Measurement of Biomarkers for Environmental Estrogen Exposure in Raccoons

by

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Abs</td>
<td>absorption</td>
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<tr>
<td>AI</td>
<td>angiotensin I</td>
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<tr>
<td>AIN</td>
<td>American Institute of Nutrition</td>
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<tr>
<td>ANGI</td>
<td>angiotensinogen</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CPN</td>
<td>ceruloplasmin</td>
</tr>
<tr>
<td>DDT</td>
<td>o,p'-dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DES</td>
<td>diethyl stilbestrol</td>
</tr>
<tr>
<td>DDC</td>
<td>o-dianisidine dihydrochloride</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EE</td>
<td>ethinyl estradiol</td>
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<td>n</td>
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<tr>
<td>NaCl</td>
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<tr>
<td>NPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyl</td>
</tr>
<tr>
<td>PD</td>
<td>p-phenylenediamine</td>
</tr>
<tr>
<td>RAS</td>
<td>renin angiotensin system</td>
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<tr>
<td>RIA</td>
<td>radio-immune assay</td>
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<tr>
<td>sc</td>
<td>subcutaneous</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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ABSTRACT

This project was conceived as a supplement to that of Dr. J. Levengood entitled "DePue WMA Biological Site Characterization: Segment III - Levels and Effects of Selected Metals in Raccoons". Dr. Levengood had proposed to collect plasma and harvest tissue from raccoons collected in the Lake DePue area, and compare xenobiotic metal levels in tissues from these animals with metal levels from a similar number of raccoons collected from a pristine reference area along the Illinois river.

In this supplemental project, we proposed to measure biomarkers for estrogenicity in plasma of these same animals, and to confirm the results through quantification of an estrogen-regulated protein, complement C3, in uterine tissue from all females collected. Raccoons are particularly suitable biomonitors when considering contamination of an Illinois lake or waterway, because of their habits and abundance.

We analyzed samples from raccoons located in polluted as well as pristine areas using the ceruloplasmin and angiotensinogen biomarkers developed in our lab. Increase in angiotensinogen was seen in raccoon living in contaminated areas suggesting exposure to estrogenic substances. However, no confirming increases were seen with ceruloplasmin. In addition, complement C3 was not detectable in uterine tissue of raccoons from either location.

We conclude that elevated angiotensinogen levels indicate an adverse effect of the polluted environment, but that other markers did not confirm that this was due to estrogenic compounds.
EXECUTIVE SUMMARY

A. Study Purpose

Dr. Levengood's report offers an extensive background into the contamination of Lake DePue and the choice of raccoons as biomonitors for evaluating accumulation of xenobiotics (Levengood 2000). He also describes how results may be used to extrapolate a measure of risk to other species that may be more sensitive, more secretive and/or more at risk for extinction from the area. However, Dr. Levengood's report does not address environmental estrogen exposure. Currently there is considerable concern about the presence of substances with estrogenic properties having been released into the environment (Weiss, 1994). This concern is for the health of humans and wildlife (Toppari et al, 1995; Tillit et al, 1992).

Several laboratory animal-based bioassays have been developed to test environmental contaminants thought to be potentially estrogenic. Use of these bioassays to test estrogenicity of specific chemicals has resulted in an extensive list of "environmental estrogens", including many chlorinated and poly aromatic hydrocarbons, such as TCDD, certain PCBS, diethylstilbestrol, kepone and o,p'-DDT. Many of these are common environmental pollutants. There is a growing literature identifying gross morphological and physiological abnormalities of the reproductive systems of both males and females, in wildlife and humans in various parts of the world. Yet the link relating the presence of specific environmental contaminants to reproductive abnormalities is weak, due to a lack of biomarkers of exposure.

Biomarkers are needed that can better link the presence of these substances in the environment with specific estrogenic effects in wildlife and humans. A biomarker exists for the evaluation of estrogenicity in fish and other oviparous animals such as birds: measurement of the egg protein vitellogenin. Correlations of vitellogenin levels with exposure to potentially estrogenic substances helps to place estimate of risk on a stronger scientific basis, particularly if
elevated vitellogenin levels correlate with other reproductive abnormalities. However, since mammals do not produce vitellogenin, this protein is limited in its use as a biomarker of estrogen exposure. Very recently, with the aid of funds from the Illinois Department of Natural Resources, we have developed a system of biomarkers to test for estrogen exposure in mammals (Dean and Jeffery 1999). The biomarkers developed are the plasma proteins ceruloplasmin and angiotensinogen, whose hepatic synthesis is upregulated by estrogen.

