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ABSTRACT

Title of thesis: Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Protects the Ovary of Mice Against Mitoxantrone Cytotoxicity

Thaddeus Nnabue, Master of Science 2016

Thesis directed by Dr. Istvan Merchenthaler, Professor of Medicine, University of Maryland.

PACAP is a pleiotropic polypeptide with cytotrophic, anti-inflammatory, and anti-apoptotic activities. Mitoxantrone (MTX) is a chemotherapeutic drug used for treatment of multiple sclerosis. My hypothesis is that PACAP will protect the granulosa cells of the ovarian follicle from cytotoxic cell death caused by MTX treatment. A total number of 27 mice were assigned to 3 groups; MTX, MTX + PACAP, and control. TUNEL staining, caspase-3 assay, and H&E staining were used to visualize the granulosa cells in ovary sections. H&E staining showed that MTX treatment reduced the number of developing follicles. Positive TUNEL in granulosa cells of ovary treated with MTX confirmed cell damage by MTX. Follicles in the MTX + PACAP group were TUNEL negative. The caspase-3 staining was negative in all groups. These observations suggest that MTX causes granulosa cell death from DNA damage but not due to apoptosis, and PACAP protects the granulosa cells from DNA damage.

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Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Protects the Ovary of
Mice Against Mitoxantrone Cytotoxicity

by
Thaddues Nnabue

Thesis submitted to the faculty of the Graduate School
of the University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
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DEDICATION

This work is dedicated in loving memory of my late father, Nze Cyprian Nnabue.

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LIST OF ABBREVIATIONS

AC; adenylate cyclase

Apaf-1; apoptotic-activating factor 1

ATP; adenosine triphosphate

cAMP; cyclic adenosine monophosphate

CIA; chemotherapy-induced amenorrhea

CNS; central nervous system

CP; cyclophosphamide

DAB; diaminobenzidine

DAP1; 4',6-diamidino-2-phenylindole

FDA; Food and Drug Administration

FEMIMS; fertility and mitoxantrone in multiple sclerosis

H&E; hematoxylin and eosin

IAUCUC; Institutional Animal Care and Use Committee

MPAPO; a new recombinant PACAP-derived peptide

MTX; mitoxantrone

PA; permanent amenorrhea

PACAP; pituitary adenylate cyclase-activating polypeptide

PBS; phosphate-buffered saline

POF; premature ovarian follicle

PSMG; pregnant mare's serum gonadotropin

SIDS; sudden infant death syndrome

TA; transient amenorrhea

TUNNEL; terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling

VIP; vasoactive intestinal polypeptide

I. INTRODUCTION

A. PACAP

A.1. The discovery of PACAP

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic peptide (Eun et al., 2014) which possesses an anti-apoptotic property and utilizes cyclic adenosine monophosphate (cAMP) as a signaling intermediate (Jozwiak et al., 2014). It has been shown that PACAP has a protective function against cellular damage from cytotoxic substances (Horvath et al., 2014; Juhasz et al., 2015). Studies also show that it is effective in reducing the cardio-, liver-, and kidney- toxicity of chemotherapeutic agents (Lee et al., 2014; Arimura et al., 2006; Khan et al., 2011). Due to its widespread physiological and pathophysiological activities, there is increased research in its application, and most recently in neuroprotection of the central nervous system, CNS (Eun et al., 2014; Jozwiak et al., 2015; Ramos-Alvarez et al., 2015).

The story of PACAP began in October 1989 when Akira Arimura (Tulane University, New Orleans) and his coworkers published an article where they reported a new regulatory peptide that was isolated from ovine hypothalamus. This peptide stimulated adenylate cyclase (AC) activity in the anterior pituitary gland of rat hence they called it “Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)”. This discovery resulted from a research which involved fractions from 4300 ovine hypothalami (Vaudry et al., 2009). The result showed that PACAP could stimulate AC by increasing cAMP formation. It is also very remarkable that the sequence of PACAP has been well conserved during evolution from the protochordates to mammals (Vaudry et al., 2009).

PACAP is produced by nervous tissues, the pituitary gland, and many peripheral organs (Rawlings and Hezareh 1996). PACAP belong to the vasoactive intestinal polypeptide (VIP)- secretin- growth hormone releasing hormone (GHRH)- glucagon superfamily (Rosselin et al., 1982). It is found in males and females, and also in infants and the elderly. It has been shown that the amount of PACAP and its receptors varies with sex, thereby explaining the different reactions between males and females to stress (Mosca et al., 2015).

A.2. PACAP receptors

PACAP has two main forms; PACAP38 has 38 amino acid residues, and PACAP27 has 27 amino acid residues (Brown et al., 2014; Juhasz et al., 2015). The PACAP receptors are of the secretin type class II G-protein-coupled receptors. The three receptors have been identified as PAC1 receptor (PAC1-R), VPAC1 receptor (VPAC1-R), and VPAC2 receptor (VPAC2-R). These receptors are present in many tissues and signal through cAMP (Eun et al., 2014). PACAP binds to these receptors with different affinities and then is able to effect cellular changes. The PACAP receptors have two types of binding sites. Type I receptors has higher binding affinity for PACAP than VIP while the type 2 receptors has equal affinity for PACAP and VIP. PAC1-R is more PACAP-specific and is widely distributed in the brain, the pituitary gland and several peripheral organs.

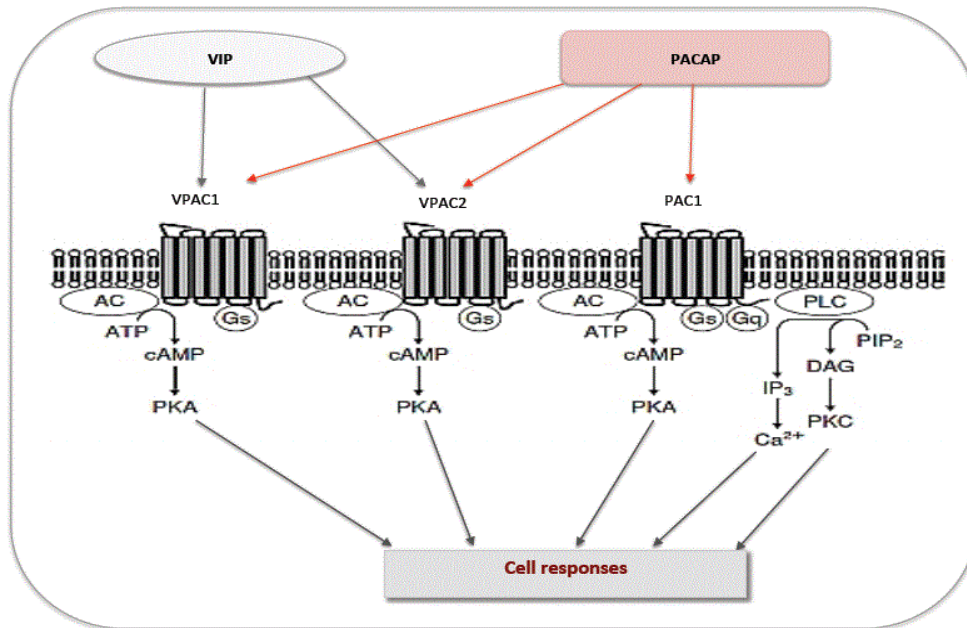


Figure 1: PACAP bind to receptors in cells. PAC1 receptor is more specific for PACAP while the VPAC receptors will bind VIP and PACAP equally. Binding to these receptors will activate ATP to form cAMP and initiate cellular response.

Diané et al., 2014

The VPAC1-R and VPAC2-R bind PACAP and VIP equally and is abundant in different organs (Vaudry et al., 2000). Cells that are distressed will display more receptors and thereby attract more PACAP to their available binding sites.

A.3. Pleiotropic functions of PACAP

PACAP has many functions in organs and tissues. It is widely distributed in the body and plays protective, regulatory, and therapeutic functions. The cytoprotective and cytotropic

effects of PACAP centrally and peripherally are primarily due to its potent anti-inflammatory activity (Brown et al., 2014; Sakamoto et al., 2015)

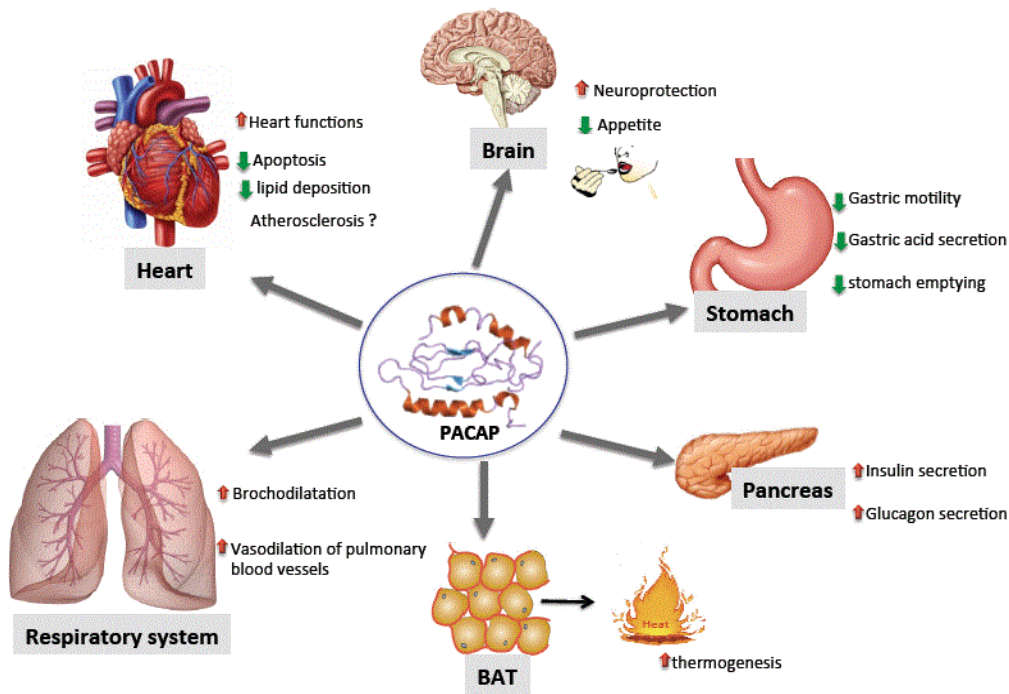


Figure 2: PACAP, a multi-functional protein. PACAP is a neurohormone and a neuroprotector in the CNS. PACAP is an anti-inflammatory agent and anti-apoptotic in the PNS. BAT is brown adipose tissue.

