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Determining the Effects of St. John's Wort on Porcine Granulosa Cells

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Appendix D - UNIVERSITY HONORS PROGRAM
SENIOR PROJECT - APPROVAL

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PROJECT TITLE: Determining the Effects of St. John's Wort on Porcine Granulosa Cells

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: ___________________________ Faculty Mentor

Date: 5/5/00

 Comments (Optional):
Determining the Effects of St. John's Wort on Porcine Granulosa Cells

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May 3, 2000

ABSTRACT

St. John’s Wort (SJW) is a popular over-the-counter herbal medicine sold in pharmacies, grocery stores, and health food stores. SJW is an herbal plant found all around the world and is being used as a popular treatment for anxiety, depression, and insomnia. Its major chemical component is hypericum. Its mechanism of treating depression is not well known, and its overall affect on different areas of the body is also unknown. In this study, the relationship between SJW and granulosa cells is studied. Granulosa cells function in the maintenance and proper growth of oocytes into mature ovum. This function is vital to proper reproductive cycles in female patients and thus, is important to study. In this study, granulosa cells are aspirated from follicular cells obtained from a local pig farm. Using tissue culture techniques and radioimmuno assays, SJW’s effect, or lack thereof can be determined. The radioimmuno assay utilizes radioactively labeled progesterone and the competitive binding between a progesterone antibody and progesterone. By analyzing the radioactivity of the sampled progesterone produced by granulosa cells, we can compare the amounts of progesterone produced by treated cells to those cells that were not treated with SJW. Further experiments can be carried out once SJW’s relationship with granulosa cells is determined.
INTRODUCTION:

St. John’s Wort (SJW) is one of many popular over-the-counter herbal medicines. St. John’s Wort is found in the United States, Europe, North Africa, and Asia, commonly referred to as “road-side weed” (1). Its major chemical components are hypericum and hyperforin (2,3). St. John’s Wort has been found, in several studies, to be an effective antidepressant for patients suffering from various degrees of depression (2,3,4). The reported side-effects of St. John’s Wort are minimal when compared to prescription antidepressants (3). Examples of such antidepressants include tricyclic antidepressants (TCA) and specific serotonin reuptake inhibitors (SSRI’s). The mechanism by which St. John’s Wort achieves its anti-depressant action, however, is not known. It is postulated to act as an uptake inhibitor of serotonin and gamma aminobutyric acid (GABA). Both serotonin and GABA are general inhibitors of the central nervous system. That is, the secretion of these neurotransmitters at neuro-muscular junctions leads to inhibition of further excitation of the nervous/muscular systems. Once these neurotransmitters are secreted into neuro-muscular junctions, they can be reabsorbed into cerebrospinal fluids and prevented from inhibiting further excitation. SSRI’s are drugs that inhibit the reabsorption of serotonin and GABA, and thus, inhibit further excitation of the central nervous systems (3,5,6). Evidence from several studies tends to support St. John’s Wort’s antidepressant activities in vitro, but in vivo action of St. John’s Wort is yet to be determined. SJW appears to affect several neurotransmitters such as serotonin, norepinephrine, and dopamine—preventing the uptake/reabsorption of each. The most obvious effect reported thus far has been SJW’s effect on GABA receptors (4). Although SJW has been shown to act similarly to other classes of prescription antidepressants, it is
not as effective as prescribed antidepressants as found in several studies. However, it also was found that side effects reported for SJW were fewer and more mild when compared to prescription antidepressants. This observation is important when considering the "drop-out" rate during depression treatment due to unwanted side effects. Because little is known about SJW’s affect on the body in addition to its mechanism of action, more research is needed.

RATIONALE:

One area of SJW’s activity that could be better defined is its effects on cells involved in reproduction. Because reproduction is of great important to many people, much research is carried out with many drugs in order to deduce their effects on the reproductive cells. For example, the anticancer drug, taxol and its effects on granulosa cells is one instance where a drug’s relationship to reproductive cells is studied (7). The limited knowledge of SJW’s activity similarly encourages study of its effects on granulosa cells and its role in reproduction. Moreover, with such abundant use of SJW, patients of reproductive age using SJW is a significant number. Thus, determining whether or not SJW has adverse effects on reproductive function is of importance.

HYPOTHESIS:

St. John’s Wort may or may not have effects on granulosa cells—those somatic cells that surround the oocyte (female reproductive cell). The purpose of this study is to determine whether or not SJW has an effect on the role of granulosa cells in reproduction. Granulosa cells function in the maintenance of oocytes that then develop into mature ovum. Once an oocyte matures into mature ovum, the male reproductive cell, sperm, can fertilize the ovum. Granulosa cells properly maintain oocyte maturation through
steroidogenesis. That is, the production and maintenance of steroids/hormones. In this study, the production of the hormone progesterone will be examined. Granulosa cells treated with SJW may or may not exhibit abnormal levels of produced progesterone.

**METHODOLOGY/DESCRIPTION:**

Two trials, A and B, were carried out for this study. The processes for both trials were identical and involved five experimental stages. The first stage involved obtaining and cleaning the porcine follicles. The second stage involved aspirating the follicular fluid from the porcine follicles—this fluid contains the desired granulosa cells. The third stage involved culturing and growing the granulosa cells. The fourth stage involved treating the granulosa cells with various levels of St. John’s Wort. The fifth stage involved the use of radio-immuno assays to determine the levels of produced hormones. Once the levels of produced hormones are determined and analyzed, conclusions and future experimentations can be planned.

**Obtain and Clean Porcine Follicles:** Porcine follicles were obtained from Wampler’s Sausage farm and cleaned of debris and excessive blood.