While their rates of synthesis are regulated by several other stimuli, the only stimulus that they have in common is estrogen. Thus if both biomarkers are elevated this can serve as a marker to suggest that the body may be producing an estrogenic response. The advantages of these biomarkers are: they can be measured in blood, a readily available tissue; they can be measured in both sexes; hospital clinics measure both of these parameters. This is in strict contrast to the classic uterine weight test, that requires sacrifice of the individual under test, and that can only test females. Here we propose to use these biomarkers to evaluate estrogenicity in raccoons from an area known to be heavily contaminated by industrial waste. Racccoons serve as excellent biomonitors of environmental contamination. In fact, environmental contaminants, including the estrogenic organochlorine insectides have been found in the omental fat of raccoons living in Kansas, Florida and Michigan (Layher et al., 1987; Nalley et al., 1975; Herbert and Peterle 1990).

B. OBJECTIVES

1. To measure ceruloplasmin and angiotensinogen in plasma of raccoons collected at Lake DePue and at a reference site on the bank of the Illinois river. These raccoons will be collected as part of the Levengood proposal to evaluate bioavailability of xenobiotic metals in raccoons. Plasma levels will be compared between contaminated and reference sites.
2. To measure uterine complement C3 protein levels in a subset of the female raccoons collected for objective 1.

C. HYPOTHESES

1. Raccoons living in areas contaminated with environmental estrogens will have increased levels of ceruloplasmin and angiotensinogen compared to raccoons living in pristine areas.

2. Raccoons living in areas contaminated with environmental estrogens will have increased levels of complement C3 compared to raccoons living in pristine areas.

D. TASKS

1. Method Development

1a. Tissue and blood collection:

Whole blood was collected from raccoons in the field, serum prepared and frozen, as described in the Levengood report (Levengood 2000). In addition, 1 g portions of uterus of all females harvested were rapidly frozen in liquid nitrogen, and kept at -80°C until analysis.

1b. Ceruloplasmin measurement:

Using a method previously developed in our lab, ceruloplasmin was estimated through measurement of its oxidase activity (Dean and Jeffery 1999). Disappearance of substrate was followed over time as disappearance of absorbance at 540 nm. Data were reported as International Units of activity/L serum, and compared to control samples.
1c. Angiotensinogen measurement:

Also using a method previously established in our lab (Dean and Jeffery 1999), a radio-immune assay (RIA) was carried out using a kit (Diasorin, Stillwater, MN) designed to measure angiotensin I. To generate angiotensin I from angiotensinogen, samples of plasma were incubated with excess renin, and samples taken from this for the RIA. Data generated was compared to a standard curve constructed from angiotensin I.

2. Complement C3 measurement:

First, frozen uterine tissue was thawed then homogenized, analyzed for protein by the BioRad method, and submitted to electrophoresis on 4-12% acrylamide gel. Proteins were then transferred to a nylon membrane for immunoblotting using a goat anti-rat complement C3 IgG antibody (ICN Biomedical, Costa Mesa, CA). This was followed by incubation of the blot with the secondary antibody, rabbit anti-goat IgG conjugated to alkaline phosphatase (ZymaXTM, San Francisco, CA) and its substrate (BCIP/N-BT; Kirkegaard and Perry Labs, Gaithersburg, MD). Our ability to measure complement C3 was assessed using uteri from rats exposed to endogenous as well as environmental estrogens.

3. Analysis of raccoon samples

In task 2, we validated our complement C3 bioassay and its ability to detect increased complement C3 levels following exposure to bisphenol A (BPA), a known estrogenic environmental pollutant. Additionally, further supporting the use of our bioassay in determining the estrogenicity of compounds, complement C3 levels were shown to be a more sensitive indicator of estrogenicity than the traditional uterine weight assay. Thus, the complement C3 bioassay and ceruloplasmin and angiotensinogen biomarkers were then ready for use with the raccoons samples collected by Dr. Levengood. All assays were carried out according to procedures reported above.
Although our systems were able to measure ceruloplasmin and angiotensinogen in raccoon plasma, no difference in ceruloplasmin was seen between animals living in pristine or contaminated areas. However, increases in angiotensinogen were seen in raccoons living in contaminated areas. Our assay did not detect complement C3 in any of the raccoon uteri.

E. CONCLUSION

Elevated angiotensinogen levels in raccoons from the polluted areas suggests that environmental contaminants are having a significant negative health impact on wildlife living in this area. However, the lack of concomitant elevation in ceruloplasmin suggests that the polluting components are not estrogenic. Additionally, the lack of elevation in complement C3 tends to confirm this suggestion. However, no positive controls, in the form of estrogen-treated raccoons, were evaluated in this study.