Diané et al., 2014

In the central and peripheral nervous system PACAP functions as a neurohormone or neuromodulator and regulates neurotransmitter release as well as neuronal/axonal growth and development (Gonzalez et al., 1997; Villalba et al., 1997; Lu et al., 1997; Dickson

and Finlayson 2009; Fahrenkrug 2006; Lee et al., 2015). For example, it regulates the release of oxytocin and vasopressin in the CNS (Ergang et al., 2015). PACAP is essential for learning, thinking and memory process (Feany and Quinn 1995). In animal models, it provides protection from apoptotic cell death in traumatic brain and spinal cord injuries (Reglodi et al., 2000; Tamas et al., 2006; Uchida et al., 1996), Parkinson's and Alzheimer's diseases (Brown et al., 2014, Eun et al., 2014), Crohn's disease (Arranz et al., 2008), and Arthritis (Abad et al., 2001; Delgado). Its effects can also be linked to the urogenital, respiratory, gastrointestinal, endocrine systems, including the sexual organs (Fung et al., 2014; Koves et al., 2014; Oka et al., 1998). Ferguson et al., (2013) demonstrated the importance of PACAP as a mediator to stress response, crucial to normal breathing in neonates. Cummings et al., in 2009 also associated PACAP deficiency to sudden infant death syndrome (SIDS) in African American neonates, and went further to suggest an association between the variant coding region of PACAP gene and SIDS. Other similar studies support the important role PACAP plays in neonatal growth and development (Barrett et al., 2013; Mosca et al., 2015).

Acting in the pancreas, PACAP stimulate insulin secretion and subsequently lowers serum glucose levels and protect from diabetic neuro- and renopathies (Ma et al., 2015; Marzagalli et al., 2015; Rossato et al., 2014; Nakamachi et al., 2012; Sanglioglu et al., 2012).

A.4. PACAP and cancer

PACAP treatment has been shown to be beneficial in the management of cancer, particularly in cancers involving hematopoietic lineages. Ji-Hae et al., in their study

published in 2014 concluded that PACAP induces apoptosis in human cervical cancer cells. They arrived at this conclusion by observing significantly reduced cell growth resulting from PACAP over-expression in human cervical cancer cells using TUNEL assay, morphology of nuclei in HeLa and HT-3 cells, and western blot analysis. This further supports that PACAP can be therapeutic and over-expression is well tolerated.

A.5. Physicochemical characteristics of PACAP

Although PACAP has many remarkable beneficial activities, the greatest limitation of its clinical use is due to its short half-life of only 3-5mins. Also, it rapidly undergoes proteolysis and is easily filtered by the kidneys. This probably explains why *in-vivo* production is insufficient during disease condition. To combat this problem scientists are still trying to stimulate more *in-vivo* production of PACAP but the best alternative is to develop more metabolically stable, and receptor subtype-specific analogs of the peptide. The challenge is to test if these PACAP analogs will perform as good as, or even better than the native peptide. Ma Yi et al., 2015 tested a new recombinant PACAP-derived peptide (MPAPO) on lacrimal wound healing. The result showed improved results of the mutant variant over the wild type PACAP which suggest that the MPAPO could indeed be a better alternative to treating patients with corneal wound while also increasing lacrimal secretions which could improve dry eyes. Also, other similar polypeptides with specificity and potency have been synthesized and studies show that the mutant type have similar functions to their natural type (Igarashi et al., 2005).

PACAP or its analogs as peptides have to be taken parenterally (subcutaneous or intramuscular injection); they cannot be taken orally. Future effort should aim to develop special formulations for prolonged absorption or even oral administration.

B. Mitoxantrone, multiple sclerosis and fertility

B.1. MTX as a chemotherapeutic agent

MTX is a topoisomerase inhibitor; it disrupts DNA synthesis and DNA repair in both healthy cells and cancer cells (Clerico et al., 2008). MTX have been in use since 1987. It was initially used to treat acute non-lymphocytic leukemia, acute myeloid leukemia and metastatic breast cancer. Immunex branded MTX as Novantrone and began selling in 1993. In 1997, Federal Food and Drug Administration (FDA) approved MTX for the treatment of pain in the late stage of prostate cancer. Again in 2002, Immunex acquired clearance from FDA to use MTX for neurological disability and for clinical relapses in MS patients. The trademark branded name Novantrone is also been sold by Serono Inc. after they obtained the rights from Amgen in late 2002. The recommended therapy is by short intravenous infusion every 3 months. As an immunosuppressor, MTX targets the T cells and macrophages, B cell proliferation, antigen presenting cells, pro-inflammatory cytokins, and DNA. The drug is eliminated through biliary excretion and urine (www.xconomy.com).

B.2. MTX as a therapy for multiple sclerosis

Although MTX is an anthracene-based anti-neoplastic drug used as chemotherapy in some cancers, it is also used as a second line treatment in MS (Cocco and Marrosu 2014). The toxicity of MTX when used as a treatment option for a patient is of main concern. It

is toxic to the heart (Dores-Sousa et al., 2015), liver and kidney (Qi et al., 2014; Rossato et al., 2013). It is also known to cause infertility in females (Koves et al., 2014). Just like other chemotherapeutic drugs, MTX has been shown to damage the ovarian follicle, thereby inducing temporary or permanent sterility in females. This is further complicated by long term use and age.

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) characterized by T-cell mediated chronic inflammation. It is an autoimmune or immune-mediated condition which attacks the myelin and nerve fibers leading to degeneration of tissue and obstruction/distortion of nerve impulse to and from the brain (Kamm et al., 2014; Alonso and Hernan 2008). Although the direct cause of MS is still unclear, it is believed that both environmental and genetic factors play a role in the progression of the disease. The prevalence of MS in the United States of America (USA) is reportedly >400,000. About 10,000 new cases are recorded yearly while over 2 million people have the disease globally (Noseworthy et al., 2000; Kabadi et al., 2014).

There is evidence that MS is more prevalent in females than males. The female-to-male ratio is between 3:1 and 2:1 (Duquette et al., 1992), with a current 2.5% lifetime risk for MS among women (Alonso and Hernan 2008). The prevalence of MS is increasing significantly largely due to increased incidence in women (Confavreux and Vukusic 2006; Koch and Sorensen 2010). Symptoms usually are evident between 20-40 years of age (Blumhardt 2004). As a result, MS is more frequent in young women of childbearing age (Cavalla et al., 2006).

The effect of MS on fertility is controversial and still not yet adequately studied. While some studies report no effect (Levi et al., 2001), others report amenorrhea and irregular hormonal imbalance (Grinsted et al., 1989). Sexual dysfunction (SD) is a major cause of dissatisfaction which leads to a negative quality of life in MS patients (Cavalla et al., 2006). SD may be a direct result of MS or could stem from the emotional/psychological problems resulting from MS. In either case, this could seriously affect fertility in both sexes. More research seems to support a direct link and hence it is worrisome that MS affect mostly women of childbearing age.

The symptoms of SD in women include reduced libido, inability to achieve orgasm, and reduction in genital sensation (Ghezzi 1999; Zorzon et al., 1999; Borello et al., 2004). A major symptom in men is erectile and ejaculatory dysfunction (Ghezzi 1999; Zorzon et al., 1999). This is partly due to hormonal imbalance, particularly reduced testosterone in males while females have higher follicle stimulating hormone (FSH) and luteinizing hormone (LH), and lower estrogen levels mainly in the early follicular stage of the menstrual cycle (Grinsted et al., 1989). Higher FSH is closely related to a reduced ovarian reserve which has a strong correlation with low fertility (Levi et al., 2001)

The treatment regimen for MS can be very exerting with serious side effects. Drugs that exert negative effects on gametes can be classified into three categories: symptomatic drugs, drugs that treat relapses, and disease-modifying therapies (Cavalla et al., 2006).