**Aspirate Follicular Fluid from Porcine Follicles:** Using a 3/8ths inch needle, gauge 26, the follicular fluid, containing the granulosa cells, was aspirated from the follicles.

![Figure 1. Photograph of inside a porcine follicle. Granulosa cells are pointed to with arrows, surrounding the follicle.](image)
Culture and Growth of Granulosa Cells: Once the granulosa cells are obtained and cleaned, they are placed in PBS/BSA medium and prepared for culture. The cells are centrifuged at 1000 rpm for 10 minutes. After centrifugation, the supernatant containing the unwanted blood cells is removed from the tubes. This process is repeated three times. Next, the tubes containing the granulosa cells and culture medium are plated onto tissue culture plates for thirty minutes. During this incubation period, macrophages found in the fluid attach to the plate while the granulosa cells remain floating in the medium. Macrophages are the self-defense cells that could interfere in the culture of granulosa cells. After macrophages attach to the plate bottom, the medium containing the wanted cells can be removed and plated onto another plate for culture. In both trials, a 48-well plate (6 X 8) was used. Figure 2a and 2b are pictures of cells from both treatments before being treated with SJW.

Figure 2a—Trial A Pretreated Cells

Figure 2b—Trial B Pretreated Cells

Treatment with St. John’s Wort: A control plus seven experimental groups, each done in sextuplets, were treated in the 48-well plate. The seven concentrations used were 20 μg/ml, 2 μg/ml, 200 ng/ml, 20 ng/ml, 2ng/ml, 200 pg/ml, and 20 pg/ml. Normal concentrations of SJW used by patients are 20 μg/ml or less.
In both trials, the cells were treated with SJW for 24 hours. After treatment with SJW, pictures of cells with notable physical differences were taken. Please see fig. 5a and 5b.

Fig. 3—Example of Plate Used for St. John’s Wort Treatment

Fig. 4—Chemical Structure of Hypericum

Fig. 5a. Granulosa Cells with 20 µg/ml

Fig. 5b. Granulosa Cells with 200 ng/ml
Comparing figures 5a and 5b to figures 2a and 2b, there is an obvious discoloration of the cells, mainly due to the color of the SJW solution itself. Also, the cells treated with SJW seemed to be more widely dispersed. Cells treated with concentrations lower than 200 ng/ml did not appear to differently than the control group cells.

Radio-Immuno Assay (RIA) of Produced Hormones: Radio-Immuno assay is a useful and widely used method for the determination of the concentrations of many substances. From the results of the assay, we can conclude that SJW either inhibits granulosa cells from producing normal amounts of progesterone (P₄), causes granulosa cells to produce more than normal amounts of P₄, or does not produce any change in the levels of P₄ produced. The RIA process involves four steps. The first step involves adding a known amount of radioactively labeled P₄ (³H-P₄) to each of the test samples of progesterone. The second step involves the addition of P₄ antibody, which binds to both the test sample of progesterone and ³H-P₄. The samples are then left overnight to allow for proper binding with the antibody. The third step involves the separation of unbound progesterone and bound progesterone. That is, any progesterone that did not bind to the P₄ antibody is separated from the progesterone that did bind. This is done using C-Dextran coated charcoal. Unbound progesterone is captured in the coated-charcoal, and the samples are then centrifuged to separate the charcoal containing the unbound progesterone from the solution containing bound progesterone. The final step involves the actual assaying of the hormones in the samples. The samples are placed in a scintillation counter, which measures the radioactivity, or counts per minute, of the samples. Scintillation fluid is added to all samples before being placed into the scintillation counter. The resulting counts per minute for each sample can be compared
to the control group of samples, which were not treated with SJW. Interpretation of the results from the radio-immuno assay can be seen in figure 6.

Figure 6. A sample with a higher CPM correlates to more radioactivity. A sample with a higher CPM contains less P₄ than a sample with a lower CPM.

- If CPM < CPM of Control Group, then
  \[ [P_4] \text{ of sample} > [P_4] \text{ of control} \]
- If CPM > CPM of Control Group, then
  \[ [P_4] \text{ of sample} < [P_4] \text{ of control} \]

RESULTS:

The results showed that the control group had a CPM of 4012.35. Cells that were treated with 20 µg/ml exhibited a sharp decrease in CPM, meaning that those samples contained a higher concentration of P₄. As the concentration of SJW decreased there was an overall trend towards a higher CPM. Figures 7 and 8 display this trend.

Figure 7.
Both figures 7 and 8 show that at 20 μg/ml, the samples had a much lower CPM than the rest of the samples. As the concentrations of SJW were decreased, the CPM values increased slightly.

**CONCLUSION:**

From our results, it can be concluded that St. John’s Wort seems to cause granulosa cells to produce more than normal amounts of progesterone at concentrations of 20 μg/ml. As levels of SJW are decreased, granulosa cells tend to produce less progesterone. Since most patients take SJW at the 20 μg/ml concentration, it would seem that the patient’s ability to produce normal amounts of progesterone is not inhibited, but rather, enhanced. However, if patients take SJW at lower concentrations than 20 μg/ml, their ability to produce normal amounts of progesterone may be slightly inhibited.

**FUTURE EXPERIMENTS:**

From this study, refinements and changes to the experiment can be made. One of the first changes would be to focus on the 20 μg/ml concentrations, focusing on concentrations in
smaller increments around the 20 μg/ml concentration. Furthermore, cells can be treated with concentrations beyond the 20 pg/ml concentration in order to see where levels of progesterone production returns to normal. After these are determined, the effect of prolonged exposure to St. John’s Wort might be considered.

THANKS:

Dr. Thomas Chen and Wampler’s Sausage Farm
References:


