Culture with Isoproterenol and Gonadotropins Restores Aspects of Steroidogenesis in Photoregressed Ovaries

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# Abstract

Adrenergic activation in the ovary is involved in the secretion of sex steroids as well as folliculogenesis.  Culture of ovaries with isoproterenol, a β-adrenergic receptor agonist, increases follicle stimulating hormone receptor and progesterone production, and *in vivo* exposure to isoproterenol can also increase androgen secretion. While the role for adrenergic stimulation in active and neonatal ovaries has been examined, the impact of adrenergic activity in the return to function of photoregressed ovaries in seasonally-breeding animals is not fully understood.  In photoperiodic Siberian hamsters (*Phodopus sungous*), exposure to long photoperiods stimulates and maintains ovarian function, whereas exposure to short photoperiods induces reduction in antral follicle formation, ovulation, and plasma estradiol concentrations. We hypothesized that stimulating photoregressed ovaries *in vitro* with isoproterenol with or without gonadotropins would potentially recapitulate aspects of photostimulated recrudescence as opposed to untreated ovaries; specifically impacting estradiol production as well as the expression of genes associated with the return of folliculogenesis and steroidogenesis. Isolated ovaries were then cultured for 10 days in one of four treatment groups: standard media (no treatment, NT); gonadotropins (GT); isoproterenol (20mM, ISO); or isoproterenol plus gonadotropins (ISO+GT). Culture with isoproterenol stimulated expression of genes involved with both steroidogenesis and folliculogenesis, along with increasing estradiol production and prostaglandins, critical for multiple aspects of ovarian function. Culture with ISO, regardless of GT exposure, increased mRNA expression of early steroidogenic enzymes (*StAR* and 3b*Hsd)* along with folliculogenic factors (*Gdf-9* and *Cox-2*). These data suggest that photoregressed ovaries are able to respond to β-adrenergic stimulation alone, particularly in aspects of steroidogenesis; however, the additional stimulation of gonadotropins is needed to restore actual production of estradiol and ovarian growth in photoregressed ovaries.

1. **Introduction**

Culture of ovaries with isoproterenol, a β-adrenergic receptor agonist, increases follicle stimulating hormone receptor and progesterone production, and *in vivo* exposure to isoproterenol can also increase androgen secretion. Nervous system activation in the ovary is involved in the secretion of sex steroids, like estrogen and progesterone, as well as the development follicles. These studies will help to better understand the role of intra-ovarian signaling in the mammalian ovary, as well as provide a unique natural model to study the return of function in quiescent ovaries, similar to what is observed in anorexic women. Although multiple studies have investigated differences between nonovulatory-SD and cycling-LD ovaries; a systematic investigation of the mechanisms that restore folliculogenesis during recrudescence had not been conducted.

In many species, oocyte maturity and associated intra-ovarian signaling does not transpire uninterrupted. In many seasonal breeding species, photoperiodic and environmental cues are used to synchronize reproductive function with seasonal changes in resources, facilitating  necessary for optimal reproductive success (Glass et al., 1986).  Siberian hamsters respond to seasonal shifts in photoperiod, the number of hours of light per days, resource accessibility, and energy exertion to physiologically induce or halt reproductive function (Reiter et al., 1980).

In many long day breeding individuals, photoperiodic Siberian hamsters (*Phodopus sungous*), exposure to long photoperiods (>12 hours of light per day) stimulates and maintains ovarian function, whereas exposure to short photoperiods (<12h light/day) depresses secretion of hypothalamic gonadotropin releasing hormone (GnRH) and adenohypophyseal gonadotropins (Salomon et al., 2018). This decline in both follicle stimulating hormone (FSH) and luteinizing hormone (LH) induces reduction in mature follicle formation, ovulation, and plasma concentrations of estradiol (Salverson et al., 2008) concentrations, and the secretion of gonadotropin- releasing hormone (GnRH). Decreased production of GnRH leads to reduced secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which subsequently compromises both ovarian folliculogenisis and steroidogenesis (Schlatt et al., 1993).

Exposure of photoperiodic adult female Siberian hamsters (*Phodopus sungous*), female Siberian hamsters to short day photoperiods (<12h light/day) show a decline in decreases numbers of antral follicles and corpora lutea numbers along with plasma concentrations of FSH, prostaglandin, and estradiol as compared to long day photoperiod exposure (Moffatt-Blue et al., 2006). In contrast, transfer of the photoinhibited hamsters to long photoperiods (>12 hours of light per day) gradually promotes restores reproductive activity through increased FSH secretion leading to ovarian recrudescence (Salverson et al.,2008).