The disease-modifying agents (DMAs) include the beta-IFN-Ia and -1b, and the anthracene-based immunosuppressive drug Mitoxantrone (MTX). Other immunosuppressive drugs that are seldom used are methotrexate, azathioprine, and

cyclophosphamide (CP). CP is closely associated to amenorrhea in women (La Mantia et al., 2002; Portaccio et al., 2003; Watson et al., 1985).

C. PACAP and prevention of MTX-induced infertility

The primary action of MTX in the ovary seems to be the granulosa cells of the developing follicles. Whether MTX damages the oocyte is not clearly understood. The toxic effects of MTX in various tissues have been studied extensively but little work has been done about how it directly causes amenorrhea and if PACAP can play any protective role to combat this side effect. This research will prove that PACAP is a good antidote to MTX cytotoxicity, and will improve fertility in females by protecting the granulosa cells of the ovarian quality. The significance of establishing such a connection is very useful in the management of patients on MTX therapy (or other chemotherapies) and particularly in women of reproductive age. Once the proof of principle that PACAP can prevent MTX-induced infertility has been established the future plan will be to test the metabolically stable and receptor subtype-specific PACAP analogs for efficacy.

Fertility and MTX in MS (FEMIMS) is an Italian collaborative study that examined the effects of MTX therapy on fertility in women with MS. The study included 20 specialists in MS, and 189 women diagnosed with MS. Age at the beginning of MTX therapy was between 14 - 45 years old with a mean age of 35 years. A standardized questionnaire was used to obtain all information from the patients and emphasis was placed on menstrual cycle irregularities (Cocco et al., 2008). The follow up period was regarded as the time of standardized questionnaire.

Amenorrhea was characterized according to clinical presentation during the course of the study and was determined by the menstrual cycle presentation. Transient amenorrhea (TA) was defined as that which stopped during follow up while permanent amenorrhea (PA) was that present during observation and lasting for more than 6 months (Yen et al., 1999). Menopause was defined as PA with hormonal changes and confirmed with ultrasound (Clemons et al., 2007). PA and menopause were classified as CIA (Cocco et al., 2008)

Table 1: Menstrual alterations in 189 women with MS. Table shows results before, during and after MTX treatment

	Before		During		After	
	N	%	N	%	N	%
No abnormalities	142	75	92	49	70	37
Transient abnormalities	47	25	40	21	28	15
Transient amenorrhea	0	0	28	15	43	23
Permanent amenorrhea	0	0	23	12	18	10
Menopause	0	0	6	3	30	16

Table from Cocco et al., 2008

Result showed that MTX therapy may affect fertility in women with MS. Also, risk of CIA was directly related to age of patients during MTX treatment with a higher risk in women above the age of 35 years old (Cocco et al., 2008)

There are a decreasing number of viable ovarian follicles with increasing age (Wallace et al., 2005; Fornier et al., 2005; Walshe et al., 2006). There is evidence that older women are more prone to ovarian toxicity from chemotherapy (Fornier et al., 2005). The follicle that is mainly affected is the primordial follicles which represent the overall follicular pool. Older women therefore suffer loss both from natural follicular depletion and that caused by CIA. This will lead to reduced fertility and complications that could arise from pregnancy due to poor ovarian quality from MTX treatment. Premature ovarian follicle (POF) is also possible in adolescents, or women maintaining a regular menstrual cycle during MTX treatment. Normal menstruation in these patients does not guarantee fertility (Ostensen et al., 2006; Fornier et al., 2005; Walshe et al., 2006). However, pregnancy and conception is still possible in women suffering from CIA, although the chances are rare (Bath et al., 2004).

Available evidence supports that the prevention of infertility from CIA is very important to many women with MS. This is because CIA may lead to other conditions of psychosocial nature. Other complications that could arise include cardiovascular diseases, genitourinary dysfunction, osteoporosis, and hot flushes (Cocco et al., 2008). It is therefore of high importance to recognize, prevent, and treat CIA especially with women of reproductive age. Choosing the best treatment with the least side effects is one way to combat this problem. Another solution will be to find a co-therapy that has the potential to prevent CIA without affecting the efficacy of treatment. PACAP could be the answer to the latter.

D. Mechanism of protection by PACAP in MTX toxicity in the ovary

It is believed that PACAP prevents CIA by protecting the granulosa cells of the ovarian follicle. The granulosa cells are important cells which surround the oocyte and provide nourishment and support from the developing follicles to the mature egg. These cells are constantly dividing and increase from a single cell layer in the primordial follicle, to a multi cellular layer in the Graafian follicle. They are often the target of MTX cytotoxicity due to their rapid cellular division, and death to these cells will mean degeneration of the oocyte. Therefore, to evaluate the protective action of PACAP on the granulosa cells it is important to understand the ovary and the various stages of follicular development.

The ovaries produce PACAP and also have PACAP receptors, thus providing a good example for an autocrine and paracrine regulation. PACAP provides protection and help stimulate cell growth and development especially in the primordial follicles. It is an ovarian regulatory peptide. PACAP is also important in germ cell migration, follicular development, atresia, and meiotic division (Reglodi et al., 2012).

The various stages of follicular development are the primordial, the primary, the secondary (pre-antral and antral), and the mature Graafian follicles.

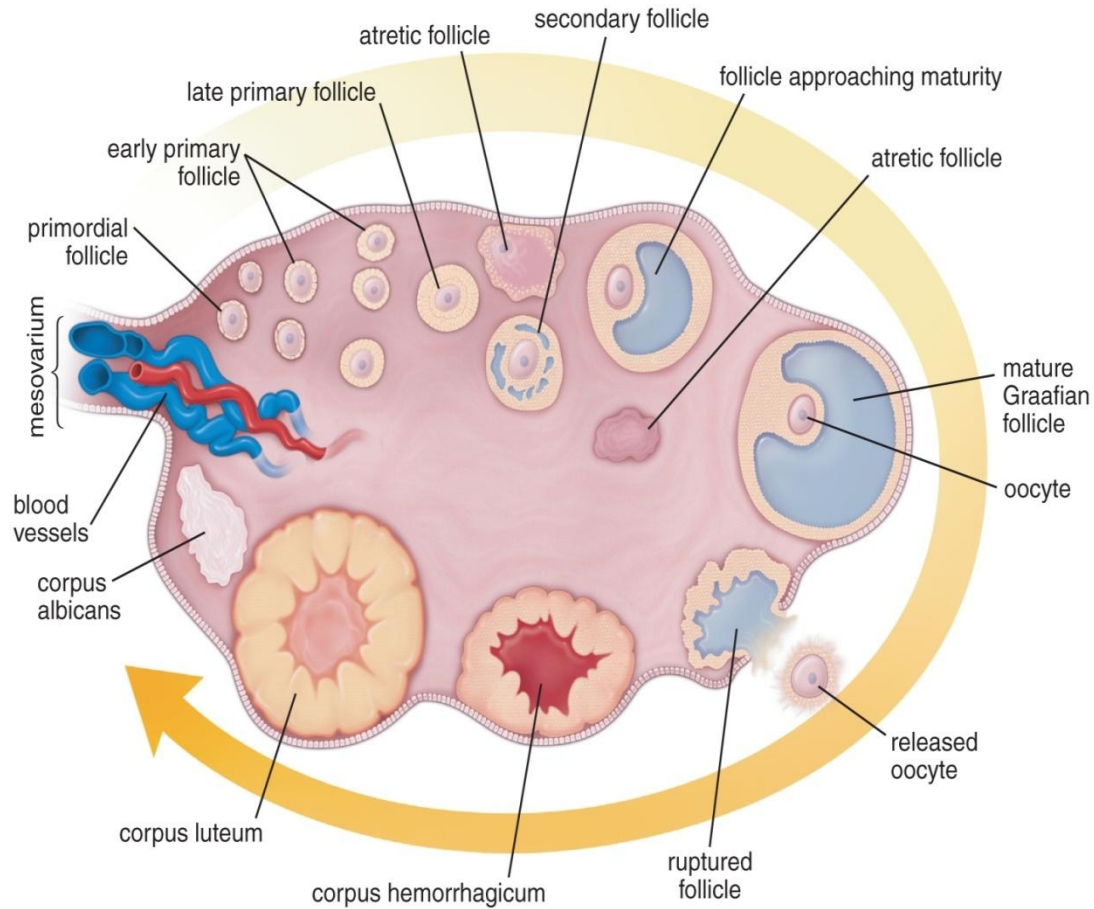


Figure 3: The ovary showing the various stages of follicular development. Each follicle begins as a primordial follicle and gradually matures to the Graafian follicle which is released as the egg. Each ovary is supplied with rich blood by blood vessels which nourish the cells. The granulosa cells surrounding the egg directly provide nourishment to the ova.

Ross et al., Histology: a Text and Atlas. 4th ed. 2003, Lippincott Williams & Wilkins

The ovaries hold about one to two million eggs during birth. Throughout life most of the eggs will die by atresia which begins at birth and ends at menopause. Only about 400,000 eggs remain at puberty and about 1000 are lost with each menstruation. The primordial follicles are the dormant subset which represents a woman's reserve. They represent the bulk that will mature to the primary, pre-antral and antral follicles. Any damage or reduction in the primordial follicles will affect the number of follicles that mature to the antral follicles. Therefore, long term preservation of these follicles is the solution to maintaining a woman's reserve and improving the stockpile.

