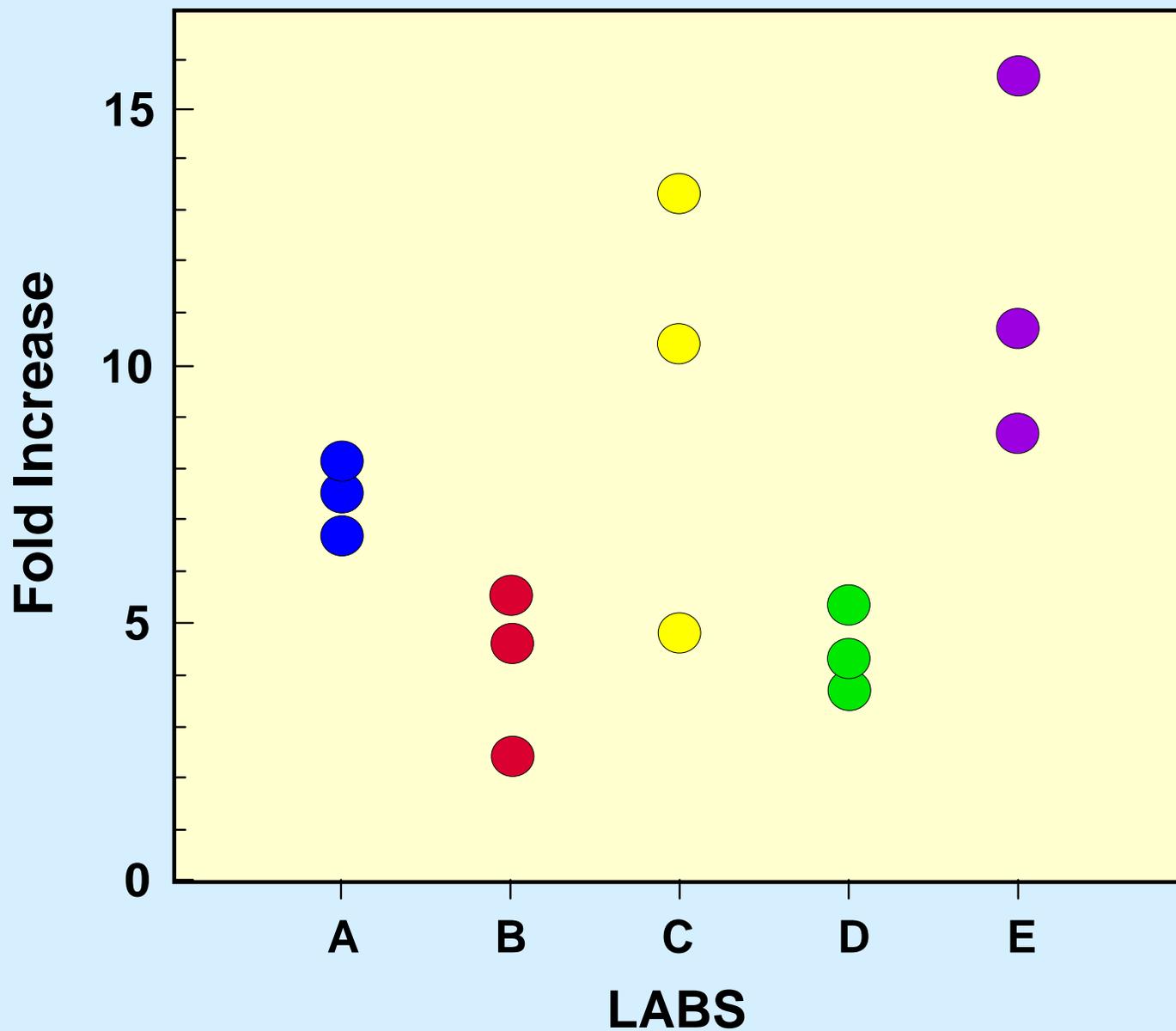
**Southern Research Institute**

## Multi-lab Comparisons

- **Media Control**
- **Media-Vehicle Control**
  - hCG / + hCG (0.1 IU/ml)
- **Aminoglutethimide**
  - 10, 100, 1000  $\mu$ M
- **Ethane dimethane sulfonate (EDS)**
  - 1000  $\mu$ M
- **3 Replications**
- **Each treatment condition within a block- n=3**  
1/2 h wash, hourly interval sampling –aliquots from each pooled.

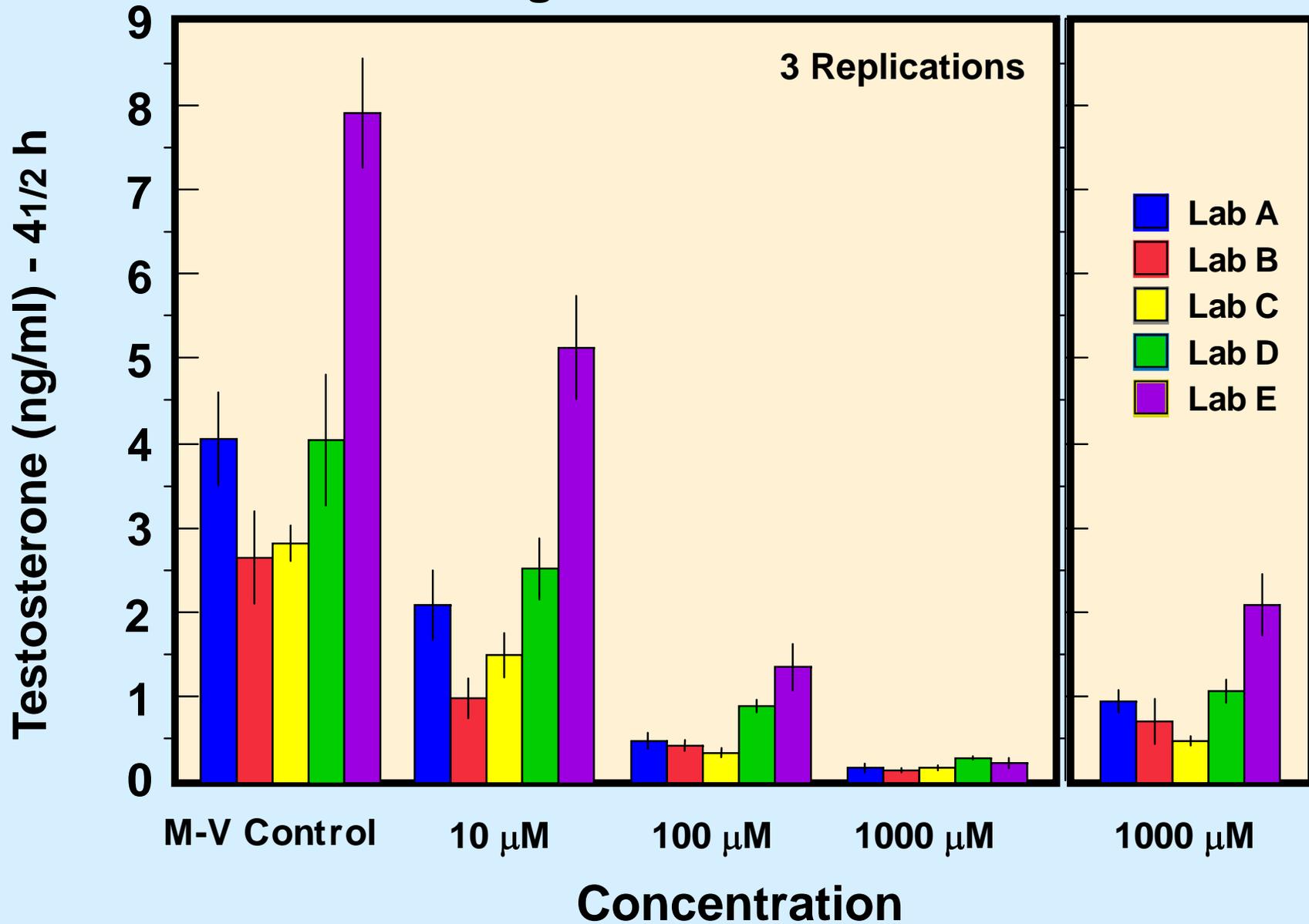


### hCG stimulation (Media-Vehicle Control)

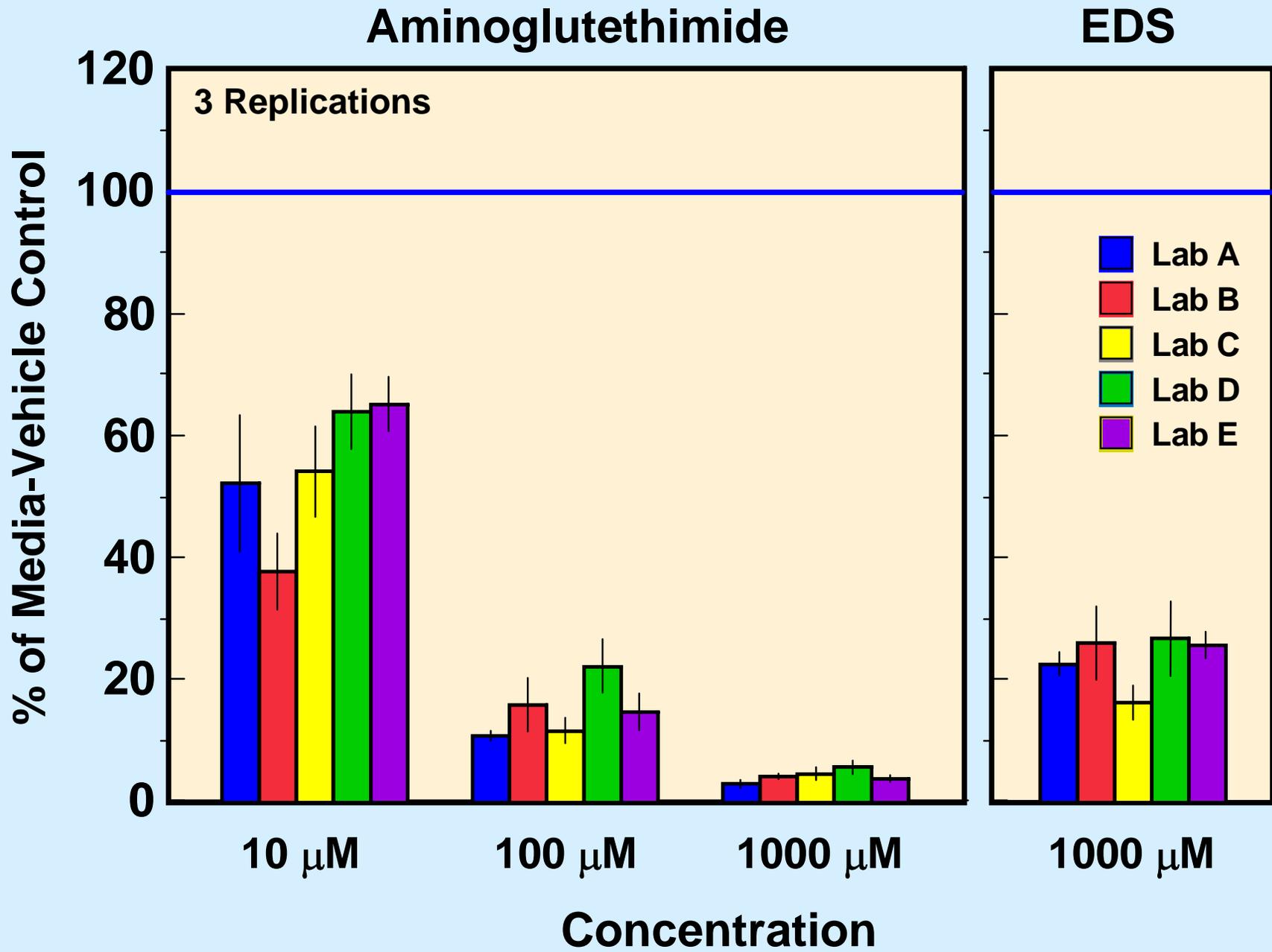


