Research Plan

1. Specific Aims

Hypertension and chronic kidney disease are more common in men than in premenopausal women at the same age. The endothelin (ET) system appears to be regulated by ovarian hormones, and has been shown to account for sex differences in Na\(^+\) regulation and blood pressure control. In parallel, our preliminary results show a sex-difference in the inner medullary ET-1 gene expression in response to salt loading to the kidney. During a high salt diet, ET-1 is released within the kidney in response to hyperosmolality promoting urinary Na\(^+\) excretion (U\(_{Na}\) V). Recent in vitro evidence suggests an association between the purinergic system and the ET system in the collecting duct. Moreover, non-renal interactions between purinergic receptors and ET-1 have been demonstrated in neurons and blood vessels. However, the in vivo interaction between these two critically important systems in the kidney has not been clarified in either sex. Understanding of the underlying mechanism of how the ET system is involved in sex-differences in Na\(^+\) excretion may lead to the development of more efficient sex-specific therapies for hypertension and kidney-related diseases. Thus, the overall goal of this fellowship is to elucidate the modulatory role of ovarian hormones in sex-related differences in Na\(^+\) handling by the kidney. Additionally we will explore sex-differences in the role of renal purinergic receptors in ET-dependent control of Na\(^+\) excretion. Specific aims to be addressed are:

**Aim 1:** To test the hypothesis that ovarian hormones regulate the response to medullary hyperosmolality through activation of estrogen receptors (ER) and/or progesterone receptors (PR). We will test this hypothesis by examining the effect of increasing the intramedullary (IM) osmolality on renal salt handling in: (1) male, (2) intact female, (3) ovariectomized (OVX), and (4) OVX rats supplemented with β–estradiol (E\(_2\)) and/or progesterone (P) in the presence and absence of their respective receptor blockers.

**Aim 2:** To test the hypothesis that purinergic receptor activation increases ET-dependent natriuresis and participates in sex-dependent differences in Na\(^+\) excretion. We will test this hypothesis by determining if: (1) IM activation of purinergic receptors increases ET-1 production, and (2) IM purinergic receptor blockade inhibits ET-1 produced in response to high salt intake. Parallel experiments will be conducted in both male and female rats.

2. Background and Significance

Premenopausal women are largely protected from hypertension and kidney-related diseases compared with men of similar age.\(^1\), \(^2\), \(^3\) This phenomenon is gradually lost after menopause, pointing to a pivotal role of ovarian hormones in the maintenance of cardiovascular and renal health in women.\(^4\), \(^5\), \(^6\) ET-1, a potent vasoactive peptide found in cardiovascular and renal tissues, has been implicated in sex-differences in blood pressure control and kidney function.\(^7\), \(^8\) The ET system plays a central role in controlling salt and water balance and blood pressure.\(^9\) High NaCl intake was reported to increase the osmolality of the renal medulla.\(^10\) Within the renal medulla, which contains the highest concentration of ET-1 in the body\(^11\), this peptide is released in response to medullary hyperosmolality\(^12\) and inhibits tubular Na\(^+\) transport promoting natriuresis mainly through activation of ET\(_B\) receptors.\(^9\) The signaling mechanism by which high extracellular osmolality translates into an increase in ET production and/or action is currently unknown. It is noteworthy that most of the published work on the ET system has been conducted in the male population or in pooled populations from...
both genders. However, some studies have attracted the attention to sexual dimorphism in the renal ET system.\(^7\), \(^{13-18}\) Additionally, ER and PR are expressed in the kidney\(^{19, 20}\), and an important role for E\(_2\) and P in the regulation of renal electrolyte homeostasis has been demonstrated.\(^{21-24}\) Thus, the current proposal seeks to elucidate the regulatory role of E\(_2\) and P in ET-dependent renal Na\(^+\) handling.

Purinergic signaling has also emerged as another important system in the renal control of blood pressure and Na\(^+\) excretion.\(^{25, 26}\) In response to increased tubular flow, ATP is released from renal tubular cells inhibiting Na\(^+\) transport along the nephron\(^{25, 27}\), mainly through P2Y\(_2\) receptor activation.\(^{28, 29}\) Both P2Y\(_2\) Knockout (KO) mice and ET\(_B\) deficient rats have been demonstrated to have salt-sensitive hypertension.\(^{13, 29}\) However, the interaction between renal ET and purinergic signaling is not clear. In collaboration with the sponsor’s lab, Dr. Kohan’s lab has recently demonstrated that renal P2Y\(_2\) receptor blockade inhibits ET-1 production in vitro (unpublished data). Furthermore, non-renal studies demonstrated that purinergic receptors contribute to ET effects in sensory neurons\(^{30}\) as well as blood vessels.\(^{31, 32}\) Collectively, there is fairly clear evidence to suggest that ET and purinergic receptors are important in controlling renal tubular reabsorption of Na\(^+\) and water, but the nature of the relationship between the renal purinergic and ET systems in the context of potential sex differences has not been defined yet. Importantly, sex hormones have been shown affect the purinergic system also.\(^{33-36}\) Studies in the current proposal are uniquely suited to elucidate the novel mechanism of purinergic receptor mediated effects on renal excretory function in male and female rats.

**Significance:** Data that have become available in recent years generally reinforce the importance of ovarian hormones in determining quality of life and prognosis of cardiovascular and renal diseases in female patients. Increasing evidence demonstrates that pathophysiology, manifestations, and complications of cardiovascular and renal diseases differ in premenopausal, hormonally supplemented and unsupplemented postmenopausal patients, indicating that female sex hormones play a central role in the maintenance of cardio-renal health in the female population. Thus, the management strategies evaluated in the general population may differ significantly between male, premenopausal female and postmenopausal female patients with and without hormonal replacement therapy. The number of trials that specifically address cardio-renal diseases in females under different hormonal status remains small. When results of needed studies of the modulatory role of gonadal hormones on critically important systems involved in cardiovascular and renal control, such as ET system, and other relevant topics become available, developing clinical practice guidelines based on a systematic evidence will be applicable. Therefore, the proposed studies will help expand our knowledge on the regulation of the renal ET and purinergic system by sex and ovarian hormones, hoping to pinpoint new sex and hormonal status-specific therapeutic approaches to manage cardiovascular and renal diseases. Additionally, the proposed studies will provide the applicant with the opportunity to master new physiological and molecular biology methodologies that will complement and broaden her current knowledge and research experience.

3. Preliminary Studies

Previous studies in our lab demonstrated that female rats have demonstrated a more robust renal ET-1 natriuretic capacity compared to males, and gonadectomy diminished this sex difference.\(^{14}\) Additionally, our lab has also shown that ET-1 mediates the diuretic and natriuretic response to increased medullary osmolality in male rats.\(^{12, 37}\) Our preliminary studies involved investigating the effect of IM infusion of isosmotic (284 mOsm/Kg H\(_2\)O) and hypersmotic (1800 mOsm/Kg H\(_2\)O) saline for in male, intact and ovariectomized (OVX) female rats for 60 min. Interestingly, during the first 30 min period of increasing the medullary
osmolality, significant diuresis and natriuresis were demonstrated only in males and OVX rats, compared with isotonic saline (Fig. 2). Females only show these responses in the second 30 min period. Blood pressure and K⁺ excretion remained constant during the experiments. Interestingly, the inner medullary ET-1 mRNA expression in male rats was significantly increased after 60 min of medullary interstitial infusion of hyperosmotic saline (Fig. 3). In contrast, ET-1 expression did not significantly change in females, indicating that an increase in renal inner medullary ET-1 could play a more important role in the diuretic and natriuretic response to IM hyperosmolality in male compared to female rats. These data shows that ovariectomy eliminated the sex difference in the diuretic and natriuretic response to IM infusion of hyperosmotic saline (Fig. 2), and directed us to focus on the ovarian hormones as potential players in the sexual dimorphism in the response to salt loading to the kidney. Therefore, Aim 1 will target studying the effect of depletion and repletion of E₂ and P on renal ET system response to IM hyperosmolality.

Purinergic receptor activation has been suggested to be a key player in control of Na⁺ excretion. It was recently demonstrated by Dr. Donald E. Kohan’s lab that purinergic (P₂Y₂) receptor blockade in collecting duct inhibits ET-1 production in vitro (personal communication). A pilot study in our lab has demonstrated that IM infusion of the purinergic agonist, UTP, produces natriuresis. Moreover, our preliminary experiments showed that suramin, a purinergic antagonist, inhibits the diuretic response to increased medullary osmolality in males (Fig. 4). Therefore, in Aim 2 we speculate that activation of renal medullary purinergic P₂Y₂ receptors should increase ET-1 production, which in turn, would inhibit Na⁺ and water excretion and produce a natriuretic and diuretic effect.

4. Research Design and methods
Aim 1: To test the hypothesis that ovarian hormones regulate the response to medullary hyperosmolality through activation of ER and/or PR.

Rationale: Sex differences in the development and progression of cardiovascular and kidney diseases have been widely reported and ET-1 is a potential mediator of this sexual dimorphism. ER and PR are expressed in the kidney⁷, ⁸, and sex steroids affect many components of ET system. However, the exact relationship between the E₂/P and ET-1 system in the kidney is not clear. Ovarian hormones are pivotal for the maintenance of renal health in females, and we propose that E₂ and/or P regulate the response of renal ET-1 system to increased medullary osmolality. Thus we will test the hypothesis that males and females respond differently to IM hyperosmolality, and that ovarian hormonal depletion by ovariectomy eliminates this sex-difference in salt handling by the kidney. Additionally, we will study whether the individual or combined supplementation of E₂ and P to OVX rats will restore sexual dimorphism in renal ET-1 system response to increased medullary osmolality. Furthermore, studies will be extended to investigate whether the modulatory effects of ovarian hormones are
mediated through ER and/or PR-dependent mechanisms. Aim 1 will be achieved by studying the effect of increasing the medullary osmolality on urine flow, Na⁺ excretion and inner medullary ET-1 gene expression in Sprague Dawley (SD): (1) male, (2) intact female, (3) OVX, and (4) OVX rats supplemented with E₂ and/or P in the presence and absence of their respective receptor blockers. Time line for IM infusion and groups used in Aim 1 are illustrated in Fig. 5. All rats in Aim 1 will be fed normal salt (0.4% NaCl) diet.

**Experiment 1.1. Do male and female renal ET-1 systems respond differently to increased medullary osmolality?**

**Experimental Design.** Male and female rats will be surgically prepared for acute IM infusion as detailed in the Methods section. Isosmotic saline (284 mosmol/kgH₂O) will be infused into the renal medullary interstitium during a 60 min equilibration period and 30 min baseline urine collection period, followed by isosmotic or hyperosmotic saline (1800 mosmol/kgH₂O) for two further 30 min periods. Urine flow, osmolality, Na⁺ and K⁺ will be measured. Blood pressure will be monitored during the experiment. At the end of each experiment, the renal inner medulla will be isolated for ET-1 mRNA assessment, and blood samples will be collected for measurement of the level of sex hormones (E₂ & P).

**Experiment 1.2. Do ovarian hormones account for the sexual dimorphism in renal ET-1 system response to increased medullary osmolality?**

**Experimental Design.** Female rats will undergo ovariectomy and will be implanted with placebo pellets (OVX), or hormonal pellets containing E₂ (0.35 mg/pellet, OVX+E₂), P (25 mg/pellet, OVX+P), or the same doses of both hormones simultaneously (OVX+E₂+P) for 21 days. Sham-operated controls are performed in parallel. Three weeks after ovariectomy or sham surgery, rats will be prepared for IM infusion technique and the effect of medullary hyperosmolality will be studied following the same experimental protocol detailed in Exp 1.1. and illustrated in Fig. 5.

**Experiment 1.3. Does blockade of ER or PR inhibit the modulatory effects of ovarian hormones on salt handling by the kidney?**

**Experimental Design.** Rats will undergo ovariectomy and will be co-supplemented with E₂ or P with its respective receptor antagonist. OVX rats implanted with E₂ pellets will receive daily doses of ICI-182780 (ER antagonist, 1 mg/kg, S.C., OVX+E₂+ICI). OVX rats implanted with P pellets will receive daily doses of mifepristone (PR antagonist, 10 mg/kg, S.C., OVX+P+Mif). The first injection of ICI-182780 or mifepristone will be given 1 h prior to E₂ or P pellet implantation, respectively, in order to provide efficiently blockade of ER and PR before ovarian hormones are supplemented. On day 21 after OVX, the last injection of ICI-182780 or mifepristone will be given 1 h prior to surgical preparation for IM infusion. After surgical preparation, the same experimental protocol detailed in Exp 1.1. and Fig. 5. will be performed.

**Anticipated results and interpretation**

Increasing medullary osmolality simulates the effect of a high salt diet and will increase Na⁺ and water excretion. We expect that ovarian hormones will regulate renal salt handling through activation of sex hormone receptors. Thus, it is expected that depletion of ovarian hormones will mask the sexual dimorphism in response of ET-1 system to medullary hyperosmolality, and repletion of ovarian hormones to hormonally depleted rats will restore this sexual dimorphism through activation of ER and/or PR. Consequently, male-female differences
in salt handling are expected to be masked again by pharmacological blockade of ER and PR. We expect that E$_2$ is the major player because of its documented interaction with the cardiovascular and renal systems. On the other hand, very little is known regarding the effect of P on cardiovascular, however a role for P in renal electrolyte homeostasis has been demonstrated.$^{21-24}$ Furthermore, the possibility that the coexistence of both ovarian hormones is mandatory for the enhanced natriuresis is also postulated and will be addressed by studying the effect of dual supplementation of OVX rats with E$_2$ and P.

**Potential Problems, Pitfalls and Solutions**

Rats have approximately a 4-day reproductive cycle (estrus cycle), which consists of 4 stages: estrus, proestrus, metaestrus, and diestrus. Although we do not expect that the estrus cycle will impact our findings, we plan to perform the experiments using intact females in a periodic manner so we will spread performing female experiments over 4 consecutive days (i.e., different estrus cycle phases will be presented). However, if the intact females show high variability in their results, we will examine whether changes in estrus cycle phases and consequent fluctuations in their plasma level of ovarian hormones are impacting our results. For this case, we will split each intact female group into two: diestrus and proestrus rats, based on microscopical examination of their vaginal smears.

To ensure sustained supplementation of E$_2$ and P to hormonally-depleted rats, OVX rats will be implanted with controlled release hormonal pellets, sustained over 21 days. We plan to conduct preliminary studies to make sure that these hormonal regimens provide a physiological level of the sex hormones by measuring serum E$_2$ and P levels in several interval points during the 3 weeks hormonal supplementation period. We specifically selected using ICI-182780 (Fulvestrant, Faslodex) because it is a complete ER antagonist for both ER-α, ER-β, with no agonist effects, which in addition, accelerates the degradation of the ER.$^{38,39}$ The selected doses ICI-182780 and mifepristone doses are reported in the literature to provide adequate blockade of ER and PR.$^{40,41}$ If our results showed that the sexual dimorphism in the renal salt handling is mediated through ER-dependent mechanism, our future studies will target investigation of the exact subtype of ER; nuclear receptors (ER-α, ER-β), and GPER (G-protein coupled ER) involved in modulating the salt handling by the kidney.

If male-female differences in ET system response to salt loading the kidney are not totally eliminated by OVX, we will study the effect of depleting testosterone by performing orchietomy. Then, we will study the effect of supplementing orchietomized rats with testosterone in the presence and absence of the androgen receptor blocker, flutamide, to verify the involvement of testosterone receptor activation in mediating sex differences in salt handling by the kidney. If the inner medullary ET-1 mRNA expression results did not provide a proper explanation of the effects of E$_2$/P on the renal ET-1 system, we plan to measure the medullary ET$_A$ and ET$_B$ receptor expression in males and females with different hormonal status. Sex differences in ET-1 receptor density, as well as ET-1 receptor subtypes, favouring the protective effective in females, have been described.$^7$

As NOS/NO system is modulated by ovarian hormones and participate in ET-dependent control of Na$^+$ excretion$^{13}$, we will monitor the changes in urinary NO metabolites (nitrites and nitrates) in our initial experiments. If we observe a sexual dimorphism in the urinary levels of NO metabolites in response to medullary hyperosmolality, we will elucidate the effect of E$_2$ & P on the renal NOS system by measuring the urinary level of NO metabolites in females with different hormonal status. We will also measure NOS isoforms in the inner medullas.

Our preliminary results also pointed to an interesting hormonally-dependent difference in the ability to concentrate urine. The urine osmolality is significantly lower in females,
compared to males, and depletion of ovarian hormones by ovariectomy restored the high urine osmolality. Thus we additionally plan to elucidate the effect of ovarian hormones on the ability to concentrate urine and whether these effects are mediated through activation of ER and PR.

Aim 2: To test the hypothesis that purinergic receptor activation increases ET-dependent natriuresis and participates in sex-dependent differences in Na\textsuperscript{+} excretion.

**Rationale:** Purinergic (P2Y) receptors are expressed along the nephron\textsuperscript{12},\textsuperscript{22,23} and play an important role in the renal control of blood pressure and Na excretion\textsuperscript{25,26}. We hypothesize that renal tubular flow and Na\textsuperscript{+} delivery along the collecting duct results in release of ATP and subsequent activation of P2Y\textsubscript{2} receptors as a means of stimulating ET-1 production. Our lab has previously published that IM infusion of hypertonic saline in male kidneys increases urine ET-1 excretion. To directly determine whether purinergic signaling could trigger the increase in urine ET-1, we will conduct IM infusion of UTP, a P2Y agonist, and measure ET-1 and Na\textsuperscript{+} in the urine. Additionally we will study the effect of purinergic receptor blockade on ET-dependent natriuretic response to high salt diet in male and female SD rats.

**Experiment 2.1. Does IM activation of purinergic receptors promote ET-dependent natriuresis in male and female rats?**

**Design:** Male and female rats will undergo uni-nephrectomy. One week later, micro infusion (iPRECIO) pumps will be implanted with a catheter connecting the pump to the renal medulla. Infusion of saline into the renal interstitium will start immediately at a rate of 9 μl/h. Rats will be allowed to recover for 24 hours postsurgical before being placed in metabolic cages. 24-h urine will be collected for 3 days to establish basal levels of ET, and Na\textsuperscript{+} excretion rate. Then, the implanted pumps will be refilled with UTP (200 pmol/kg/min) and 24-h urine samples will be collected for another 3 consecutive days. To confirm that UTP effects are through P2Y\textsubscript{2} receptor activation, we will infuse two additional groups of male and female rats with the specific P2Y\textsubscript{2} receptor antagonist, ARC-118925, 24 h before starting UTP infusion. All urine samples will be analyzed for measurement of osmolality and electrolytes and ET-1 levels. Rats will be maintained on normal salt diet. At the end of each experiment, inner medullas will be collected for assessment of ET-1 gene expression levels and blood samples will be collected for measurement of E\textsubscript{2} & P levels. Time line for Exp.2.1. is illustrated in Fig. 6.

**Experiment 2.2. Does purinergic receptor blockade inhibit ET-1 produced by increasing medullary osmolality in response to high salt diet in male and female rats?**

**Design:** Male and female rats will undergo the same surgical preparation as in Exp. 2.1. The implanted pumps will infuse suramin (0.75 mg/kg/min), a purinergic blocker, or saline into the renal interstitium. One day postsurgical, the rats will be placed in metabolic cages on regular normal salt diet for 3 days. After 3 days, rats will be placed on a high salt diet (4% NaCl) for 3 additional days. 24-h urine will be collected across the 6 days for assessment of urinary ET-1 and electrolyte levels.
Water and food intake will be monitored during this experiment. At the end of each experiment, inner medullas and blood will be collected for assessment of ET-1 gene expression and hormonal levels, respectively. Time line for Exp.2.1. is illustrated in Fig. 6.

**Anticipated results and interpretation**

Our hypothesis predicts that UTP-induced natriuresis is associated with an increase in urine ET-1 levels in males and females. We expect this to be inhibited by purinergic receptor antagonism. Additionally, we expect that females have a greater natriuretic response to purinergic receptor activation compared to males as our lab have previously demonstrated that IM infusion of ET-1 causes more robust natriuresis in females, in comparison to males. However, differences in the natriuretic effects of exogenous versus endogenous ET-1 are also possible. The high salt diet is expected to enhance the urinary excretion ET-1 and Na⁺ as our lab has observed many times in males. We expect that this effect will be more robust in females compared to males. We also expect that purinergic receptor blockade will attenuate the ET-dependent natriuretic response to high salt diet.

**Potential Problems, Pitfalls and Solutions**

One major point regarding the acute IM infusion technique in Aim 1 is the low urine yield obtained during the 30 min urine collection periods. This small urine volume together with detection limit considerations does not allow the accurate measurement of ET-1 from urine samples obtained from acute experiments with kits currently available in the market. Thus, Aim 2 is designed to involve a metabolic cage study to allow measuring of urinary ET-1.

The purinergic agonist, UTP, was chosen as a first approach because of its relative specificity for P2Y₂ and P2Y₄ receptors. In addition, while evidence suggests that the P2Y₂ receptor is most likely responsible for stimulating ET-1 production, there remains the possibility of a full range of purinergic receptors that could be involved. Thus, if our initial experiments with ARC-118925 do not inhibit the UTP response, we will move to a more general purinergic blocker such as suramin. Furthermore, while we are using UTP in our initial experiments, it will be important to explore whether the less selective ligand, ATP, can be infused to produce a similar effects. If the natriuretic response to IM infusion of UTP or the anti-natriuretic response to ARC-118925 or suramin decline with time (i.e. the urinary Na⁺ excretion on day 2 and 3 is less than that on the first day), this may be related to the stability of these purinergic drugs in solution at body temperature. Thus, we will consider loading the iPRECIO pumps with fresh solutions on shorter time intervals (every 12 or 24 hours).

Our pilot studies revealed that an IM dose of 200 pmol/kg/min of UTP produced a significant natriuresis and that an IM dose of 0.75 mg/kg/min of suramin blocked the diuretic effect of hyperosmotic saline (Fig. 4.). We know from *in vitro* studies that 10 μM concentration of ARC-118925 is a maximally effective concentration to block P2Y₂ receptors. However, the *in vivo* dosing with ARC-118925 has not been well established. We propose to use a starting dose of 1 μmol/kg/min of ARC-118925. A question surrounding Exp. 2.2. is the lack of specificity of suramin for P2Y₂ receptors. We propose to first use suramin in order to maximize our chances of seeing an effect. If justified, we will follow up with ARC-118925. Alternatively, we can use the P2Y₂ KO mice.