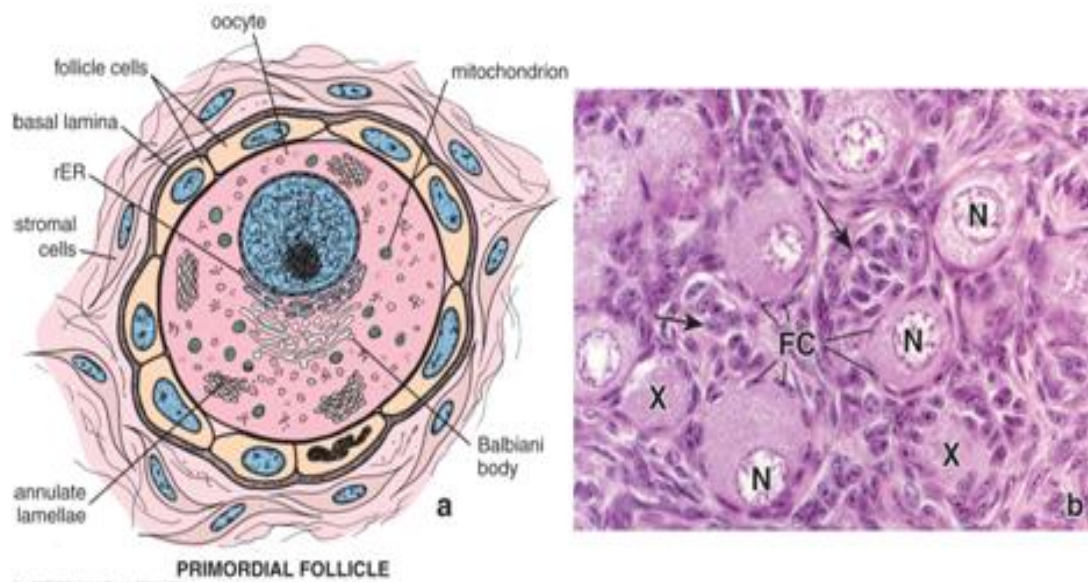


Figure 4: Primordial follicle. (a) is a schematic diagram showing a flat single layer of follicular cells (granulosa cells) just after the cytoplasm of the egg. (b) is a photomicrograph of primordial follicles showing the oocyte surrounded by single layer of flattened granulosa follicular cells (FC). The nucleus (N) is slightly eccentric, and X marks oocytes with missing nuclei. The arrow shows 2 follicles in which the granulosa cells are enclosed.

Ross et al., Histology: a Text and Atlas. 4th ed. 2003, Lippincott Williams & Wilkins

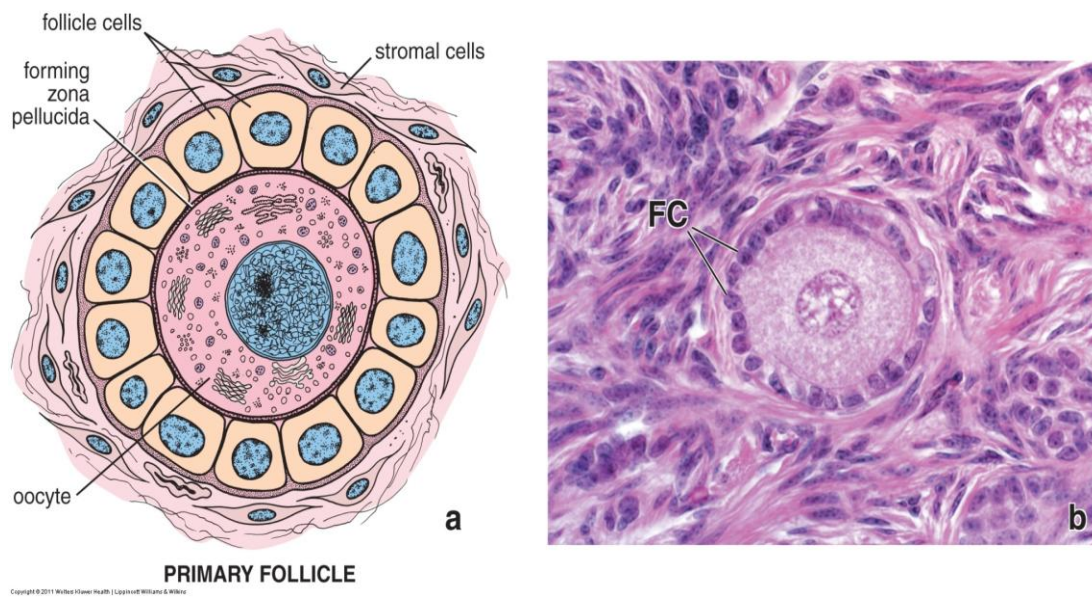


Figure 5: Primary follicle. (a) and (b) is a schematic diagram and a photomicrograph of a single primary follicle at an early stage of development. Notice the single cuboidal follicular cells (FC) and the early formation of the zona pellucida.

Ross et al., Histology: a Text and Atlas. 4th ed. 2003, Lippincott Williams & Wilkins

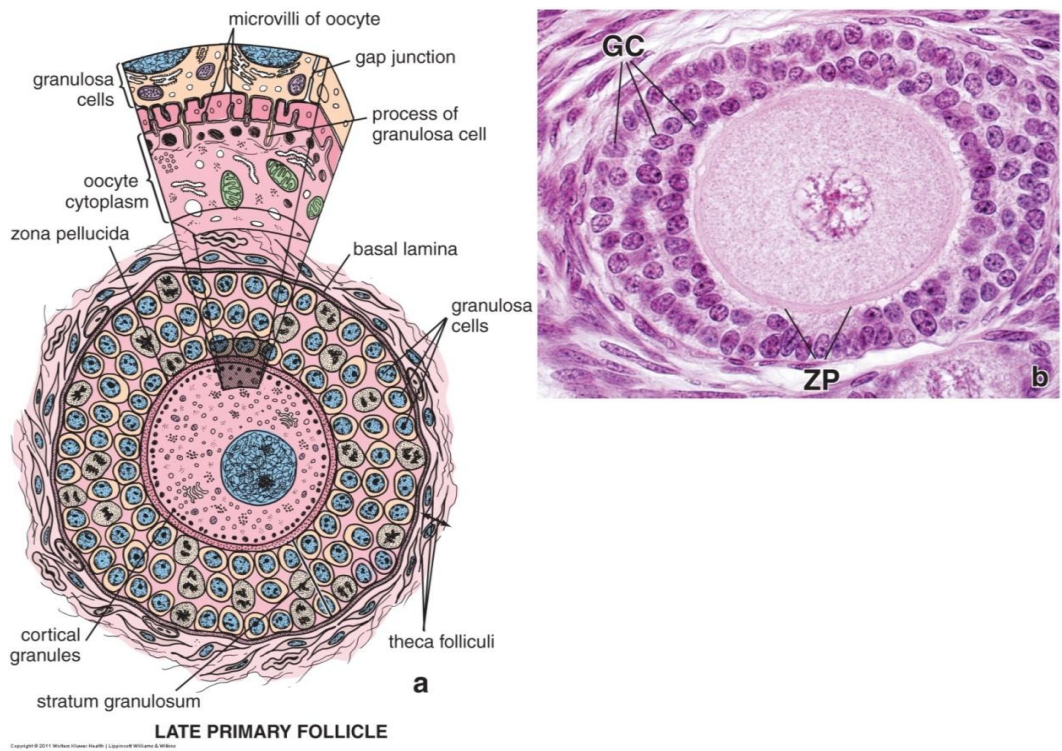


Figure 6: Late primary follicle. (a) and (b) is a schematic diagram and a photomicrograph of a late primary follicle showing the multilayered mass of granulosa cells (GC) surrounding the oocyte and the prominent zona pellucida (ZP).

Ross et al., Histology: a Text and Atlas. 4th ed. 2003, Lippincott Williams & Wilkins

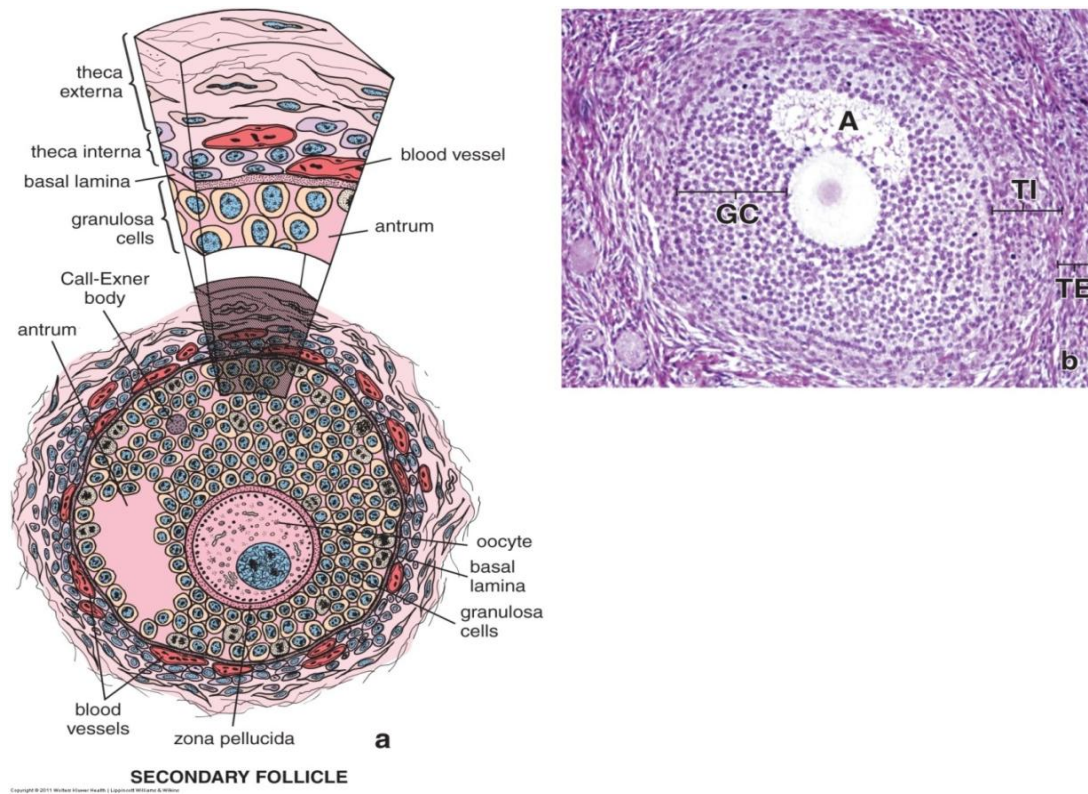
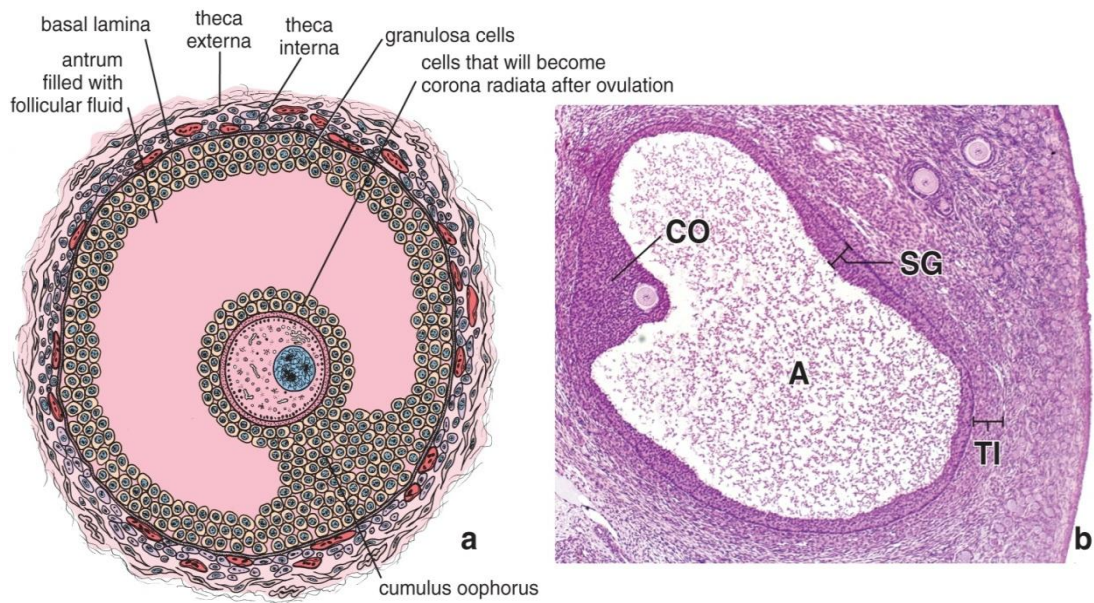


Figure 7: Secondary follicle. (a) and (b) is a schematic diagram and a photomicrograph of a secondary follicle. The antrum (A) is formed when the granulosa cells die due to condensation and poor nutrition. This leaves a space where some cells are closely attached to the oocyte while others are some distance away. The antrum continues to widen until the mature follicle is formed. TI is the theca internal, and TE is the theca external.

Ross et al., Histology: a Text and Atlas. 4th ed. 2003, Lippincott Williams & Wilkins



MATURE GRAAFIAN FOLLICLE

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Figure 8: Mature Graafian follicle. (a) and (b) is a schematic diagram and a photomicrograph of a mature Graafian follicle. Follicle has a large antrum containing an oocyte embedded within the cumulus oophorus. The antrum is filled with fluid and the egg is surrounded by layers of granulosa cells, now called the corona radiata, which remains with it after ovulation. SG is the stratum granulosum.

Ross et al., Histology: a Text and Atlas. 4th ed. 2003, Lippincott Williams & Wilkins

It is believed that CIA caused by MTX is the result of direct damage to the primordial follicles (Edan et al., 2004). Damage to these follicles is irreversible and cannot be cured even with the most sophisticated treatments currently available (Levi et al., 2001). The key of this research is to prove that MTX causes death of the follicles and that PACAP can improve fertility by protecting the granulosa cells and thus improve the chances of pregnancy in these women.

With all the side effects of MTX there is need to find an antidote that could offer some protection from cytotoxic damage. The cytoprotective functions of PACAP could be a useful factor as a co-therapy. An interesting fact is that most body cells, including the ovary, have PACAP receptors hence it is realistic that this combination maybe the key to improving the quality of life for MS patients. If this theory proves true the same hypothesis could be extended to pilot other similar studies utilizing other chemotherapeutic agents.

E. Preliminary data

Dr. Merchenthaler and his colleagues (University of Maryland Baltimore) in 2015 showed that PACAP protects the granulosa cells against MTX toxicity *in-vitro*. The experiment utilized rats that were divided into 3 groups; MTX, MTX + PACAP, and control. Immature rats were stimulated with pregnant mare's serum gonadotropin (PSMG) and granulosa cells were harvested. The cells were later treated with MTX for the MTX group, MTX and PACAP for MTX + PACAP group, and saline for control.

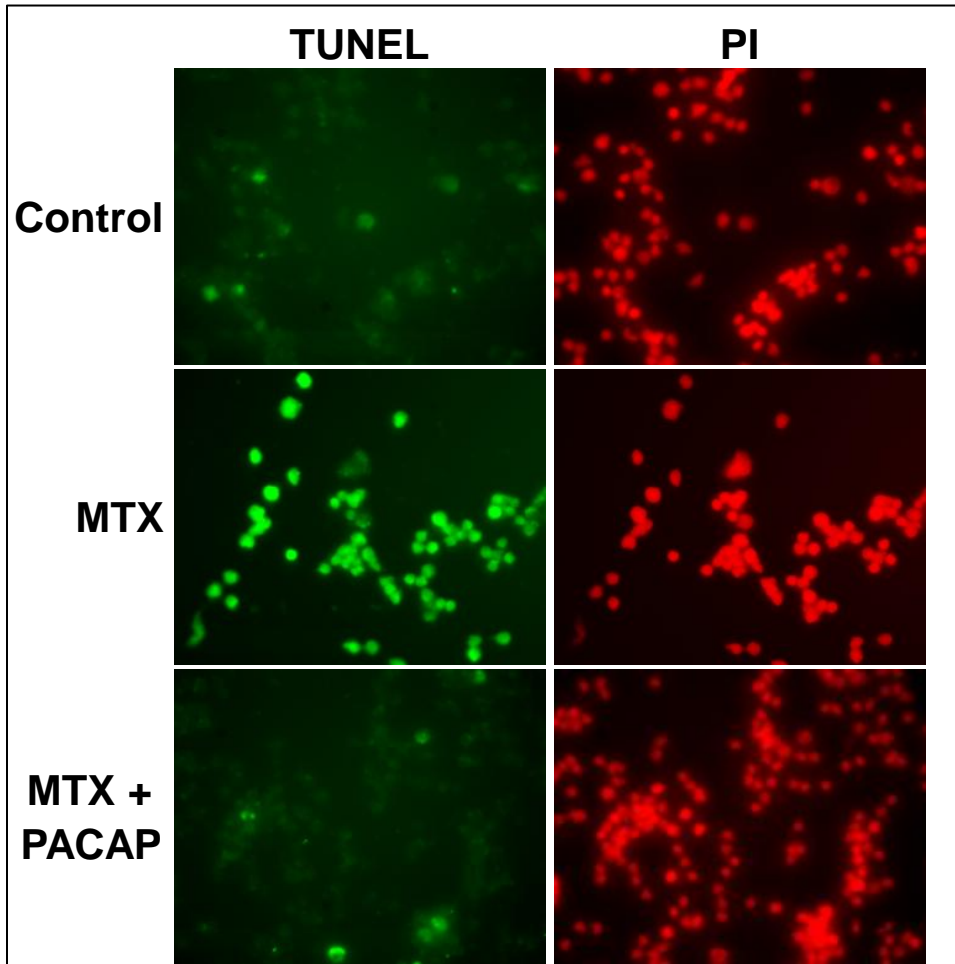


Figure 9: MTX induces and PACAP prevents granulosa cell apoptosis. Immature rats were stimulated with PMSG and granulosa cells harvested 48hrs later. Cells were cultured and incubated with MTX or MTX + PACAP for 24hrs at 37°C. Cells were pre-incubated for 1hr with PACAP before the addition of MTX. TUNEL label/apoptotic cells are green, and the propidium iodide (PI) counter stain is red.