While the neuroendocrine regulation of gonadal regression and recrudescence has been well characterized, little is known about intra-ovarian pathways that mediate the seasonal transition between a non-functional ovary to a functional gonad. Our aim was to analyze stimulating photoregressed ovaries *in vitro* with isoproterenol, a neural system stimulant, with or without hormones gonadotropin stimulation of that stimulate follicle growth and estradiol production. We hypothesized that culture with ISO, would potentially recapitulate aspects of photostimulated recrudescence as opposed to untreated ovaries; specifically impacting estradiol production as well as the expression of genes associated with the return of folliculogenesis and steroidogenesis.

1. **Materials and Methods**

*2.1 Animals*

Female Siberian hamsters (n=16) obtained from our breeding colony were treated in compliance with California State University Long Beach and NRC guidelines for the use of laboratory animals, and under the requirements of approved CSULB IACUC protocol #316. All animals were housed in individual polypropylene cages prepared with bedding and tap water, and were given *ad libitum*  access to food ( Laboratory Rodent Diet 5001 from LabDiet, St. Louis, MO) and water. Adult female Siberian hamsters were exposed to short day (8L:16D) photoperiods for 16 weeks to induce gonadal regression. At week 16 tissues were collected, ovaries were weighed and dissected. Isolated ovaries were then cultured for 10 days in one of four treatment groups: standard media (no treatment, NT); gonadotropins (follicle stimulating hormone and luteinizing hormone, GT); isoproterenol (20mM, ISO); or isoproterenol plus gonadotropins (ISO+GT). One ovary was fixed in 10% neutral buffered formalin for several days; the contralateral ovary was flash frozen for mRNA extraction.

*2.2 cDNA synthesis and RT-PCR*

Trizol LS reagent (Invitrogen Life Technologies, Carlsbad, CA) was used to extract total RNA from the ovaries as per manufacturer’s protocol. Samples were treated with DNase to remove all potential DNA contamination. One microgram of total RNA was used for cDNA synthesis using the ImProm Reverse Transcription System according to manufacturer’s instructions. (Promega, Madison,WI). For PCR analysis, cDNA was diluted 1:5 with DNase/ RNase free water. Relative real-time PCR System (Applied Biosystems Thermo Fisher, Waltham, MA) with no changes to dNTP or MgCl2 concentrations. The PCR reaction mix contained 1 μl cDNA + 1 μl each of forward and reverse primers (80nM concentration) + 6 μl SYBRgreen mix +3 μl water to a total volume of 12 μl. PCR cycles consisted of a 15 min hold at 95 °C (1 cycle), then 40 amplification cycles(e.g, amplification number closer or above 40 indicates low gene expression) at an appropriate melting temperature, extension (1 min at 72 °C) followed by dissociation. Melt curves for all products were produced to identify the pretense of non-specific products, and efficiency ranged from 90 to 96% for all reactions. Non-template negative controls were included in each PCR analysis. For the standard curve, cDNA from all samples was pooled and a 4-point curve was included with each run. The standard curve was used to calculate the relative amounts of mRNA expression. Ratios of the values for the genes of interest compared to the mRNA expression of reference glyceraldehyde 3-phospate dehydrogenase (Gapdh) were analyzed for statistical differences.

*2.3 Follicle stimulating hormone and estradiol immunoassays*

Follicle stimulating hormone and estradiol levels were determined by enzyme immunoassay using FSH ELISA and Estradiol ELISA kits (Caymen Chemical, MI) following all provided instructions. All plasma samples and standard curves were run in duplicate per kit instructions, and R2 values from the standard curves were 0.97 and 0.98 for FSH and 17b-estradiol, respectively. Reported intra-assay coefficient of variation (%CV) values are between 8.5–9.4 for FSH and 7.8–18.8 for 17b-estradiol. Hormone concentrations were calculated using previously reported methods (Shahed and Young, 2011; Shahed et al., 2015).

*2.4 Data*

Ovarian expression of steroidogenic acute regulatory protein (*Star*), 3β-hydroxysteroid dehydrogenase (3β*Hsd*) and growth and differentiation factor-9 (*Gdf-9*) mRNA, assessed using real time PCR.

All data were analyzed by a one-way ANOVA using the PRISM statistical software package (GraphPad Prism 6, San Diego, CA).

# Results

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Figure 1. *Mean mRNA ± SEM expression of* Cox-2/Gapdh and Gdf-9/Gapdh *across NT,GT, ISO, and ISO+GT culture groups.*

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Figure 2. *Mean mRNA ± SEM expression of* Star/Gapdh and 3β-*Hsd* /Gapdh *across NT,GT, ISO, and ISO+GT culture groups.*

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Figure 3. *Mean mRNA ± SEM expression of* ovaries *across NT,GT, ISO, and ISO+GT culture groups.*

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(ELISA estradiol and prostaglandins analysis ,graph similar to Fig 1-3, would also be in this results section)

# Discussion

I previously hypothesized that stimulating photoregressed ovaries *in vitro* with isoproterenol, a neural system stimulant, with or without hormones that stimulate follicle growth and estradiol production, would potentially recapitulate aspects of photostimulated recrudescence as opposed to untreated ovaries; specifically impacting estradiol production as well as the expression of genes associated with the return of folliculogenesis and steroidogenesis.

The expression of Gapdh mRNA did not change significantly between different photoperiod exposure groups when quantified by real-time RT PCR, as we have reported previously (Shahed and Young, 2013).

Ovarian expression of steroidogenic acute regulatory protein (*Star*), 3β-hydroxysteroid dehydrogenase (3β*Hsd*) and growth and differentiation factor-9 (*Gdf-9*) mRNA, was increased in the ISO and ISO+GT groups, whereas cyclooxygenase-2 (*Cox-2*) mRNA increased in GT, ISO, and ISO+GT groups as compared to NT.  Estrogen receptor 1 (*Esr1*) mRNA expression increased in the ISO+GT group as compared to all other groups, whereas expression of *Esr2* mRNA did not differ significantly across treatments.  Culture with isoproterenol stimulated expression of genes involved with both steroidogenesis and folliculogenesis, along with increasing estradiol production and prostaglandins, critical for multiple aspects of ovarian function.  Culture with ISO, regardless of GT exposure, increased mRNA expression of early steroidogenic enzymes (*StAR* and 3b*Hsd)* along with folliculogenic factors (*GDF-9* and *Cox-2*).  In contrast, only the combination of ISO+GT increased *Esr1* mRNA, ovarian mass, and media concentrations of estradiol and prostaglandins.

(All data were analyzed by a one-way ANOVA using the PRISM statistical software package (GraphPad Prism 6, San Diego, CA). goes into data analysis)

These data suggest that photoregressed ovaries are able to respond to β-adrenergic stimulation alone, particularly in aspects of steroidogenesis; however, the additional stimulation of gonadotropins is needed to restore actual production of estradiol and ovarian growth. While β-adrenergic stimulation advanced some aspects of recrudescence, gonadotropins as well as exposure to additional factors are likely necessary to fully restore function in photoregressed ovaries.

During this study only a few genes which indicate early steroidogenic enzymes and early folliculogenic factors were tested. In future studies more genes associated with late and early steroidogenic enzymes and early and late folliculogenic factors will be tested under NT, GT, ISO, and ISO+GT culture. This same study will also be carried out under long day photoperiod (>12 hours of light per day) conditions to further understand the data collected thus far.

# Conclusions

Adrenergic activation in the ovary is involved in the secretion of sex steroids as well as folliculogenesis.  Culture of ovaries with isoproterenol, a β-adrenergic receptor agonist, increases follicle stimulating hormone receptor and progesterone production, and *in vivo* exposure to isoproterenol can also increase androgen secretion. We hypothesized that stimulating photoregressed ovaries *in vitro* with isoproterenol with or without gonadotropins would potentially recapitulate aspects of photostimulated recrudescence as opposed to untreated ovaries; specifically impacting estradiol production as well as the expression of genes associated with the return of folliculogenesis and steroidogenesis. Culture with ISO, regardless of GT exposure, increased mRNA expression of early steroidogenic enzymes (*StAR* and 3b*Hsd)* along with folliculogenic factors (*GDF-9* and *Cox-2*).  In contrast, only the combination of ISO+GT increased *Esr1* mRNA, ovarian mass, and media concentrations of estradiol and prostaglandins. While β-adrenergic stimulation advanced some aspects of recrudescence, gonadotropins as well as exposure to additional factors are likely necessary to fully restore function in photoregressed ovaries.

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