F. RECOMMENDATIONS

1. The complement C3 bioassay may be used in place of the classic uterotrophic assay, in rats, for assessing the estrogenicity of compounds. However, further work needs to be done to optimize the assay for measuring complement C3 in raccoon uterus.

2. Angiotensinogen, ceruloplasmin, and/or other estrogen-regulated plasma proteins synthesized in the liver, and immediately secreted into plasma, should be developed as reliable biomarkers of estrogen exposure in humans and wildlife.
Chapter 1. INTRODUCTION

A. Environmental Estrogens

Environmental estrogenic pollution has gained enormous attention in the last half-century in the United States, as well as abroad. Indeed, a large number of chemically diverse environmental contaminants have been shown to have estrogen-like effects. Examples of environmental estrogens include the pesticides kepone (Hammond et al., 1979), methoxychlor (Bulger et al., 1985; Tullner 1961) and \( o,p' \)-DDT (Cecil et al., 1971; Dodds et al., 1938; Rooryck et al., 1987; Robison et al., 1984; Singhal et al., 1970; Welch et al., 1969), polychlorinated biphenyls (PCBs; Korach et al., 1988; Jansen et al., 1993), bisphenol A (a breakdown product of plastics; Ashby and Tinwell 1998; Krishnan et al., 1993), zearalenone (a fungal product; Sheehan et al., 1984), alkylphenolic compounds (Soto et al., 1991; White et al., 1994); and phytoestrogens produced by a variety of plants (Cheng et al., 1953; Dees et al., 1997; Zava et al., 1997).

In some cases, these xenoestrogens may be potent estrogens which have been specifically developed for their hormonal activity (e.g. DES), while in other cases, they are weak estrogens found to exert hormonal activity as an unexpected side effect (e.g. DDT). Additionally, the wide distribution of weakly estrogenic flavonoid pigments in food crops (e.g. genistein in soy) and medicinal plants raises additional questions about the possible health benefits and risks of these compounds.

While most of these environmental contaminants elicit estrogenic effects, their biological actions are usually 1000 times less active than estradiol (Nelson 1974; Korach et al., 1978; Korach et al., 1988). This tends to suggest that weak environmental estrogens might be without physiological consequence. However, estrogenic compounds warrant concern because most of these environmental contaminants are lipid-soluble and ubiquitous, and can accumulate in the food chain. Also, since these contaminants mostly exist as mixtures in the environment, the additivity of adverse effects of the various contaminants
could reach a threshold for toxicity even when the separate components are not in sufficient quantity to be
toxic or even readily measured in environmental samples.

B. Health Effects of Environmental Estrogens

Evidence of deleterious estrogenic effects in humans from xenoestrogens comes from studies of young boys and girls living in industrial countries. Prepubertal breast development was observed in young boys and girls, and the young girls started menarche much earlier than expected (Perez-Comas 1982). Additionally, estrogenic compounds have been linked with hepatocellular, cervical, testicular, ovarian, and breast cancers in experimental animals (Preat et al., 1986; Watanabe and Kobayashi 1993) and humans (Christopherson et al., 1977; Herbst et al., 1971; Yager et al., 1991). However, increased incidence of cancers is not the only health effects seen with estrogenic compounds. In 1947, crop dusters handling DDT had decreased sperm counts (Singer 1949) and workers in a kepone plant suffered low sperm counts, impotence, and loss of libido (Guzelian 1982). These effects have been confirmed in laboratory animal studies. DES and 4-octylphenol, a phthalate plasticizer, caused a decrease in testicular size and sperm production in rats (Sharpe et al., 1995). In addition, reproductive changes such as infertility and male feminization in wild species have been attributed to environmental estrogens. Adverse effects have been seen in a variety of wild and domestic species including fish (Jobling et al., 1996), cheetahs (Setchell et al., 1987), quail (Leopold et al., 1976), and alligators (Guillette et al., 1994).

Without a doubt, environmental estrogens have the potential to cause serious adverse effects that disrupt reproduction and threaten species survival. Assessment of the estrogenicity of chemicals is needed to make judicious waste management decisions. Furthermore, no methods exist to determine whether people are exposed to effective doses. Thus, a bioassay is needed, whose endpoints can be used as biomarkers of environmental exposure to estrogenic substances. Here we propose such an assay.
C. Need for Bioassay/Biomarkers

Most studies on the estrogenic activity of compounds have been performed using a bioassay that focuses on the uterus. In the classic uterotrophic assay in rodents, estrogens cause an increase in uterine weight, fluid retention and cell proliferation (Dodds et al., 1938; Welch et al., 1969). Although the uterotrophic response assay is widely used for identifying estrogenic compounds, hypertrophy and hyperplasia of the uterus can be induced aspecifically by non-estrogenic compounds including corticosteroids and antiestrogens. Moreover, the precision of the test is affected by surgical techniques (e.g. bleeding of the animal) and the variability of uterine weight due to nutritional status and estrus cycling. Furthermore, uterine weight cannot be used as a biomarker in wildlife or humans, since this endpoint requires sacrifice of the exposed subject. Finally, uterine weight is gender-specific which renders half the population unavailable for assessment of exposure. Therefore, while this bioassay has its advantages for use, the fact that it requires sacrifice of the animal precludes its useless as a nonlethal biomarker of estrogen exposure. These limitations have caused some researchers to begin looking at other target organs for estrogenic effects. An area that has been gaining a lot of attention involves assessing the hepatic effect of estrogens.

D. Use of Liver Proteins as Biomarkers of Exposure to Estrogenic Compounds

Several in vivo studies have demonstrated a direct relationship between estrogen levels and the production of several liver plasma proteins (Kendall and Rose 1992; Klett et al., 1992; Kneifel and Katzenellenbogen 1981; Middleton and Linder 1993; Sunderman et al., 1971). We have developed a bioassay based on the demonstrated response of the liver to estrogenic compounds. This method is based on the estrogenic upregulation of the hepatic production of the two plasma proteins, ceruloplasmin (CPN) and angiotensinogen (ANGI). These two proteins have been shown to be predominantly synthesized in
the liver and to be upregulated by estrogens in both sexes in experimental animals and humans (Hongbrown and Deschepper 1993; Middleton and Linder 1993; Kneifel and Katzenellenbogen 1981; Sunderman et al., 1971). Because neither protein is stored in the liver, but secreted immediately upon synthesis, non-specific necrotizing hepatotoxic compounds will not increase their plasma levels. As a result, our method could serve as a bioassay, and yield proteins that could serve as biomarkers to effectively identify exposure to estrogenic substances.

1. Ceruloplasmin

Ceruloplasmin functions in the transport of copper. It is produced in the liver and immediately secreted into the systemic circulation. Several studies have established that CPN levels increase following exposure to estrogens in both humans and laboratory animals. In fact, because CPN is a liver-derived plasma protein, increases in its levels have helped in establishing the liver as a target organ of estrogens.

The possibility that estrogens caused increased levels of CPN began to surface in the mid 1950s. It was noted that rats treated with estrogens had increased levels of copper and that this increased copper level was attributable to increased levels of CPN (Meyer et al., 1958; Turpin et al., 1952). Studies in rats treated with DES, estradiol, or ethinyl estradiol (EE) have all shown increases in both serum and mRNA levels of CPN (Clemente et al., 1992; Kendall and Rose 1992; Middleton and Linder 1993; Musa et al., 1965; Sunderman et al., 1971). Additionally, it was also noted that increased levels of CPN were observed in humans exposed to estrogenic compounds; specifically in women who were taking oral contraceptives (Carruthers et al., 1966; Musa et al., 1965) or who were receiving estrogen replacement therapy (Clemente et al., 1992).
2. Angiotensinogen

Angiotensinogen is a glycoprotein that is mainly synthesized by the liver and secreted into the bloodstream. It is a central component of the renin-angiotensin system (RAS) and serves as the sole substrate for renin in its generation of angiotensin I which is then converted to angiotensin II, a potent vasoconstrictor.

The ability of estrogens to increase levels of ANGI has been well documented. Studies examining the correlation between estrogen exposure and increased levels of ANGI have been reported since the late 1930s (Dodds et al., 1938). Treatment with DES, estradiol or EE has been shown to result in a positive correlation between exposure and increased levels of ANGI protein and mRNA in laboratory animals (Gontar et al., 1984; Gordon et al., 1992; Helmer and Griffith 1952; Klett et al., 1992; Kneifel and Katzenellenbogen 1981). Similar increases have also seen in humans (Schunkert et al., 1997).

3. Proposed Biomarkers

Since CPN and ANGI are secreted directly into the plasma where their concentrations increase directly in response to estrogens, they should serve as excellent endpoints for a bioassay in test animals and reliable biomarkers both in wildlife and humans. Moreover, the regulatory region of the ANGI gene has been reported to contain a functional estrogen responsive element, stressing the link between the expression of this protein and a direct estrogenic action (Feldmer et al., 1991).

The concomitant use of two proteins as biomarkers significantly decreases the risk of misinterpretation of the data which can occur due to non-specific upregulation which is unavoidably linked with single endpoint bioassays. In the uterine weight assay, lack of specificity for estrogenicity is a serious confounding effect, since uterine weight increases in response to many non-estrogenic compounds. Admittedly, CPN synthesis is also upregulated by many non-estrogenic agents including copper, indomethacin and phenytoin. Likewise, ANGI responds to increased plasma NaCl, isoproterenol, and
guanfacine. However, the only known common pathway of induction for these two proteins is a response to estrogen stimulation.

E. Complement C3 as a Novel Bioassay of Estrogenicity

Most studies of the estrogenic activity of compounds have been performed using a bioassay that targets the uterus. In the classical uterotrophic rodent assay, estrogens caused an increase in uterine weight, fluid retention and cell proliferation. Although the uterotrophic assay is widely used for identifying estrogenic compounds, hypertrophy and hyperplasia of the uterus can be induced aspecifically by non-estrogenic compounds including corticosteroids and antiestrogens. Moreover, the precision of the test is limited by excision technique and fluctuations in the control weights, due to nutritional status and estrous/menstrual cycling.

Additionally, although this assay is considered the gold standard for testing the estrogenicity of compounds, it has not been standardized and has actually yielded conflicting results, especially in regard to weak environmental estrogens. For example, *in vivo* studies looking at increases in uterine weight with bisphenol A have given contradictory data. One study showed that treatment of intact immature female Sprague-Dawley rats with BPA (dose) for 3 days had no effect on uterine weight, in contrast to the effects of estradiol (Gould et al., 1998). However, other studies treating immature AP rats with 400-800 mg/kg BPA for 3 consecutive days (Ashby and Tinwell, 1998) or adult, ovariectomized Swiss-Webster mice that received 100 g/kg BPA daily for 3 weeks (Mariotti et al., 1998) have shown increases in uterine weight following BPA treatment.

Therefore, there is a need to identify other parameters of estrogenicity that can be used, along with uterine weight, to identify estrogenic compounds. One of the objectives of this study is to determine the feasibility of using uterine complement C3 levels, as a measure of an estrogenicity.
Complement C3 is a good target to use in conjunction with uterine weight because it would not require taking additional tissue and it would yield a biochemical analysis in addition to the increase in uterine weight since it looks at a specific protein level. Increased synthesis and secretion of complement C3 has been shown to occur following exposure to potent estrogens such as estradiol and diethylstilbestrol (Komm et al., 1986; Kuivanen and DeSombre 1985; Sunderman et al., 1989). However, in vivo assays that would assess the ability of environmental estrogens to increase complement C3 have not been done. Thus, here we address the feasibility of using complement C3 levels as an indicator of estrogenicity.
CHAPTER 2. METHODS

A. Measurement of Complement C3 Following Endogenous Estrogen Treatment

1. Introduction

Increased synthesis and secretion of complement C3 has been shown to occur following exposure to estradiol. Thus, here we plan to use complement C3 levels as an indication of exposure of raccoons to environmental estrogens. A search of the literature has been unsuccessful in yielding work that would indicate that complement C3 has been examined in raccoons. Additionally, since this technique has not previously been used to evaluate environmental estrogens, we first measured complement C3 by western blot using rat uterus. Uteri were from adult ovariectomized Sprague-Dawley females (7.5 week, 175-200 g) exposed to one dose of ethinyl estadiol (100 μg). The uteri were harvested at 16 hours, defatted, snap frozen in liquid nitrogen and stored at -80°C until use.

2. Western Blot Analysis

a. Tissue Preparation

Western blots were carried out on previously frozen rat uterine tissue. The uterine tissue (~50 mg) was homogenized in 100 μl of homogenization buffer for 30 seconds on ice. The homogenization buffer contained aprotinin (50 μg/ml), leupeptin (5 μg/ml), sodium chloride (0.1 M), Tris (0.01 M), and ethylene diamine trichloric acid (0.001 M). The samples were then centrifuge for 10 minutes at 10,000 rpm. The supernatant was removed and placed in a new microcentrifuge tube.

b. Protein Analysis

Total protein concentration of the homogenized uterine tissue was determined by the Biorad method. Bovine serum albumin was used to produce a standard curve, with samples compared to this
curve. Uterine homogenates were diluted 1:40 and 1:80 to determine protein levels which fell within the linear part of the standard curve.

c. Western Blot

Western blot analysis was carried out on previously frozen uterine tissue. The uterine tissue was homogenized, protein content determined, then electrophoresed on a 4-12% acrylamide gel. To prepare samples for electrophoresis, 20 μg of total protein from the uterine sample was mixed with 10 μl loading dye (0.4% bromophenol blue in glycerol) then brought to a total of 20 μl with phosphate buffered saline. The samples were placed in boiling water for 3 minutes. A molecular weight standard similarly prepared.