Picture used with permission from Dr. Merchenthaler, University of Maryland Baltimore

Terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling (TUNEL) analysis showed significant staining of the granulosa cells in the MTX group when compared to the other 2 groups. Propidium iodide (PI) stain was used to confirm the presence of the granulosa cells. The Figure above shows a comparison between all three groups in the experiment. Apoptosis/DNA induced fragmentation is absent in the MTX + PACAP group when compared to the MTX group by itself. Therefore TUNEL stain is only positive in the MTX group. This experiment is important because it provides proof of principle on which this present study is built.

II. HYPOTHESIS

The progression of MS can be improved with MTX but MTX, among other side effects, causes infertility. We hypothesize that PACAP could prevent the side effects of MTX on the ovary. The hypothesis is based on observation indicating that the side effects of MTX can be protected in the heart, liver, and kidney by PACAP (Dr. J. Maderdrut, Tulane University; personal communication). If PACAP would provide the expected protection in the ovary, it is very likely that it could protect against the side effects of other chemotherapeutic agents.

The overall hypothesis of this research is that PACAP acting, via PACAP receptors, will reduce the reproductive toxicity of MTX by protecting granulosa cells of the ovarian follicles from cell death.

III. SPECIFIC AIMS

Aim 1; To establish a concentration of MTX that is cytotoxic to mice without killing not more than 10% of the experimental group.

Approach; mice were injected weekly with 2 or 3 mg/kg i.p. of MTX, and then they were inspected daily for any visible side effects. These doses are comparable with those used in women (60 - 150 mg/m²). Also, weights were monitored and any animal in distress was isolated. At least 90% of the MTX treated mice survived the treatment regime.

The doses were chosen based on similar studies from other research using animal models (Weibach et al., 2004; Piao et al., 2007) which provided preliminary data that support the toxicity of MTX on granulosa cell cytotoxicity *in-vivo*.

Aim 2: To demonstrate that 10 ug/mouse of PACAP can protect ovarian follicles against the harmful effects of MTX on mice.

Approach; MTX-treated mice were injected with 10 ug of PACAP 1hr before MTX and then 24 and 48hrs thereafter. The dose of PACAP was based on data from other groups (Mori et al., 2010; Khan et al., 2013; Li et al., 2008; Ji et al., 2013; Li et al., 2006; Kato et al., 2004). The treatment regimen was repeated for four weeks while the conditions of the animals were monitored. After euthanasia, the ovaries were collected and processed for histological evaluation including hematoxylin and eosin (H&E) staining, and staining for apoptotic markers (caspase-3 and TUNEL).

IV. MATERIALS AND METHODS

A. Materials, methods and study design

The research animal was mice. A total number of 27 female mice (two experiments with two different doses of MTX) that are known to have a regular reproductive estrous cycle were randomly assigned into 3 groups. Group 1 received only MTX, group 2 received MTX and PACAP (MTX + PACAP), while group 3 was the control group and received saline. Experiment I utilized only two groups; group 1 and group 3, while experiment 2 utilized all three groups. Mice were treated with 2 mg/kg of MTX for experiment 1 while 3 mg/kg of MTX was used for experiment 2, PACAP38 10 ug/mouse, and normal saline for the control group. The weights of the animals were also recorded on alternate days to monitor the effect of the treatment and easily identify any mice in distress.

Table 2: Experimental groups and number of mice. Table shows the two different experiments with the treatments used. Experiment 1 used 2 mg/kg while experiment 2 utilized 3 mg/kg of MTX per mouse

Group	Experiment 1	Experiment 2
MTX	5	5
MTX + PACAP	0	5
Control	6	6
Total	11	16

Treatments were administered by intra-peritoneal injections up to four weeks, and mice were closely observed for any physical abnormality like hair loss, tumor, inactivity, and change in eye color. 24hrs after the last day of treatment, all mice were euthanized and their bodies perfused with paraformaldehyde. The ovaries, kidney, and liver were dissected out, collected in 4% paraformaldehyde and later embedded in paraffin. For the current study, only the ovaries were processed for histological evaluations including H&E staining, staining for apoptosis marker (caspase-3), and TUNEL.

Treatment paradigm for a single week					
Treatment:	PACAP (P)	MTX	P	P	P
Time (hrs)	-1	0	12	24	48

Figure 10: Treatment paradigm. MTX group received 2 or 3 mg/kg of MTX per mouse once weekly. MTX + PACAP group was treated with 10 ug/mouse of PACAP 1 hr before MTX treatment, and then again after 24hrs and 48hrs weekly. Control received saline. This treatment was repeated weekly for 4 weeks.

Mice were purchased from Jackson Laboratories. The mice strain selected for this research was 2 months old BALB/c strain because it is well suited for both cancer and immunological work and our collaborators at Tulane University used this strain in their studies. Animals were properly housed and all basic living requirements, including diet

and water, were provided. MTX was purchased from Sigma Laboratories while the human variant of PACAP38 was obtained from Tulane University.

The number of mice per cage was not more than five. This is to meet the regulation of Institutional Animal Care and Use Committee (IAUCUC). Daily observation and administration of treatment was done under a certified biohazard cabinet. PACAP, MTX and saline were injected intra-peritoneally without any anesthesia by using insulin syringes and needles. The weight of each mouse was taken and monitored to identify any animal in critical health.

Following euthanization, animals were perfused in 1% followed by 4% paraformaldehyde before being embedded in paraffin wax. Tissue sections were cut with a rotary microtome and collected in microscopic slides for staining. Ovary sections were analyzed using TUNEL staining, Caspase-3 assay, and H&E stain. TUNEL staining cocktail mixture, and Caspase-3 assay (#9661; #D-1775;D3RG9; and 8G10) were purchased from Cell signaling Company.

B. Methods

B.1. Caspase-3 immunohistochemistry

Caspase-3 belongs to the cysteine-aspartic acid protease, otherwise known as the caspase family. Caspase-3 is encoded by the caspase-3 gene and is present as a pro-enzyme which undergoes proteolysis and dimerize to produce the active enzyme. Its active site consists of cysteine and histidine residuals. These residuals are the key to its high specificity and function.

Caspase-3 is very important for the apoptotic process and normal brain development. Its role in cell death is characterized by chromatin condensation and DNA fragmentation in all cell types. Among other caspases, caspase-3 plays a dominant role in apoptosis and cleaves caspases 6 and 7, and is required for the normal function of caspases 8 and 9. Two apoptotic pathways have been identified with caspase-3 action. The intrinsic activation utilizes cytochrome c from the mitochondria, together with caspase 9, apoptotic-activating factor 1 (Apaf-1), and adenosine triphosphate (ATP) to activate caspase-3. The extrinsic pathway is signaled by granzyme B which activate caspases and triggers the apoptotic cascade of caspase-3. The caspase-3 zymogen is inactive until it is activated by either of the two pathways. High levels of fragmented caspase-3 in the blood or tissue is an indication of infarction or tissue damage from apoptosis.

In the activated caspase-3 immunohistochemistry assay, specific antibodies to caspase-3 and caspase cleaved cytokeratin were used. The primary antibodies, rabbit anti-cleaved caspase-3 antibodies, were used at 1:100 dilution and sections incubated in a humid chamber. Following wash in phosphate-buffered saline (PBS), the sections were incubated with secondary antibodies (donkey anti-rabbit IgG conjugated with biotin) for 1hr at room temperature (RT). After washing the tissues with PBS, the sections were incubated with peroxidase-conjugated streptavidin (1:500, 1hr at RT). Sections were again washed in PBS and then treated with diaminobenzidine (DAB) containing hydrogen peroxide. The antigenic sites were expected to be labeled with brown DAB precipitate.

B.2. TUNEL staining

TUNEL means terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling. It is employed to identify DNA fragmentation from apoptotic cells. It is similar to caspase-3 immunocytochemistry but less sensitive. The first stage was dewaxing and rehydration of tissue in a series of xylene and alcohol. The tissue sections were later treated in a proteinase digestion for permeability of the cells. The sections were next stained using the TUNEL cocktail and incubated in a humidifier for an hour at 37⁰C in the dark. The final step was counterstaining with 4',6-diamidino-2-phenylindole (DAPI) using fluorescent staining procedure. Microscopy was done using blue light excitation, with wavelength 450 – 500 nm, to view the green fluorescein emission.

B.3. Hematoxylin and eosin Staining (H&E)

This is a very popular histological stain used to assess structural arrangement and tissue damage. It has been used since the 1970's and is still a very relevant stain till date. The major stain is Harris's hematoxylin which contains hemalum that stains cell nuclei blue. The counter stain is eosin Y which stains eosinophilic structures as pink, red or orange. The staining process is quite simple and only requires dipping slides into stain for a few minutes followed by water.

H&E is excellent for embedded tissues and cytological specimen. It clearly outlines cellular architecture and abnormalities/malignancies can easily be visualized by a microscope after staining. It is most ideal for cancer studies where cell morphology is paramount for identification. The quality of staining is directly related to the quality of the tissue and the embedding process hence, it is advisable to always handle sections for

H&E with care. Also, hematoxylin should be stored in the dark to prevent the continuous oxidation to hematein.