To ensure the correct delivery of saline and drugs into the renal medulla, the tip of the catheter will be implanted about 5 mm into the normal kidney or about 7 mm into the enlarged kidney, after nephrectomy of the other kidney. At the end of every single experiment, infused kidneys will be collected and sliced open for visual inspection of the proper positioning of the IM catheter. We have already tested the applicability of using the micro infusion pumps (iPRECIO, programmable and refillable pumps) for IM infusion in a pilot trial in our lab. The trial
involved programming those pumps for drug delivery with a rate of 9 μl/h, and the pre-programmed pumps were used for chronic IM infusion of drugs to uninephrectomized rats. We were able to refill the pump reservoir percutaneously via a re-sealable septum according to the manufacturer instruction. Finally, the experimental design of Aim 2 will cause a dramatic decrease in the number of rats employed by half because each rat will be employed in 2 groups. In Exp. 2.1., the rat will serve as a control while the pumps are filled with saline and then the pump will be refilled with a purinergic drug to study its effect. In Exp. 2.2., each rat will be kept first on normal salt to serve as control, then high salt diet will be introduced to study the effect of salt loading.

**General Methods**

**Acute IM Infusion:** A tracheotomy will be performed to facilitate breathing. A catheter will be inserted into the femoral vein infusing 3% bovine serum albumin in PBS to replace fluids lost, and another catheter will be inserted into the femoral artery to monitor blood pressure. Urine will be collected from the left kidney via a catheter placed in the left ureter. A catheter will be inserted into the left kidney, infusing isosmotic saline directly into the renal medulla (0.5 ml/h).

**Nephrectomy:** Uni-nephrectomy of the left kidney will be performed through a left lateral incision of the abdominal wall, and the left kidney will be removed after renal vessel ligation.

**Chronic IM Infusion Technique and Pump Implantation:** One week after performing nephrectomy, micro infusion pumps (iPRECIO) will be implanted dorsally, and connected to catheters inserted into the renal interstitium of the right kidney. Pump refilling will be performed under isoflurane anesthesia.

**Ovariectomy:** Bilateral ovariectomy will be performed through dorsal bilateral incisions. Ovaries will be exposed, tied off and removed. **Sham Operation:** Sham rats will undergo dorsal bilateral incisions and ovarian exposure without removal of the ovaries.

**Pellet Implantation:** At the time of ovariectomy, controlled-release pellets (Innovative Research of America) will be implanted subcutaneously in the lateral side of the neck between the ear and the shoulder, as recommended by the manufacturer.

**Animal Tissue RNA Isolation, cDNA Synthesis, and Real Time PCR:** Tissue RNA from renal inner medullas will be isolated using Purelink Mini RNA extraction kit (Ambion) according to manufacturer’s instructions. Then, the isolated RNA will be reverse transcribed using QuantiTect Reverse Transcription kit (Qiagen). Finally, the resulting cDNA will be used to quantify mRNA by RT-PCR (CFX96 Real-Time System, BIORAD) using TaqMan primer gene expression assays. Gene expression will be quantified relative to GAPDH using 2^ΔΔCt method.

**Statistics:** For comparisons of groups with a single variable, data will be analyzed by one-way ANOVA. Multiple comparisons vs. control will be analyzed by Dunnett’s post-hoc test. For experiments involving 2 variables (i.e. sex or hormones plus IM osmolality, diet plus receptor antagonist), data will be analyzed by two-way repeated measures ANOVA, and Bonferonni’s post-hoc test for comparisons between groups. A probability of p<0.05 will be considered significant.

**5. Ethical Aspects of the Proposed Research.**

This proposal investigates whole animal physiology and complex interactions between ovarian hormones, renal ET-1, and purinergic systems. Because of this, the aims of this proposal can only be addressed using intact animals and isolated tissues. The choice of number of animals is selected on the basis of the minimal numbers needed to obtain statistically meaningful results. Use of rats in this study is guided by principles established by the “American Physiological Society’s Guide to the Care and Use of Laboratory Animals”. See attached “Vertebrate Animal Subjects” for further details.