The proteins were loaded into a precast 4-12% Tris-Glycine ready made gel (Bio-Rad #161-0906) and the gel run at 200 volts for 30-45 minutes. The proteins on the gel were then transferred to a nylon membrane for immunoblotting. The membrane (Westran #78377) was first equilibrated in 100% methanol, then the transfer apparatus (Mini-PROTEAN 11) setup and ran overnight at 35 volts.

The membrane was then incubated with the appropriate antibodies using the following procedure. The membrane was initially submerged into 25 ml of blocking buffer (5% bovine serum albumin) for 40 minutes. The blocking solution was removed and the membrane submerged directly into primary antibody solution (1:5000 dilution of goat anti-rat complement C3 IgG antibody) and placed on a shaker for 1.5 hrs. The membrane was then rinsed 4x with washing buffer (0.1 M sodium chloride, 0.05 Tris-base, and 0.05% Tween-20) for 15 minutes each. The membrane was then with the secondary antibody (1:3000 dilution of rabbit anti-goat IgG conjugated to alkaline phosphatase) for 1.5 hrs then washed as previously stated. Finally, the membrane was immersion in the substrate solution (0.21 g/L 5-bromo-4-chloro-indolyl-phosphatase and 0.42 g/L nitroblue tetrazolium) for 5 minutes then rinsed in deionized water.
3. Results

Figure 1 shows a significant increase in complement C3 in rats treated with EE compared to the control group.

4. Conclusion

As indicated, the major complement C3 band corresponds to 180,000 kb which is consistent with what previously been reported in the literature. Additionally, the results show that complement C3 levels do increase following treatment of rats with an endogenous estrogen such as ethinyl estradiol.

B. Measurement of Complement C3 Following Environmental Estrogen Treatment

1. Introduction

Our previous study used rat uterine tissue for measuring C3 in order to ensure that we could reproduce the measurements reported in the literature. This served as a means of method development, but also as a quality control step to ensure that we had achieved the appropriate sensitivity and specificity for this assay.

In this experiment, we will assess the ability of our assay to detect complement C3 in response to a particular environmental estrogen, bisphenol A. Bisphenol A (BPA) has been identified as a contaminant in food and water consumed by humans and animals. It is a byproduct in the manufacture of plastics and other products and has been found to leach out of polycarbonate flasks during autoclaving. Additionally, several in vitro studies have shown that bisphenol A has estrogenic activity, thus it has been classified as an environmental estrogen that should warrant concern (Krishnan et al., 1993). The use of BPA allows us to test the ability of our complement C3 assay to detect the estrogenicity of environmental estrogens.
Figure 1. Uterine complement C3 level in rats treated with one dose of ethinyl estradiol (EE, 100 μg) or vehicle only (Co). *denotes significant at p=0.05.
should warrant concern (Krishnan et al., 1993). The use of BPA allows us to test the ability of our complement C3 assay to detect the estrogenicity of environmental estrogens.

2. Materials and Methods

a. Animals and Treatments.

Intact immature (21 day old, 45 g) female Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Animals were maintained in a climate controlled room on a 12-hr light/dark cycle and fed an AIN-76A diet. Animals were given free access to food and water. The animals were given 3 daily subcutaneous injections of 0, 150, 300 or 600 mg/kg bisphenol A (Aldrich Chemicals, Milwaukee, WI) or diethylstilbestrol (DES, 0.05 mg/kg) in corn oil or corn oil alone (vehicle control). Rats were anesthetized with pentobarbital sodium for blood withdrawal then euthanized by cervical dislocation.

b. Tissue Preparation.

The uteri were excised, cleaned of adhering connective tissue and fat, wet weighed then snap-frozen in liquid nitrogen and stored at -80°C until complement C3 analysis by western blotting. (All chemicals used were obtained from Sigma Chemicals (St. Louis, MO) unless otherwise specified).