All embedded tissues were first cut and sections collected in microscopic slides. The next step was to dewax/hydrate sections before staining. Microscopic sections were first dipped in a jar of hematoxylin for 5 mins. After rinsing in water, slides were then placed in another jar containing eosin stain for 3 mins. Slides were washed with water and dried prior to microscopy.

V. RESULTS

A. Demographics

During the entire study period, all mice were closely monitored for any sign of distress due to MTX toxicity. 1 death was recorded from the total number of 27 mice (3.7%). The dead mouse was from the MTX group bringing the death percentage in all 15 MTX treated mice to (6.6%). The survival rate of all mice in the 3 groups was 96.2%. Apart from the dead mice no other animal showed any physical sign of distress.

Following euthanasia, and perfusion, all ovaries appeared normal and showed no necrosis or trauma. 3 mice in the MTX group and 1 mouse in the MTX + PACAP group showed significant white necrotic patches in the heart.

B. Experiment 1

B.1 TUNEL staining

In experiment 1, TUNEL staining was used to determine DNA damage of the granulosa cells in microscopic sections of the ovary. The stain was well tolerated by the tissue sections. The saline control group did not show any significant TUNEL staining and hence was negative. All mice (100%) in the MTX group were TUNEL positive evident from the staining shown below.

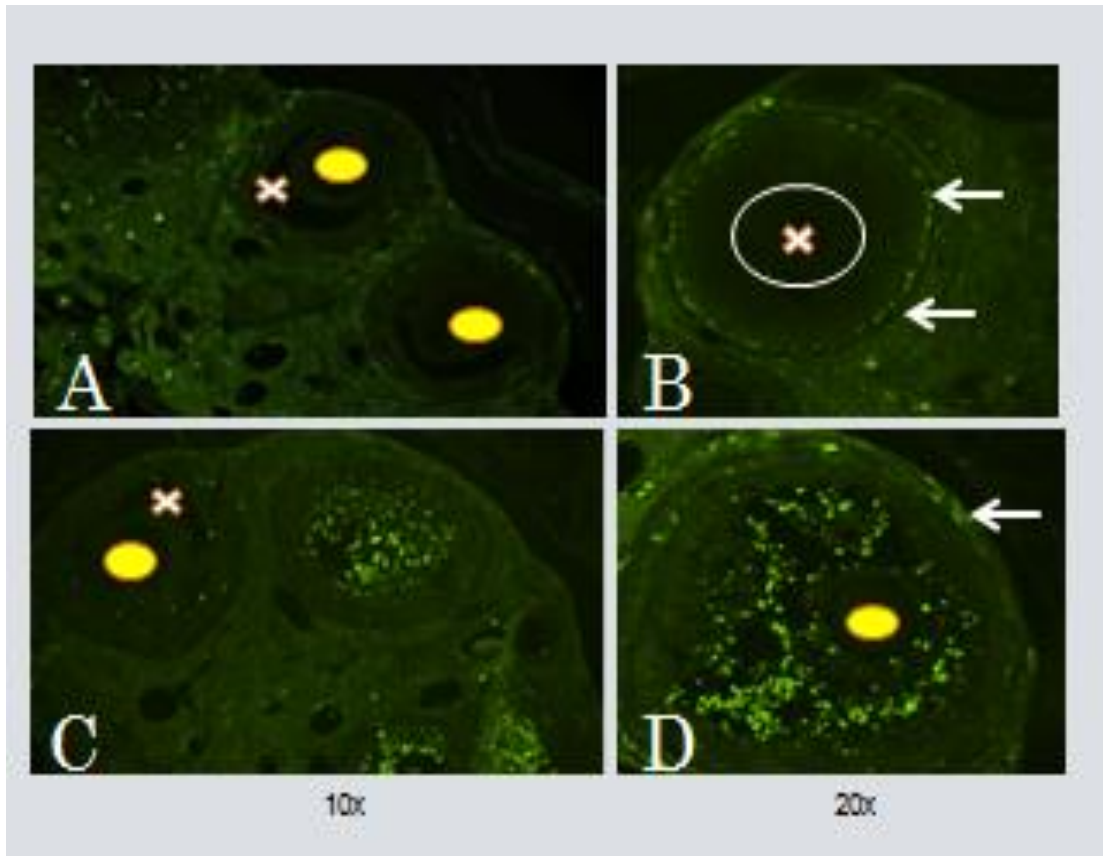


Figure 11: TUNEL labeling in the ovaries for experiment 1. Picture shows control (A,B) and MTX-treated (C,D) mice with two different magnifications. DNA fragmented granulosa cells are present primarily in antral follicles of MTX-treated mice. Only a few theca cells are stained with this marker (white arrows). The number of TUNEL-positive granulosa cells is much higher in MTX-treated than control mice. The eggs are indicated by yellow circle and the antrum of the follicles with a white cross.

B.2. Follicular count

Table 3: The follicular count. Table shows the approximated number of the primordial, primary, pre-antral, and antral follicles of the ovary. MTX group shows marked reduction in all follicular type compared to control due to cytotoxicity and cell death.

Follicle	Control	MTX	Difference	% Difference
Primordial	2000	1250	750	23
Primary	750	500	250	20
Pre-antral	200	100	100	33
Antral	350	200	150	27

The follicular count of the ovary at the different stages of follicular development was performed on H&E-stained, 10 um thick paraffin sections. The results clearly showed a significant reduction in the MTX group compared to the control group in all follicular stages; primordial, primary, pre-antral, and antral follicles.

From the table above, the highest count difference of 750 was seen in the primordial follicle while the highest percentage difference (33%) was observed in the pre-antral follicular count.

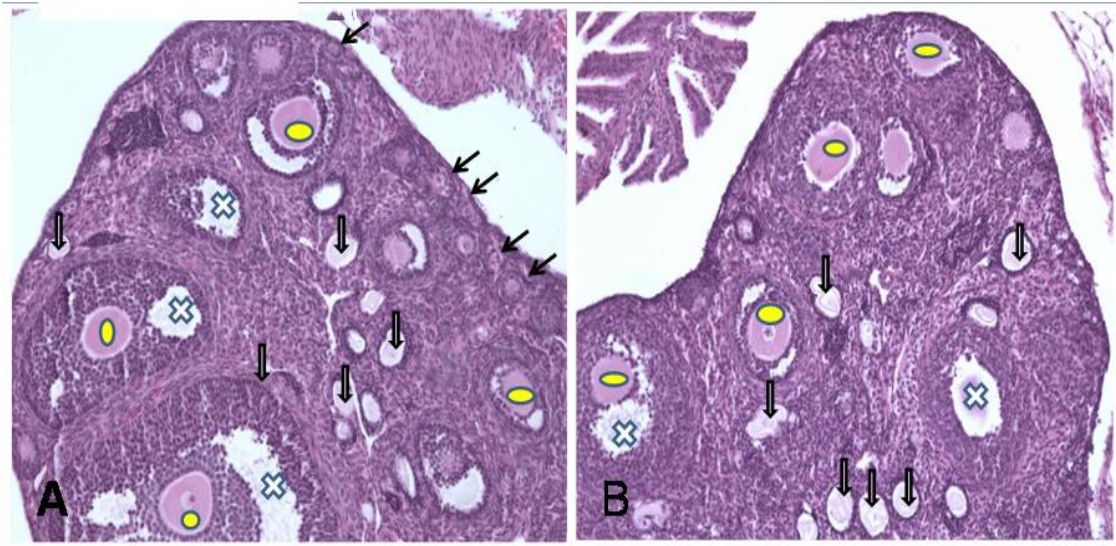


Figure 12: H&E stained paraffin sections. Result from intact (A) and MTX-treated (B) mice. MTX depletes primordial follicles which are present under the capsule of the ovary in intact animals (solid black arrows in A). Black arrows with white center label atretic eggs and/or follicles, yellow discs indicate the eggs, and X shows the antrum of a follicle.

The picture above shows the H&E staining of the capsule of an ovary section for both groups. The primordial follicles are predominately distributed at the base of the capsule and are usually the most abundant of all the follicular types. Staining reveals a more even distribution of these follicles in the control group but an absence (reduced number) in the MTX treated group. The bar chart shown below gives a more descriptive comparison of the follicular count.

