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PRINCIPAL INVESTIGATOR: Thomas Register, Ph.D.

CONTRACTING ORGANIZATION: Bowman Gray School of Medicine
Winston-Salem, North Carolina 27157

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Thomas Register, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Bowman Gray School of Medicine
Winston-Salem, North Carolina 27157
E-MAIL:
register@wfubmc.edu

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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of the proposed studies was to determine the role that hypoandrogenemia plays in the effects of oral contraceptives (OC) on bone metabolism and peak bone mass (PBM) in young female rats. Intact, adolescent/young adult Sprague-Dawley rats were treated with 1) placebo, 2) OC therapy, 3) OC supplemented with an androgen (methyltestosterone), or 4) anti-androgen therapy (bicalutamide) to determine the potential role that suppression of androgens plays on bone metabolism, bone architecture, and the attainment of PBM. Our specific aims were to determine:

1. If oral contraceptive steroid (OC) use leads to decreased peak bone mass in young intact female rats. *Findings: OC use decreased the peak bone mass of young intact female rats.*
2. If the addition of a non-aromatizable androgenic steroid to OCs prevents the detrimental effects of OC use on peak bone mass. *Findings: The non-aromatizable androgenic steroid did not prevent the adverse effects of OCs to the growing skeleton of young rats at the dose used.*
3. If the effects of OC use on peak bone mass are equivalent to the effects caused by anti-androgen use. Findings: *The anti-androgen used did not mimic the adverse effect of OCs on the growing skeleton of young rats.*

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Thomas C. Reynolds 9/21/99

PI - Signature Date

Table of Contents

INTRODUCTION

The central hypothesis underlying the proposed study was that oral contraceptive (OC) treatment of adolescent and young adult females causes an abnormal depression of circulating androgens which results in a depression of bone gain during this critical period. The end result may be a reduction in peak bone mass and an increased risk of stress fractures and osteoporosis. Similar results might be observed by suppression of androgen activity in intact animals in the absence of OC therapy. Conversely, supplementation of OC-treated females with an androgen may result in restoration of normal bone maturation. The overall goal of the proposed study was to determine the role that hypoandrogenemia plays in the effects of OC on bone metabolism and on peak bone mass in young female rats. For these studies, we used Sprague-Dawley rats, a well-characterized animal model of ovarian hormone effects on bone metabolism. These animals were examined while in the adolescent and young adult age range. We treated intact animals with 1) Placebo, 2) OC therapy, 3) OC supplemented with an androgen (methyltestosterone), or 4) Anti-androgen therapy (bicalutamide) to determine the potential role that suppression of androgens plays on bone metabolism, bone architecture, and the attainment of PBM.

BODY

Statement of Work Conducted During the First Year

Year 01

Month 1-3 *Sept 1, 1998 to Nov 1, 1998*

Task 1

- We accepted Dr. Erni Sulistiawati as an Indonesian D.V.M. who is a Ph.D. candidate enrolled at the Institut Pertanian Bogor (IPB). She started in October 1, 1998. All graduate school arrangements were arranged via telephone and E-mail with Dr. Dondin Sajuthi who acts as Dr. Sulistiawati's mentor in Indonesia.

- As a post-doctoral fellow under Dr. Jayo's mentorship, Dr. Uriel Blas-Machado was included in the project as of September 1, 1998. Dr. Blas-Machado's salary was supported by a Training Grant from the NCRR, NIH, held by Dr. Jayo

- For technical assistance, Mrs. Pam Louderback and Mr. Sam E. Rankin were hired.

- We arranged with the Wake Forest University Animal Resources Program (Ms. Vickie Hardy and Patricia Wood, and Dr. Jan Wagner) the acquisition of rats and proper housing. Due to quarantine issues in building 10 and the occupation of rooms by primates to be sent to Dr. O'Sullivan this process had to be coordinated and monitored closely. Pilot rats (n=7) were ordered to be received in October. Study rats (n=65) were ordered to be received in November.

- The availability and scheduling of the Hologic® DEXA scanner in building 27 was discussed and the dates proposed. On October 9, 1998, a memo was sent to Drs. Brommage, Hotchkiss, and Lees for the dates to use the DEXA scanner for pilot and final studies. Seven rats (10% of total approved by the institutional Animal Care and Use Committee [ACUC]) were received to conduct the pilot project (TABLE 1). This project allowed us to test the palatability and the feasibility of procedures (sedation, bleeding, densitometry, etc) to be conducted in the live animals.

TABLE 1. PILOT PROJECT

Exp Time	*Week*	*Age (days)*	*Comment*	*Date*
-1	1	63		26-Oct-98
Start diet	0	70	DXA 1	02-Nov-98
1	3	77		09-Nov-98
2	4	84	DXA 2	16-Nov-98

Based on our previous work with non-human primates (Register et al., 1997), the food consumption and body weight gains during the pilot project, no additional palatability issues were considered and the go ahead for the proposed experiment was given.