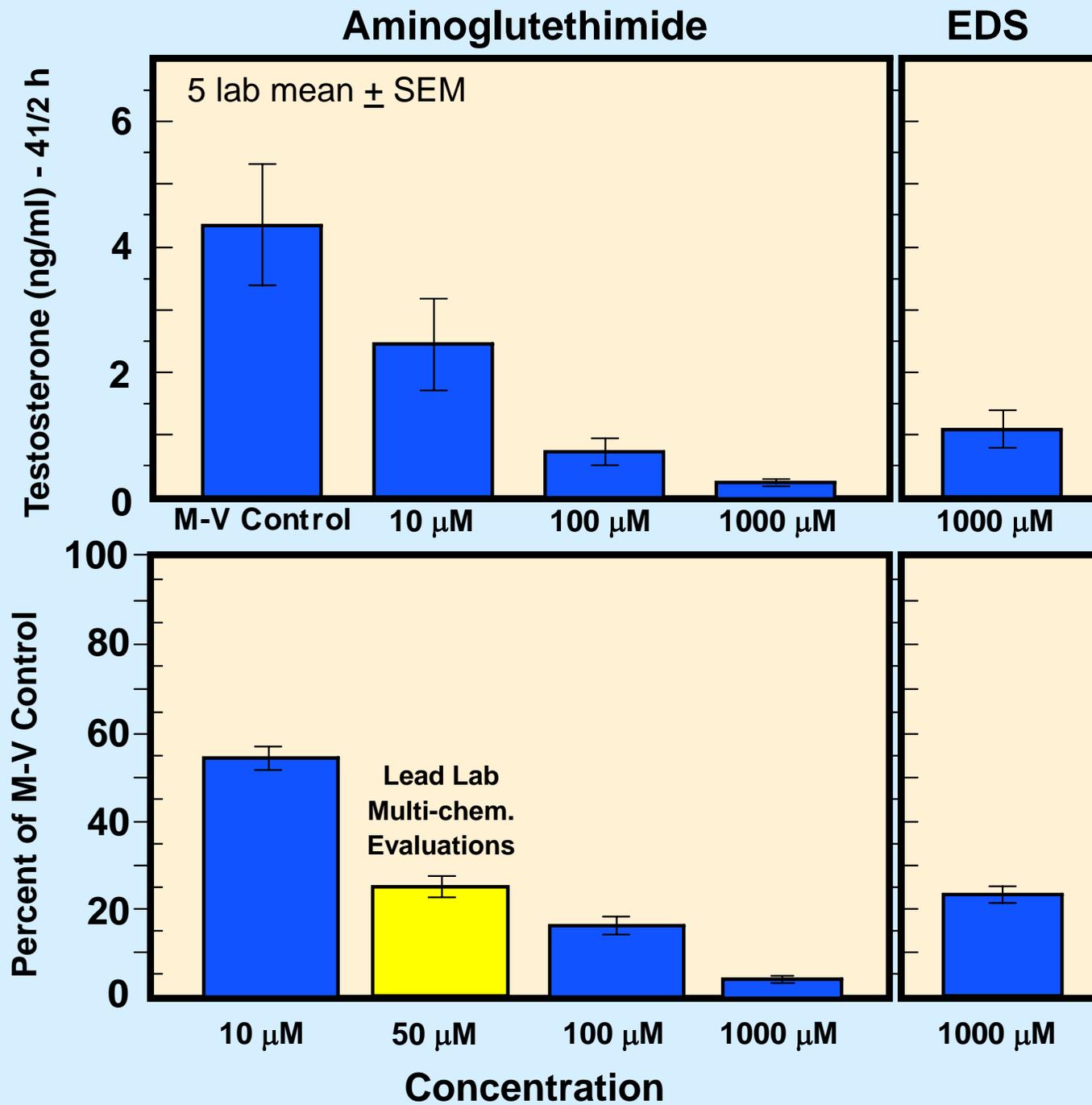
# Aminoglutethimide\*

# EDS\*



\* negative in LDH assay





# Cytotoxicity

## Selection of a Measure: Reconsideration

### Factors

- Sensitivity
- Appropriate for Tissue Fragments
- Ease of Use / Availability as Commercial Kit
- Distinctive from Effects on Steroidogenesis

# Cytotoxicity Assays Tested

## Lactic Dehydrogenase

- Secreted from dying cells
- Spectrophotometric Assay
- Ease of Use / Kits Available

## MTS Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)

- Based on the ability of viable mitochondria to produce formazan
- Kits Available / Required adaptations for use with tissue
- Spectrophotometric Assay
- Earlier stage event

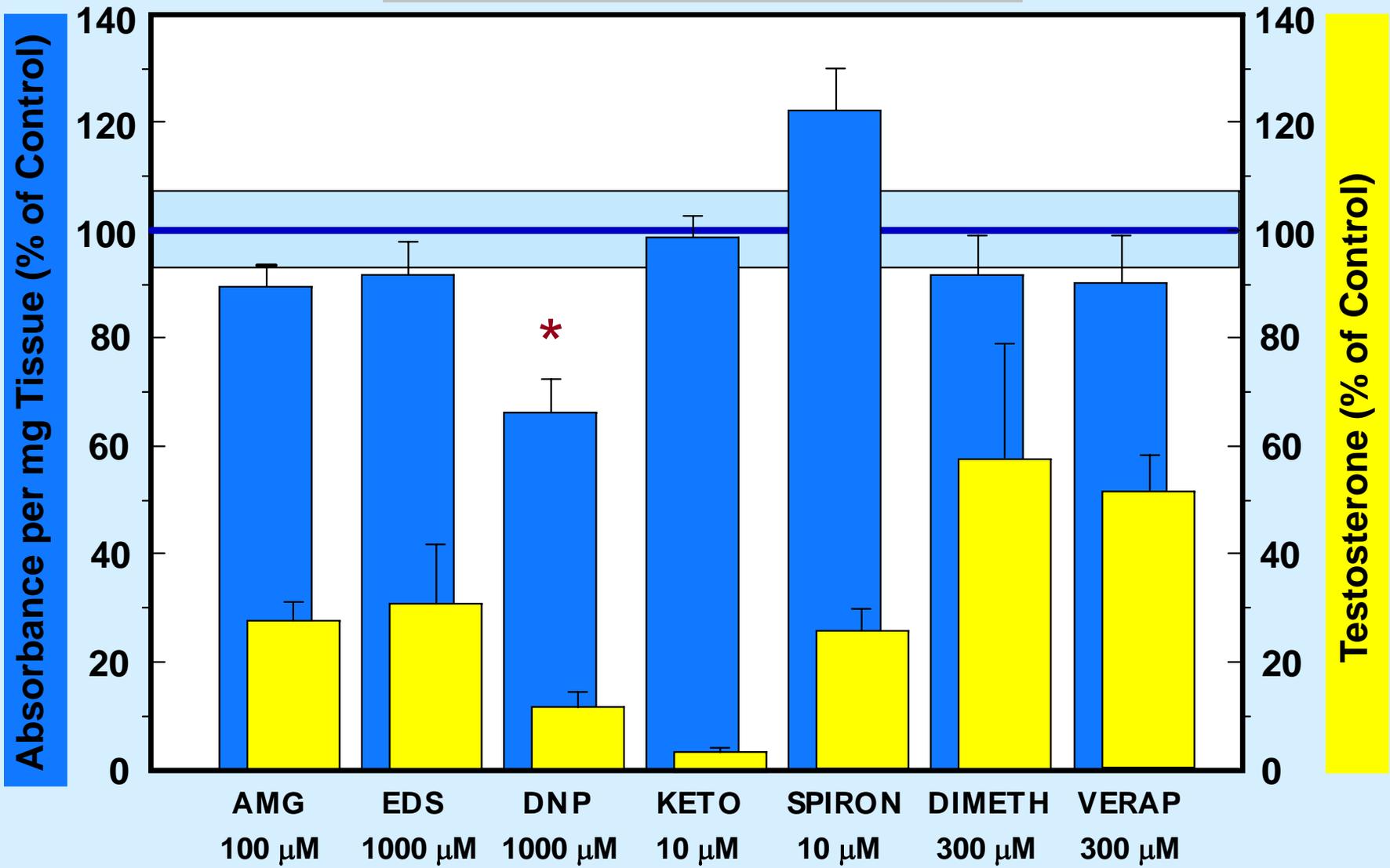
## ATP Bioluminescence

- Assessment of toxicant reductions in ATP
- Involvement in Steroidogenesis a confound  
(However- tested with dinitrophenol cytotoxicant)