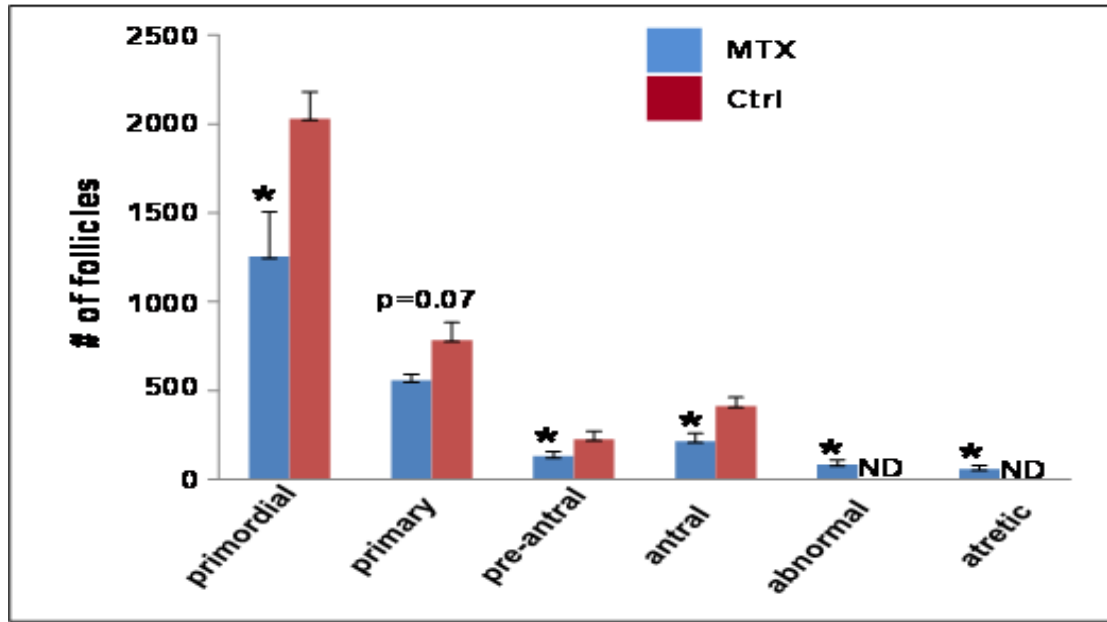


Figure 13: Follicle numbers at different stages of follicular development. Results are a comparison between intact control versus MTX-treated mice. Ovaries were serially sectioned and follicles counted in every 10th section. MTX affects ovarian reserve by reducing the number of primordial follicles and, to a lesser extent, the number of antral follicles. * = significantly different from controls (t-test, n=3, 8). ND = not detected.

These results are consistent with the hypothesis that MTX reduces the follicular count, particularly of the primordial follicles. The p-value was significant in each stage of the follicle except for the primary follicles where the p-value was 0.07. No abnormal and atretic follicles were detected in the control group.

C. Experiment 2

C.1 TUNEL staining

Three experimental groups were used here. TUNEL staining was well tolerated by the tissue sections. The saline control group did not show any significant TUNEL staining

and hence was negative. The MTX group showed the most staining relative to the MTX+PACAP group. All mice (100%) in the MTX group were TUNEL positive. In the MTX + PACAP group, all mice (100%) were TUNEL negative and only showed few insignificantly staining.

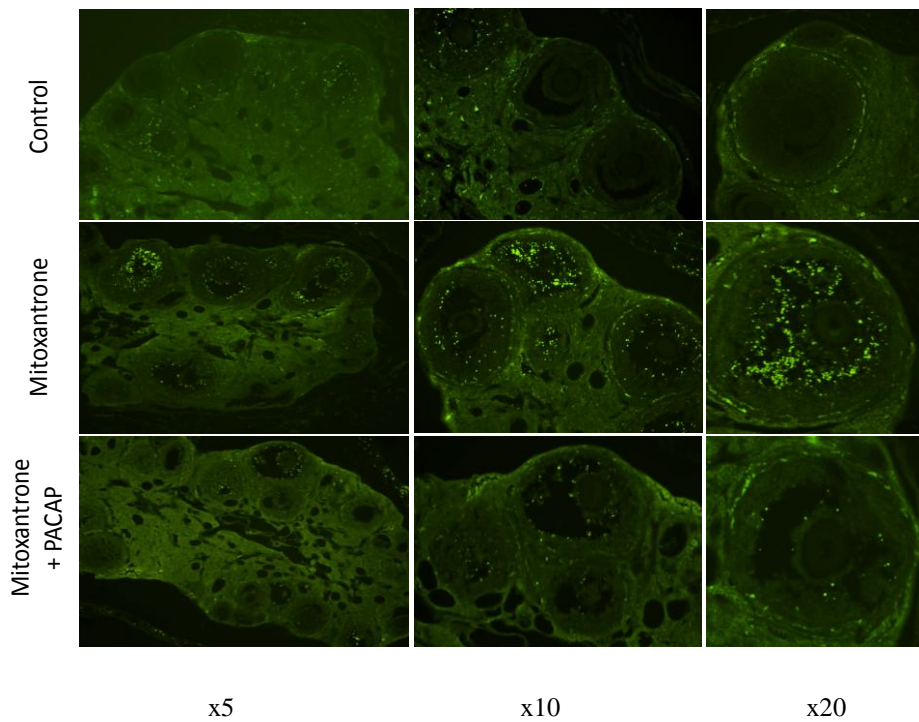


Figure 14: TUNEL staining of mouse ovary in experiment 2. Picture compares the three groups at three different magnifications. Notice the marked granulosa cell staining in the MTX group compared to MTX + PACAP, and Control. PACAP significantly reduced MTX cytotoxicity in granulosa cells of ovarian follicle

D. Caspase-3 staining

Four different caspase-3 antisera were used to stain sections of ovary of all three experimental groups; MTX, MTX + PACAP, and Control. All sections stained negative for caspase-3 for all three experimental groups in the two experiments conducted. Microscopic examination of the sections revealed normal tissue architecture. The quality and potency of the caspase-3 antisera were tested against known positive control sections of apoptotic cells (retina of an animal exposed to optic nerve damage). Only one of the four antisera showed a positive staining in these positive control sections.

VI. DISCUSSION

The preliminary data generated by Dr. Merchenthaler and colleagues at University of Maryland Baltimore shows evidence that PACAP provides *in-vitro* protection against DNA damage from MTX treatment. The result of this study is similar, only that the focus of this research is to access *in-vivo* protection from MTX toxicity.

PACAP has been shown to provide protection from apoptosis and DNA damage in many organs but little is known about protection of the ovary especially at the level of the granulosa cells. Therefore, the choice of the ovary in this study is quite novel and important especially to woman of reproductive age suffering from MS and on MTX therapy. The observed results in this study will prove the positive role PACAP plays as a cellular protector that can be used as a co-therapy with MTX treatment.

In the observed results, granulosa cells were TUNEL positive in the MTX group. This is evidence that MTX causes DNA damage of the granulosa cells. Significant protection from DNA damage was observed in the MTX + PACAP group (negative TUNEL) compared to the MTX group by itself. However, the Caspase-3 staining, which is a strong indicator of cellular apoptosis, was negative. It should be emphasized however, that the negative staining with the four antisera against active caspase-3 does not mean that the granulosa cells did not have this enzyme. The failure to achieve positive staining with them might be due to technical issues (e.g., the antisera did not recognize the antigen whose tertiary structure was modified by fixative, or the antisera did not recognize the mouse sequence of caspase-3, etc.).

We can only conclude here that the cellular damage that was observed in this study was probably due to DNA fragmentation and not apoptosis. Even though other studies have observed apoptosis due to MTX toxicity in other tissues, the same did not apply to the granulosa cells tested here.

The follicular count of the ovary revealed a significant reduction in the MTX group compared to the control in all follicular types. This supports other similar work which concludes that MTX causes a reduction in the ovarian follicular number and causes CIA (Cocco et al., 2008; Wallace et al., 2005, Fornier et al., 2005, Walshe et al., 2006). The progressive reduction in the ovarian count (from the primordial to the antral follicle) show a trend which will ultimately lead to infertility from MTX cytotoxicity.

Our goal in Aim 1 was to establish a toxic dose of MTX which kills less than 10% of the animals. The dose of 2 and 3 ug/kg mice of MTX proved to be toxic at the level of the granulosa cells of the ovarian follicle. The ovary sections of the MTX group showed significant TUNEL staining to prove DNA damage. This agrees with other studies were similar concentration was used (Weibach et al., 2004; Piao et al., 2007).

The follicular count is also proof of MTX concentration toxicity directly on the ovarian follicle. The count, which was higher in the control group compared to the MTX group, is important because MTX causes CIA by reducing the follicular count. The reduced count will translate into infertility from reduced quality and quantity of ovary. The fact that the dose of MTX did not kill more than 10% of the experimental group is important to recommend as an acceptable treatment regime in mice.

The limitation of this work is failure to predict how this dose will translate to humans and if this dose regime will also apply to other animal models. Also, failure to establish dose response curves of various concentrations of MTX so that a better comparison on how dosage affects treatment can be established.

Our goal in Aim 2 was to provide evidence for protection by PACAP against MTX toxicosis. In experiment 2, we showed that PACAP at 10 ug/mouse protected against cytotoxic cell destruction from MTX. The MTX + PACAP group clearly showed reduced TUNEL staining compared to the MTX group. This is very important because it directly relates to the hypothesis behind this study.

Another limitation is that the follicular count was not done in experiment 2 to compare how the MTX + PACAP group will differ from the MTX, and control groups. We cannot conclude that PACAP, when combined with MTX, will improve the follicular count but can only suggest so from the results.

The overall limitation of this study was the limited number of mice used for this experiment. We therefore suggest a more comprehensive study utilizing a larger sample size to obtain a more analytic data. Also, a blind study to eliminate any bias is the best alternative to a more reliable data and result interpretation.

The application of PACAP in the management of many diseases is fascinating. The limitation of its use is the short half-life of only about 3-5 minutes. This study used the endogenous PACAP38 which has a short half-life. Future studies will use metabolically stable PACAP analogs with a half-life of 2-4 hours, and analogs which are receptor subtype-specific.

In addition, the protective effect of PACAP analogs will be expanded to other chemotherapeutic agents. In these future studies the protective effects of PACAP analogs will also be expanded to functional outcomes including estrous cyclicity and fertility.

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