- As part of the annual review, on October 19, 1998, the ACUC Protocol A97-147 was approved for extension until October 20, 1999.

- In coordination with the Wake Forest University School of Medicine and the Baptist Hospital pharmacies, we obtained the schedule III drug Android® (ICN Pharmaceuticals Inc, methyltestosterone), and the prescription drugs Casodex® (Zeneca Pharmaceuticals, bicalutamide) and Levlen® (Berlex Laboratories, levonorgestrel and ethinyl estradiol).

- In coordination with Ms. Diane Wood and Kyrun Martin we started preparing the specialized diets. Ms. Wood and K. Martin were made aware of experimental rationale and of the fact that these drugs are to handled carefully since these substances could penetrate and be absorbed thru the skin.

- We ordered supplies (microscopic slides, pipettes, stains, etc).

- We arranged, with in-house Data Management System, data storage bank accounts.

- We purchased three computers using other sources of funding (Dr. Jayo's unrestricted funding) for PI, co-investigator (Dr. Register), and fellow (Dr. Blas-Machado). Provided graduate student and staff with other computers.

- Weekly meetings were scheduled with the staff and students.

Month 4 *Nov 1, 1998 to Dec 1, 1998*

Task 2

- We received the animals for the start of the proposed experiment on November 16, 1998. However, 54 animals finished the project. The loses (mortality) were due to high ambient temperature during recovery phase of sedation. The animals which finished the experiment had an average body weight of 131.98± 0.92 (mean ± sem) on November 17, 1998.

- Due to DEXA scheduling we did **not** have to train feed or reverse room light 12-hour cycle (day to night).

- Daily weighing and recording of data. Conducted daily from November 17th to 30th.

- Daily vaginal cytology were conducted daily at weighing and feeding.

- Semipurified food (with hormones) was prepared (Table 2) and keep frozen until ready to use. Once open, it was kept refrigerated.

Table 2. Semi-purified diet, designed to contain no isoflavones. Each 100 g of semi-purified high-fat diet contained the following products.

Food	***(g)***
Casein, USP	10.5
Lactalbumin	10.0
Dextrin	30.6
Sucrose	28.0
Alphacel	10.0
Lard	5.20
Safflower Oil (linoleic)	1.00
Choline Bitartrate	0.20
Vitamin Mixture, AIN-76A	1.00
Mineral Mix, AIN-76	3.50

- On Nov 17, 1998, we responded via fax and mail to a question asked by Major Ruble who is Chief, Animal Care and Use Review Division in Fort Dietrick (with respect to animal numbers that had been used). A copy of our most recent USDA inspection report were provided.

- On Nov 18, 1998 ACUC approved amendments for the ACUC Protocol No. A97-147.

Month 5 *Dec 1, 1998 to Dec 31, 1998*

Task 3

- Daily weighing and recording of data continued.
- Data to calculate parameters for randomization was entered in tabulation form.
- We divided rats into groups and start treatment
- Daily vaginal cytology was stopped.
- Baseline serum was collected.
- First and second DEXA scans were done. Sample 1 on December 1-4, 1998 and Sample 2 December 15-18, 1998. Both samples were baseline samples to provide evidence of growth. The group equivalency and randomization was conducted based on both body weight rate of change and bone density rate of change. Treatment groups were assigned using random group assignment and were separated by diet color, Group 1 (blue, oral contraceptive), Group 2 (vanilla, control), Group 3 (green, oral contraceptive plus methyltestosterone), and Group 4 (red, Cas).
- On December 18, 1998 a meeting was held in which a discussion of the experimental and technical staff coordinating responsibilities since holidays were upon us. As a group we discussed with Ms. Vickie Hardy that our group was in charge of all daily monitoring and feeding. The Animal Resource Program caretaking staff was to sweep the floors daily, and change the bedding twice a week. Weekend schedules were coordinated among ourselves due to vacations and holidays. Due to the fact that the diets contained the steroid and anti-steroid treatment, we discussed with Ms. Hardy the fact that the caretaker staff should be careful and aware of the fact that these substances could penetrate and be absorbed thru the skin. Therefore, for their own protection, gloves were to be worn always, and different gloves were to be used with each colored treatment group to prevent cross-contamination.
- On December 21st, 1998 the experimental diets containing the steroids were given for the first time to the animals.

Month 6 Jan 1 - Jan 31, 1999

Task 4

- Daily weighing and recording, and diet was made routinely.
- In contrast to our rat pilot information and previous monkey data, the rats were not eating as expected (Table 3). After review, feed was to be produced every other week to maintain palatability. The differences in total consumption were dramatic, on average 3 to 4 g of food per day were not consumed by the OC and OC+MT groups (Table 3).