## Caspase-3

- Part of apoptotic caspase cascade
- Cascade requires ATP - so questionable utility  
(However- tested with dinitrophenol cytotoxicant)

### MTS Cytotoxicity / Testosterone



## Sliced Testis Steroidogenesis Assay

### Strengths

- Only a modest degree of difficulty involved.
  - Tissue quickly obtained & readied for incubation.
- Short-term assay
  - 4 1/2 h in culture
- Compared to *in vivo* approaches, there is a reduction in animal use.
- Fragments from a single testis distributed across controls & treatment concentrations.
- Assay is able to identify clearly compounds having a known impact on testosterone secretion.

### Limitations

- In comparison to purified Leydig cell preparations, use of tissue fragments shows increased variability.
  - Fragments from different testes may be under variable degrees of initial stimulation. Amount of secreted testosterone can vary from testis to testis.
- Cell population is mixed, with Leydig cells only a minor portion of the whole. Presumption that cytotoxicity representative of a comparable effect on all cells.
- Animal usage is reduced, but not eliminated

## Performance Criteria

### Sliced Testis Procedure

- |   |  |
|---|--|
| ● Testis weight   | 1.2 - 2.0 g  |
| ● hCG stimulation   | $\geq$ 5-fold  |
| ● Media-vehicle control                                       | Cytotoxicity negative  |
| ● Positive control  | Decrease in testosterone $\geq$ 60%  |
| ● Negative control  | Not significantly different from M-V control<br><i>(% change in T from Media-Vehicle Control) &lt; 30%</i> |
| ● <i>Relationship- Media control to Media-Vehicle control</i> | <i>Not significantly different from one another</i>  |

### Testosterone Assay

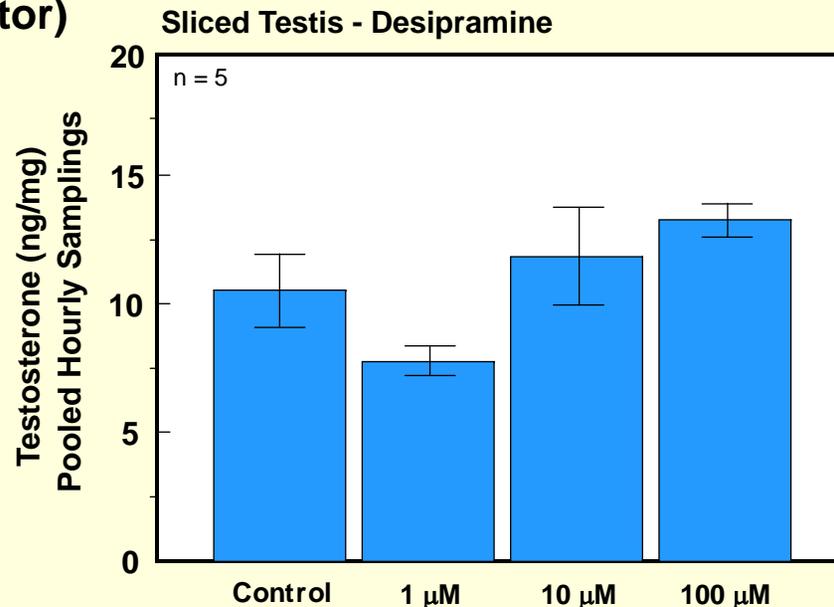
- |                          |   |
|--------------------------|---|
| ● QC samples for T assay | At least 2 (in duplicate) at separate points on the curve. Inter- / intra-assay variation $\leq$ 10%. |
| ● Assay Interference     | Compound-spiked medium should not interfere with testosterone assay. If so, must be reported.         |