Western blots were carried out on previously frozen rat uterine tissue. The uterine tissue (~50 mg) was homogenized in 100 μl of homogenization buffer for 30 seconds on ice. The homogenization buffer contained aprotinin (50 μg/ml), leupeptin (5 μg/ml), sodium chloride (0.1 M), Tris (0.01 M), and ethylenediaminetetraacetic acid (EDTA, 0.001 M). The samples were then centrifuge for 30 minutes at 10,000 rpm. The supernatant was removed and placed in a fresh microcentrifuge tube. Total protein concentration of the homogenized uterine tissue was determined by the Biorad method.
c. Western Blot.

Homogenized tissue was subjected to electrophoresis on a 4-12% acrylamide gel. To prepare samples for electrophoresis, 20 μg of total protein from the uterine sample was mixed with 10 μl 2X loading dye (0.4% bromophenol blue in glycerol) and brought to a total of 20 μl with phosphate buffered saline. The samples were placed in boiling water for 3 minutes. A prestained molecular weight standard (BioRad, Hercules, CA) was similarly prepared. The proteins were loaded into a precast 4-12% Tris-Glycine ready made gel (Bio-Rad, Hercules, CA) and the gel run at 200 volts for 30-45 minutes. The separated proteins were then transferred to a nylon membrane for immunoblotting. The membrane (Westran #78377) was first equilibrated in 100% methanol, then the transfer apparatus (Mini-PROTEAN II) setup and run overnight at 35 volts.

The membrane was then incubated with antibodies using the following procedure. The membrane was initially submerged in blocking buffer (5% bovine serum albumin) for 40 minutes. The blocking solution was removed and the membrane submerged directly into primary antibody solution (1:5000 dilution of goat anti-rat complement C3 IgG antibody, ICN Pharmaceuticals, Aurora, OH) and placed on a shaker for 1.5 hrs. The membrane was then rinsed 4 times with washing buffer (0.1 M sodium chloride, 0.05 M Tris-base, and 0.05% Tween-20) for 10 minutes each. The membrane was then incubated with the secondary antibody (1:3000 dilution of rabbit anti-goat IgG conjugated to alkaline phosphatase, ZYMED Laboratories Inc., San Francisco, CA) for 1 hr then washed as previously described. Finally, the membrane was immersion in the substrate solution (made with SIGMA FAST BCIP/NBT tablets) for 5 minutes then rinsed in deionized water.
d. **Statistical analysis.**

Uterine weight and complement C3 levels from the different treatment groups were compared by student's t-test with $\alpha=0.05$ considered significant. To quantify complement C3 levels, an external control was added to all the blots and used to normalize the data.

3. **Results**

Significant ($p=1.6 \times 10^{-8}$) increases in uterine weight was seen following treatment with the DES, the positive control (Figure 2). Additionally, a increase in uterine weight was also seen following treatment with 600 mg/kg BPA ($p=0.001$, Figure 2). However, no other increases in uterine weight were seen with smaller doses of BPA.

Increased uterine complement C3 levels was also seen with DES ($p=0.007$) and the highest dose of BPA ($p=0.01$). However, unlike with uterine weight, lower doses of BPA also resulted in significantly ($p=0.02$) increased levels of uterine complement C3 (Figure 3). This indicates that complement C3 is a more sensitive indicator of estrogenicity than uterine weight with the environmental estrogen BPA.

4. **Conclusion**

It is clear from these reports that the uterine complement C3 assay is fully operational in our lab. Additionally, the fact that our system can evaluate complement C3 levels following exposure to both natural estrogens (like estradiol, see previous experiment) and environmental estrogens (like BPA), indicates that it can serve as an excellent measure of exposure to estrogenic pollutants. We are now ready to process raccoon uterine tissue for complement C3 measurement.
Figure 2. Uterine weight (in mg) of 21 day old female Sprague-Dawley rats following exposure to bisphenol A (BPA) or diethylstilbestrol (DES) for 3 days. The letters above the bars indicate different levels of significance at p=0.05.
Figure 3. Uterine complement C3 levels in 21 day old female Sprague-Dawley rats following exposure to bisphenol A (BPA) or diethylstilbestrol (DES) for 3 days. The letters above the bars indicate different levels of significance at p=0.05.
CHAPTER 3: PILOT EXPERIMENT

Once we had established methods for measuring complement C3, we then were ready to assess the ability of this assay to detect complement C3 in raccoons, along with angiotensinogen and ceruloplasmin, as biomarkers of exposure to environmental estrogens.