Table 3. Average (AVE, g) feed consumption per day during the experiment

Group	AVE ± SD
Control	20.02 ± 1.94
OC	16.79 ± 4.80
OC+MT	17.09 ± 5.16
Cas	19.35 ± 2.09

The average feed consumption varied with the contraceptive schedule (3 days on and 1 day off, to mimic a woman's pill cycle) as shown in Table 4:

Table 4. Average (g) feed consumption per day-cycle

Group	Day 1	Day 2	Day 3	Day 4 (*NO STEROIDS*)
Control	19.71	20.18	19.79	20.42
OC	12.49	15.03	15.39	24.29
OC+MT	11.96	15.41	16.46	24.53
Cas	18.91	19.47	18.99	20.03

- Third DEXA scan (3 weeks after initiation of treatment) and serum sample were obtained.

Month 7 Feb 1 - Feb 28, 1999

Task 5

- Daily weighing and recording continued. Diet preparation and feeding was continued.
- 4th DEXA scan (6 week) and collection of serum.

Month 8 Mar 1 - Mar 31, 1999

Task 6

- Daily weighing and recording continued. Diet preparation and feeding was continued.
- 5th DEXA scan (March 4-9, 1999)
- Two fluoroscein bone labels were ordered and given (demeclocycline and calcein).
- Necropsies (March 16-19, 1999) and collection of tissues. Type and number of tissues per animal collected processed, sectioned, stained (H&E), and histologically evaluated included: ovaries (2), uterus and horns (2), vagina, cervix and urinary bladder (2), liver lobes (3), spleen and kidneys (3), adrenal

glands (2), thyroids, thymus, and pancreas (3), heart (2), lungs (2), brain (2), mammary gland (2), pituitary (1), left femur (1) and L2 vertebra (1).

- At necropsy, the right tibia and L3 vertebrae were collected, the soft tissue cleaned, and the bones placed in dark-brown stained 30 ml glass bottles containing 70% alcohol (ETOH). The right tibia's tuberosity was shaved with a sharp scalpel blade for proper fixation and the dorsal arches of the lumbar L3 vertebra removed.

- Bones were packaged and sent to Pathology Associates International (PAI) in Frederick, MD for plastic bone histologic processing. *Histomorphometry:* PAI will process, embedded in methyl methacrylate (MMA), and section at 5-10 μm, and mounted unstained or stained with modified tetrachrome with Von Kossa method. *Standard histomorphometry*: The abbreviations used are based on the ASBMR standard nomenclature (1). Structural and dynamic parameters are to be measured.

Month 9 Apr 1 - Apr 30, 1999

Task 7

- All live animal aspects of the experiment were terminated.

- Abstract was written and submitted to the Annual American Society of Bone Mineral Research (ASBMR) to be held in St. Louis, MO (Sept 30 to Oct 3, 1999).

- Soft and hard tissues were fixed, processed, embedded, section and stained for evaluations by Drs. Jayo and Blas-Machado.

- *Ex vivo* primal and distal pQCT scanning tibia.

Methods

After necropsy, the right tibia was kept frozen at -20°C until scanned using peripheral quantitative computed tomography (pQCT). The Norland Stratec XCT960 pQCT Bone Densitometer (Ft. Atkinson, WI) was used for pQCT measurements. Although methodology differed slightly from other reports, precision was similar to that previously reported (Gasser 1995, Sato 1997). A voxel size of 0.148 mm and a threshold for cortical bone of 500 was selected throughout the scans (Contour Mode 1, Peel mode 2, Cortical mode 4). Scans were taken at the proximal (metaphyseal and cancellous rich) and distal (primarily cortical) portions of the tibia. Based on previous reports and histological evaluations, pQCT scans were taken for proximal tibia at a constant 5 mm distance from the knee joint. Distal tibia evaluations were taken at a constant 1 mm proximal to the fibulo-tibial junction. For both sites, measurements included Cancellous Bone Mineral Content (Cn.BMC, in mg/mm [trab_cnt]), Cancellous Bone Mineral Density (Cn.BMD, in mg/mL [trab_dn]), Cancellous Bone Area, (Cn.B.Ar, in mm^2, [trab_a]), Cortical Bone Mineral Content (Ct. BMC, in mg/mm, [crt_cnt]), Cortical Bone Mineral Density (Ct.BMD, in mg/mL, [crt_den]), Cortical Bone Area, (Ct.B.Ar, in mm^2, [crt_a]), Cortical Thickness (Ct.Th., mm, [crt_thk]), Periosteal perimeter (Ps.Pm, mm, [peri_c]), Endosteal

Perimeter (Ec.Pm, mm, [endo_c]), Polar Moment of Inertia (P.M.I., mm^4, [ip_cm_w]), and Moment of Resistance or the (P.M.R., mm^3, [rp_cm_w]).

Statistics

All QCT raw data is expressed as mean ± SEM (Table 5). All statistical analyses were conducted using version 7.0 BMDP Statistical Software (Los Angeles, CA). Data was subjected to one-way analysis of variance (ANOVA) and post hoc pairwise comparisons utilizing Tukey's test. The letter symbol in all tables and graphs indicate the level of significance compared to Control animals ($^a p<0.05$; $^b p<0.01$).

Table 5. pQCT measurements taken from the right proximal tibia of young female rats at a constant 5 mm distal site from the joint space.

Parameter	*Control*	*OC*	*OC+MT*	*Casodex*	*p-value*
N	14	14	14	12	x
Cn.BMC	1.10 ± 0.15	1.46 ± 0.08	**1.61 ± 0.06^b**	1.11 ± 0.14	0.0023
Cn.BMD	308 ± 9.04	**270 ± 9.77^a**	**254 ± 10.8^b**	305 ± 6.40	0.0002
Cn.B.Ar	3.72 ± 0.55	5.50 ± 0.39	6.46 ± 0.32	3.68 ± 0.48	0.0000
Ct. BMC	9.65 ± 0.25	**7.74 ± 0.19^b**	**7.56 ± 0.23^b**	9.34 ± 0.23	0.0000
Ct.BMD	922 ± 13.31	920 ± 9.56	917 ± 10.67	909 ± 19.30	NS
Ct.B.Ar	10.5 ± 0.33	**8.42 ± 0.24^b**	**8.24 ± 0.20^b**	10.3 ± 0.39	0.0000
Ct.Th	0.78 ± 0.03	**0.65 ± 0.01^b**	**0.63 ± 0.02^b**	0.77 ± 0.02	0.0000
Ps.Pm	15.9 ± 0.22	**14.9 ± 0.22^b**	**15.0 ± 0.16^a**	15.9 ± 0.26	0.0012
Ec.Pm	11.0 ± 0.26	10.8 ± 0.20	11.1 ± 0.17	11.0 ± 0.24	NS
P.M.I.	36.7 ± 1.29	**27.6 ± 1.26^b**	**27.3 ± 1.05^b**	35.8 ± 1.31	0.0000
P.M.R	11.3 ± 0.34	**8.90 ± 0.36^b**	**8.78 ± 0.33^b**	11.1 ± 0.41	0.0000

Four groups of rats were compared and included a Control, an oral contraceptive (OC), an oral contraceptive plus methyltestosterone (OC+MT), and a Casodex group. Level of significance for ANOVA and compared to Control animals ($^a p<0.05$; $^b p<0.01$).

Results

None of the QCT-derived parameters measured at the distal tibia (cortical) were significantly different among groups. Therefore, these are not listed. However, significant differences were detected at the proximal tibia in both cortical and cancellous parameters. These are listed on Table 1. None of the measurements were significantly different between Control and Casodex groups.

Conclusions

OC use in growing rats, at a woman's dose which is 25% lower than that recommended for contraception, caused bone deficits at the proximal tibia compared to Control animals. This bone deficit was not prevented by OC supplemented with the androgen methyltestosterone. Surprisingly, and in contrast to previous reports (Lea et al., 1996), the nonsteroidal anti-androgen bicalutamide (Casodex) ingestion in growing rats did not cause significant bone changes compared to Control rats. Lea et al., (1996) gave rats Casodex SQ at 20 mg/kg/day for 21 days (420 mg total). Our dose tried to mimic a human dose of 50 mg/day translating to 0.89

mg/100 g of BW. Although our rats consumed Casodex for 105 days, they only ingested a tenth of Lea's (4) dose per day (approximately 280 mg total or half).

Month 10 Jun 1 - Jun 30, 1999

Task 8

- Dr. Blas-Machado accepted a faculty position at the Department of Pathology at Oklahoma State University, Stillwater, OK and departed on July 15, 1999.
- Dr. Jayo accepted a position as Senior Pathologist with Pathology Associates International. He will maintain an adjunct Associate Professor of Pathology position at the Wake Forest University School of Medicine. He will be in charge of the bone histomorphometry completion at PAI and complete soft tissue pathology. His last day as PI of the grant was June 30, 1999.
- Decalcified, processed, embedded and sectioned distal femur for histomorphometry.

Table 6. The total of distal femur metaphysis (bone + marrow) was identical for all groups (3.73 mm^2).