## Considerations in the Selection of a Screen to Evaluate Toxicant Effects on Steroidogenesis- Results from the Pre-validation Study

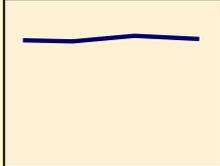
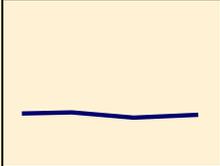
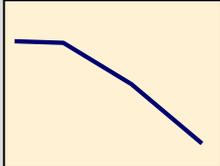
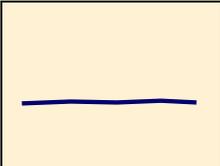
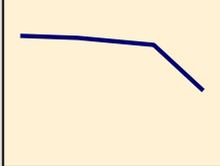
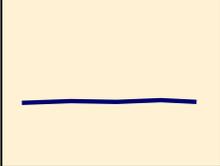
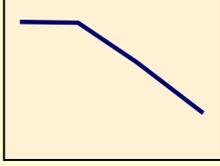
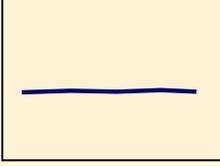
- |   |                                 |
|---|---------------------------------|
| ● <b>Predictiveness</b> - Is the screen able to identify an effect?   | <b>YES</b>                      |
| ● <b>Sensitivity</b> - Is the test sufficiently sensitive at lower concentrations of exposure?                                  | <b>Lower conc. needed</b>       |
| ● <b>Ease of Use</b> - Does the assay involve specialized training beyond general laboratory competence?                        | <b>Some</b>                     |
| ● <b>Variability</b> - Would variations (intra- & interlaboratory) within group data preclude detecting an effect?              | <b>Modest Inhibition needed</b> |
| ● <b>Standardization</b> - Can the assay be sufficiently standardized with clear guidelines for its conduct?                    | <b>YES</b>                      |
| ● <b>Animal usage</b> - Can the use of animal subjects be refined, <b>reduced</b> , replaced, or even eliminated?               | <b>YES</b><br>(Reduced)         |
| ● <b>Costs</b> - Are the costs in equipment or personnel required to perform the assays at a manageable level?                  | <b>YES</b>                      |
| ● <b>Time requirements</b> - Is the time required to perform the assay reasonable to maintain the stability of the preparation? | <b>YES</b>                      |
| ● <b>Throughput</b> - Can sufficiently large numbers of samples be assayed concurrently?  | <b>No</b><br>(Modest Nos.)      |
| ● <b>Metabolic activation</b> - Does the assay permit any necessary metabolic activation?                                       | <b>Small amt. possible</b>      |
- 

## Adjustments (Ongoing/Suggested) to the Protocol

- Shorten times (previously < 1 hr from testis removal to slicing)
  - euthanasia until placement of testis in buffer (3 min or less)
  - Initiation of sectioning of each testis until incubation of fragments (6 min or less)
- (Suggested) Lower concentrations of strongly positive/non-cytotoxic compounds (aminoglutethimide- 0.1, 1  $\mu$ M, or spironolactone- 0.1  $\mu$ M) to assess assay sensitivity / variability (~75% of Media-vehicle controls)
- (Suggested) Substitute MTS (or MTT) for LDH as measure of cytotoxicity
- Incorporation of negative control (e.g., 100  $\mu$ M desipramine – norepinephrine reuptake inhibitor)



## Results - Categorizations

<u>Result</u>	<u>Positive</u>	<u>Equivocal</u>	<u>Negative</u>	Testosterone	Cytotoxicity
No differences in T, no cytotoxicity			X		
T decreased only for concentrations showing a positive cytotoxic response			X		
Clear Dose Response (decrease at multiple concentrations). No cytotoxicity	X				
T decreased only at highest concentration. No cytotoxicity		X - Repeat -			
Cytotoxicity at highest concentration T decreased at mid & highest conc.	X—?—X - Repeat with lower conc.-				
No Dose Response, but T decreased at low or Mid concentration. No cytotoxicity		X Repeat -			

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## **Southern Research Institute**

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