A. Experimental Design

1. Animal Collection

Raccoons were collected using box (live) traps from two Illinois locations, one considered pristine (from University of Illinois farms) and one considered heavily contaminated (Lake DePue). Traps were checked as soon as possible after sunrise, and the location of trap stations on Lake DePue recorded.

2. Plasma Collection

Animals were then transferred to a coated-wire handling cone, anesthetized with 3.0 mg/kg ketamine and 0.1 mg/kg medetomidine using sterile needles and syringes. Whole blood was drawn via cardiac puncture, transferred to 2 ml EDTA-treated blood collection tubes and the blood then centrifuged for 10 minutes to obtain plasma. The plasma was then drawn off, transferred to a 1 ml plastic microcentrifuge tube, and frozen in liquid nitrogen until performance of ceruloplasmin and angiotensinogen assays.

3. Uterus Collection

To collect uteri, animals were euthanized using a chemical agent approved for use by the Laboratory Animal Care Advisory Committee. The uteri were collected and weighed, then rapidly frozen in liquid nitrogen and kept at -80°C until complement C3 analysis. Listed below is the raccoons collected along with the tissue collected and their site of collection.
a. University of Illinois farms

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B. Results

Increased levels of angiotensinogen was noted in plasma obtained from raccoons located in the Lake DePue area compared to the pristine area along the Illinois River (Figure 4). However, no similar
increases were seen in ceruloplasmin levels (Figure 5). No detectable amount of complement C3 was observed in any of the samples.

C. Conclusion

Elevated levels of angiotensinogen suggest that the polluted environment adversely affected the health of animals living in the contaminated area. However, the pollutants may not be estrogenic since no changes were seen in the levels of ceruloplasmin. The lack of increase in ceruloplasmin in animals living in contaminated areas may be due to a number of causes. A major possibility is that the pollutants affecting angiotensinogen are not estrogenic. However, increases in ceruloplasmin have only been reported following exposure to very potent estrogens. For example, increases in ceruloplasmin have been reported in the literature in response to the estrogens like ethinyl estradiol and DES. The fact that most environmental estrogens are usually only moderately to weakly estrogenic and usually have a 1000-10,000-fold decreased affinity for binding to the estrogen receptor may result in their inability to elicit increases in ceruloplasmin. Although a range of estrogenic substances should be tested to evaluate this possibility, preliminary work in our lab have shown a similar pattern. Rats treated with DDT, a prototypic environmental estrogen, have not shown increases in ceruloplasmin, while those treated with ethinyl estradiol and DES showed increases.

The inability of complement C3 to be detected in any of the uterine samples probably indicates that the western blot procedure needed to be further optimized for use with raccoon uteri. Because no raccoon anti-complement C3 antibody is available, we have been using rat antibody. Thus, this antibody may have decreased affinity for raccoon complement C3. As a result, to overcome this obstacle, one may need to increase the total amount of protein loaded per lane and/or increase the concentration of rat anti-complement C3 antibody used as the primary antibody. Such studies are underway.
Figure 4. Angiotensinogen (ANGI) plasma levels in raccoons living near University of Illinois farms (pristine) and in Lake DePue area (contaminated). *denotes significant at $p=0.05$. 
Figure 5. Ceruloplasmin (CPN) plasma level in raccoons living near University of Illinois farms (pristine) and in Lake DePue area (contaminated). *denotes significant at $p=0.05$. 
CHAPTER 4: RECOMMENDATIONS

This project is being performed as a supplement to a study by Dr. J. Levengood. He proposed to measure and compare metal levels in tissue and plasma from raccoons collected from a relatively pristine site in Illinois to those living in a heavily contaminated area. His plan was to use the information gathered to estimate risk to other species inhabiting these areas.

To supplement the work of Dr. Levengood, we proposed to use our previously developed biomarkers of environmental estrogen exposure to assess the impact of contamination in these areas. Additionally, we also proposed to develop a bioassay using complement C3 measurement to assess estrogenicity in these animals. This allows us to correlate the biomarkers angiotensinogen and ceruloplasmin, which do not require sacrifice of the animals, with a more precise measure of estrogenicity, which does require sacrifice of the animals. The fact that increases in angiotensinogen were seen in raccoons from contaminated areas indicates environmental estrogens. However, the lack of increase in ceruloplasmin, suggests that the contaminants may not be estrogenic. Additionally, the fact complement C3 was not detected in any of the uterine samples may confirm the lack of estrogenic contaminants. Alternatively, lack of complement C3 response may indicate that further optimization of the bioassay is needed for use in raccoons.
CHAPTER 5: REFERENCES