Parameter	*Group*	*Mean*	*SEM*	*P-value*
BV	OC	0.94	0.11	0.000
	Control	1.22	0.11	
	OC+MT	0.70	0.07	
	Casodex	1.30	0.13	
BS	OC	24.63	1.75	0.001
	Control	28.65	1.13	
	OC+MT	21.20	1.27	
	Casodex	30.34	2.23	
BV/TV	OC	25.24	2.84	0.000
	Control	32.68	2.96	
	OC+MT	18.70	1.77	
	Casodex	34.83	3.37	
Tb.Th.	OC	57.25	3.25	0.005
	Control	65.07	3.89	
	OC+MT	50.44	2.24	
	Casodex	65.05	3.26	
Tb.N.	OC	4.20	0.30	0.001
	Control	4.89	0.19	
	OC+MT	3.62	0.22	
	Casodex	5.18	0.38	
Tb.Sp.	OC	204.75	29.72	0.059
	Control	143.93	12.51	
	OC+MT	240.16	20.37	
	Casodex	158.23	42.43	

- Prepared soft tissues for embedding and histological evaluations

Ovaries

Ovaries were evaluated by counting the number of primary, growing, and antral follicles. Corpora lutea (CL) were counted and classified into atretic CL, hemorrhagic CL, and mature CL.

Primary (ANOVA p=0.260)

	OC	**Control**	**OC+MT**	**Casodex**
N	13	14	14	12
Mean	31.615	26.000	35.000	21.833
STD	17.868	19.896	20.840	10.338
SEM	4.956	5.317	5.570	2.984
Min	82.000	69.000	74.000	35.000
Max	11.000	4.000	5.000	6.000

Growing (ANOVA p=0.312)

	OC	**Control**	**OC+MT**	**Casodex**
N	13	14	14	12
Mean	7.000	4.786	6.214	4.417
STD	3.851	3.043	3.641	3.397
SEM	1.068	0.813	0.973	0.981
Min	14.000	9.000	15.000	11.000
Max	3.000	0.000	2.000	0.000

Antral (ANOVA p=0.448)

OC		**Control**	**OC+MT**	**Casodex**
N	13	14	14	12
Mean	12.077	13.143	9.357	11.750
STD	5.283	7.833	5.733	5.895
SEM	1.465	2.094	1.532	1.702
Min	22.000	26.000	19.000	24.000
Max	5.000	4.000	0.000	1.000

Atretic (ANOVA p=0.823)

OC		**Control**	**OC+MT**	**Casodex**
N	13	14	14	12
Mean	12.077	13.143	9.357	11.750
STD	5.283	7.833	5.733	5.895
SEM	1.465	2.094	1.532	1.702
Min	22.000	26.000	19.000	24.000
Max	5.000	4.000	0.000	1.000

Month 11-12 Jul 1 - Aug 31, 1999

Task 9

- On a letter dated July 23, 1999 by Jane E. Aubin, we received notification that the abstract had been accepted for the 21st meeting of the ASBMR. Poster #SU323 was assigned.
- Ordered kits for serum biomarkers.
- Ordered kits for hormone assays
- Carried out RIAs for serum hormones (estradiol, ethinyl estradiol, testosterone)
- Carried out RIAs for serum osteocalcin (see p. 15 for results)

August 30, 1999 - Dr. Erni Sulistiawati returned to Indonesia to complete graduate work.
September- Abstract referenced in "Reportable Outcomes" was published (see appendix, page XX).

Year 02

Month 1 Sept 1 to present

- Carried out analysis and compilation of data to date from the experiment (see page15).
- Prepared poster presentation for ASBMR Meeting in St. Louis (September 30 - October 4). (See Appendix B)
- Prepared Progress Report

Tasks to be accomplished

Months 1-6

Task 1:Plastic embedding, sectioning, and staining of 4 bones/rats
Urine Biomarkers
Serum IGF-1 Determinations
Meet with graduate committee
Bone histomorphometry training
Soft Tissue Analyses

Months 6-12

Task 2:Bone Histomorphometry
Analysis of Frozen Skeletal Samples
Data analysis and statistics
Attend ASBMR and present poster

Year 03

Months 1-12

Task 1:Prepare manuscript for publication
Take photomicrographs, make graphs and tables for publication
Submit manuscript
Make necessary changes to document and resubmit
Finish graduate course work.

Figure 1: Effects of Treatments on Body weight and Bone Density Changes in body weight (BW) and lumbar spinal bone mineral density (BMDs) were observed across time. All the animals were growing before and during the experiment. All groups gained significant ($p<0.05$) BW and spinal BMD through time. ***Control*** and ***Cas*** animals gained more BW and BMD than ***OC*** and ***OC+MT*** groups ($p<0.05$).

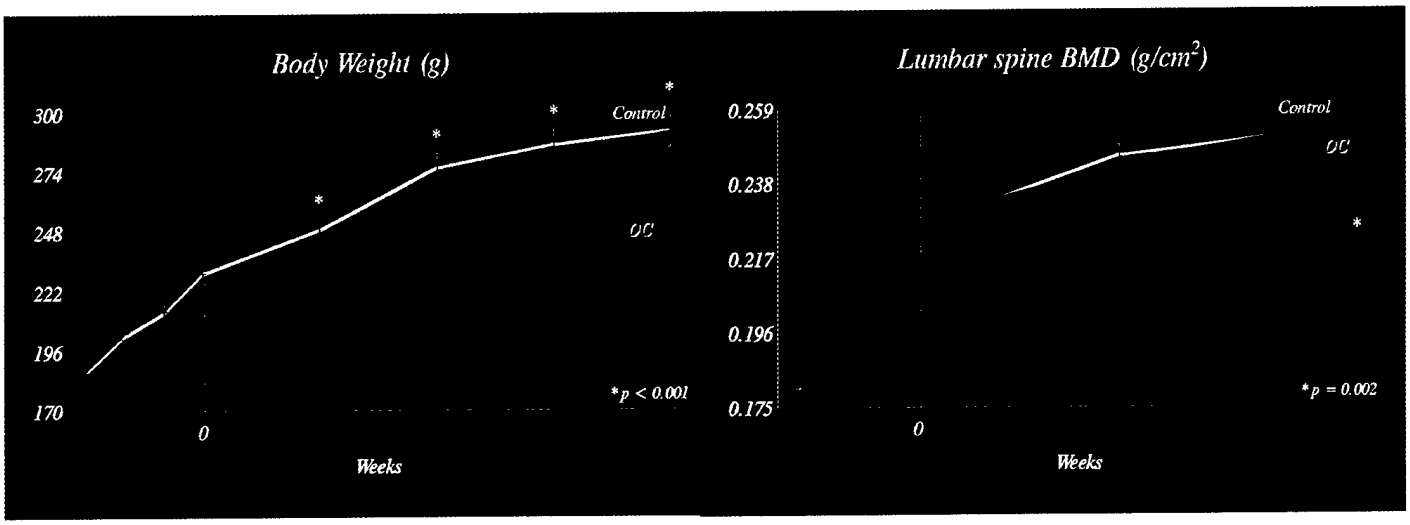


Figure 2. Effects of Treatments on Bone Biomarkers Across Time Osteocalcin and ALP significantly decreased ($p<0.05$) with time in all four groups, consistent with an age dependent decline in these markers. ***OC+MT*** had higher levels of ALP and ALT at intermediate time points (liver effects) and lower levels of osteocalcin (bone effects) than ***Control*** and ***OC*** groups.

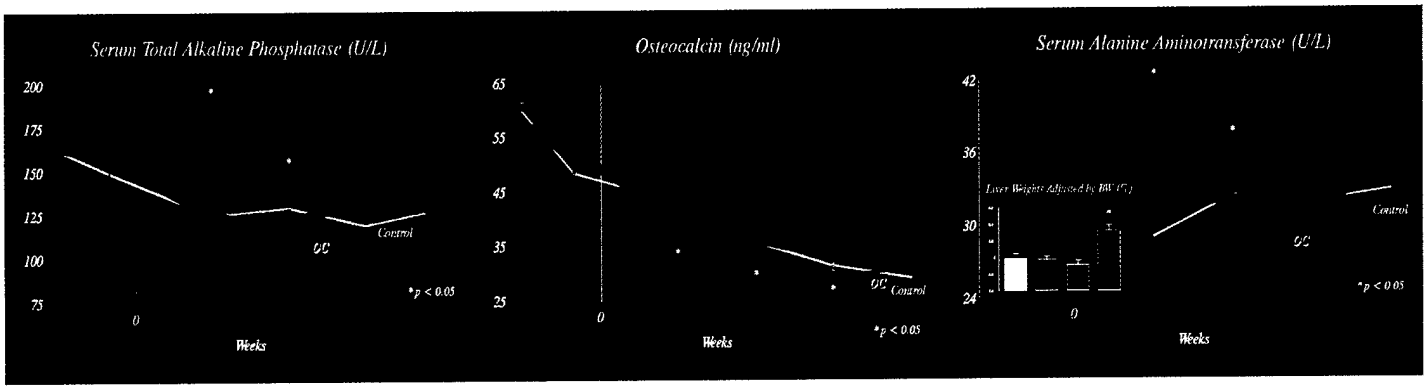
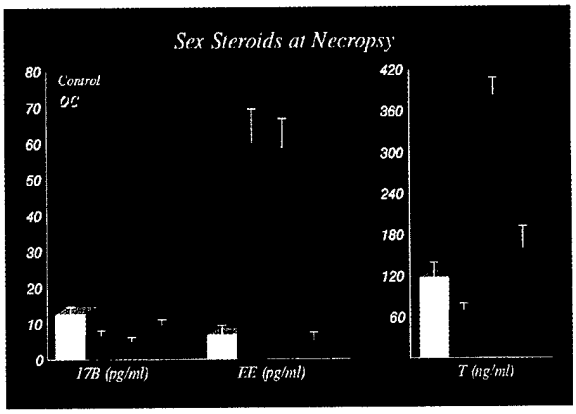


Figure 3 Effects of Treatments on Sex Steroids across time- At necropsy, the ***OC*** and ***OC+MT*** groups had significantly ($p<0.05$) lower serum levels of endogenous 17-B ($p<0.05$). EE levels were significantly higher in the ***OC*** and ***OC+MT***, and levels of T were significantly lower in ***OC*** and higher in ***OC+MT*** groups when compared to ***Controls***.



KEY RESEARCH ACCOMPLISHMENTS

The key findings of the study are:

- ✦ OC use decreased the peak bone mass of young intact female rats, similar to the findings in cynomolgus monkeys.
- ✦ Addition of a non-aromatizable androgenic steroid to OCs, at the dose provided, did not prevent the adverse effects of OCs to the growing skeleton of young rats.
- ✦ Anti-androgen treatment did not cause an adverse effect on the growing skeleton of young rats at the achieved dose, contrary to the hypothesized effects.

REPORTABLE OUTCOMES

The following abstract of data from this study has been published as follows (see also Appendix A and B).

SU323. Oral Contraceptives and Androgen: Effects on Bone Mass Acquisition in Female Rats. *MJ Jayo DVM, PhD[1], TC Register PhD[1], CL Hughes MD[2], PhD, U Blas-Machado DVM[1], PhD, E Sulistiawati DVM[1], PW Louderback BS[1], SE Rankin BA[1]. [1]Pathology/Comparative Medicine, Wake Forest University, Winston-Salem, NC and [2]Center for Women's Health, Cedars-Sinai Medical Center, Los Angeles, CA, USA.* J Bone Min Res 14(Suppl 1):S512., 1999. (See Appendix A and B)

CONCLUSIONS

This study suggests that OCs may inhibit bone metabolism and the acquisition of peak bone mass in rats, in part confirming the previous finding in cynomolgus macaques (Register et al., 1997). The addition of a non-aromatizable androgen (MT) to the OC did not counteract the effect of OC treatment on the skeleton. Androgens, natural or synthetic, are not part of any OC therapy available to women, and to our knowledge, this is the first time that anyone has evaluated the effects of addition of androgens a low-estradiol containing OCs with or without on bone tissues of skeletally immature and reproductively sound subjects.

The bone density results obtained in this study, at least as far at the effects of OC treatment, are somewhat confounded by the failure of the rats to consume their diets containing the OC. The differences in diet consumption led to differences in body weight, which is generally associated with bone mass and density. Such alterations in diet consumption were not observed in the previous study (Register et al., 1997) in cynomolgus monkeys which served as the stimulus for this initiative, nor in a pilot study we carried out prior to this experiment. The finding that the rats in this study did not eat equivalently the diets containing the hormones has some precedent, despite our pilot studies which suggested otherwise. Manoharan, et al (1970) used diet as the method for OC delivery which led to less food consumption and lower BW. Interestingly, SQ injections of OC also have led to reductions in BW (Lea et al., 1996). Regardless as to cause, lack of appetite and/or food aversion, BW were significantly reduced in the OC and OC+MT groups. Nevertheless, the addition of the non-aromatizable androgen to OC treatment did not affect diet consumption relative to the OC only group, neither did the addition of the androgen antagonist relative to the control group receiving no hormone therapy.

It should be noted however that the amount of diet and drug consumed was sufficient to provide for measurable differences in circulating sex hormones, and liver and bone biomarkers.

Addition of MT to OC caused liver effects (ALP and ALT) and bone effects (osteocalcin).The liver effects were not seen grossly (see liver weight bar graph) or histologically (not presented). Peak circulating levels of osteocalcin in the rat are found at about 21 days of age and rapidly and significantly decrease to a nadir by 16 weeks of age (Liu and Lin, 1970) . We saw similar results, but also found that addition of MT to OC treatment significantly suppressed osteocalcin levels. Young women who take OCs have reduced serum levels of bioavailable sex hormones, by direct and central negative feedback and by indirectly affecting the circulating levels of SHBG. Consequently, the level of bioavailable androgen and estrogen at the tissue level may be modulated with OCs. Determination of the effects of these treatments on other hormone sensitive organs (endometrium and mammary gland) are underway. In the OC-treated rats, serum ALP and osteocalcin levels (which had been significantly suppressed in OC-treated monkeys) were not affected, suggesting species differences in the response to OC or a dose-dependent effect since comparatively the rats here received 30% less than the human dose (based on consumption).

Summary

Although interpretation is somewhat complicated by the BW effects, our findings support the previous finding that OC use by young individuals appears to prevent proper bone accrual and maximal peak bone mass (PBM) (Register et al. 1997, Polati et al. 1995, Kreipe et al., 1993). OCs, at the dose and route given, negatively affected acquisition of PBM and skeletal integrity in young rats. Supplementation of OCs with androgens, in the dose and form of MT, failed to prevent the OC-induced bone effect. Use of the anti-androgen Casodex®, at the dose provided, did not cause adverse skeletal effects. Bone deficits have been reported in rats at a Casodex® dose of 25 mg/kg (Lea et al. 1996), which was approximately 3 times higher to ours. Future studies to examine the effects of these treatments on bone metabolism as determined by histomorphometric analyses may provide new insights into the effects of OCs and androgens on the skeleton. Nevertheless, the adverse effects of these treatments on diet consumption and body weight suggest that the effects of androgen supplementation on OC suppression of bone accretion may require reevaluation in a primate.

REFERENCES

Gasser JA. Bone 17(4):145S-154S, 1995.

Kreipe RE, Hicks DG, Rosier RN, et al. J Adolescent Health. 14(4):319-24, 1993.

Lea C, Kendall N, Flanagan AM. Calcif Tissue Int 58:268-272, 1996.

Liu FTY, Lin HS. PSEBM. 133(4):1354-7, 1970

Manoharan K, Yang MG, Mickelsen O. PSEBM. 133(3):774-9, 1970

Modrowoski D, del Pozo E, Miravet L. Horm Metab Res 24(10):474-477, 1992.

Parfitt AM, Drezner MK, Glorieux FH, et al. J Bone Mineral Res 1987;2:595-610.

Polatti F, Perotti F, Filippa N, et al. Contraception. 51(4):221-4, 1995.

Register TC, Jayo MJ, Jerome CP. Osteoporosis Intl. 7(4):348-53, 1997.

Sato M. Bone 17(4):157S-162S, 1995.

APPENDICES

A. Copy of Abstract submitted to American Society of Bone and Mineral Research (ASBMR).

B. Copy of Poster Presented at ASBMR

C. Letter from Dr. Manuel Jayo at Pathology Associates International (PAI) indicating the status of the processing of the bones for the project.

APPENDIX A

SU323

Oral Contraceptives and Androgens: Effects on Bone Mass Acquisition in Female Rats.

M. J. Jayo,[1] T. C. Register,[1] C. L. Hughes,*[2] U. Blas-Machado,*[1] E. Sulistiawati,*[1] P. W. Louderback,*[1] S. E. Rankin.*[1]
[1]Pathology/Comparative Medicine, Wake Forest University, Winston-Salem, NC, [2]Center for Women's Health, Cedars-Sinai Medical Center, Los Angeles, CA.

Oral contraceptives (OC) significantly inhibit normal bone acquisition in intact young adult female monkeys (*Register TC, et al. Osteoporosis Int 7:348-353,1997*). The OC effect on bone mineral accrual may be due to hypoandrogenemia, a well-known side effect of OC use. This experiment was designed to test if androgen supplementation during OC use may prevent the inhibition of bone mass acquisition in young subjects. Seventy-day-old intact virgin female Sprague-Dawley rats were randomized to four groups based on body weight (BW) and lumbar spine bone mineral density (BMD) by DEXA. Groups were treated with or without drugs mixed in their diet for 15 weeks: (1) ***Control,*** (2) ***OC*** (levonorgestrel + ethinyl estradiol at 0.0310 mg and 0.00619 mg per 100 g of diet, respectively), (3) OC + methyltestosterone (MT) at 0.516 mg/100 g of diet (***OC+MT***), and (4) Casodex (***Cas***), an antiandrogen, at 10.33 mg/100 g of diet. Food consumption and BW were measured daily. Spinal BMD, serum osteocalcin, alkaline phosphatase (ALP) and aspartate aminotransferase (AST) were measured every three weeks. Data were analyzed by analysis of covariance correcting for baseline values. All groups gained significant ($p<0.05$) BW and BMD through time. ***Control*** and ***Cas*** animals gained more BW and spinal BMD ($p<0.05$) than ***OC*** and ***OC+MT*** groups ($p<0.05$). Osteocalcin and ALP decreased with time in all four groups, consistent with an age-dependent decline in these markers. ***OC+MT*** had higher levels of ALP and AST at intermediate time points (liver effects) and lower levels of osteocalcin (bone effects) than ***Control*** and ***OC*** groups. Tibia lengths were significantly shorter in ***OC*** and ***OC+MT*** compared to the ***Cas*** group ($p<0.05$), and tended to be shorter than ***Controls***. OCs, at the dose and route given, negatively affected BMD and longitudinal bone growth in these young rats. The observed bone effect may relate to differences in BW gain, which was influenced by lower diet consumption in the ***OC*** and ***OC+MT*** groups. OC did not affect serum osteocalcin levels, which are significantly suppressed by OC in both women and monkeys. In the rat model, oral supplementation of OCs with androgens, in the form of MT, failed to prevent the OC-induced osteopenia. Based on the difference in BW and biomarker changes between primate and rodent models in the response to OC, future studies to examine effects of androgen supplementation on bone formation may require reevaluation in a primate.

WAKE FOREST UNIVERSITY
SCHOOL of MEDICINE
THE BOWMAN GRAY CAMPUS

SU323. Oral Contraceptives and Androgen: Effects on Bone Mass Acquisition in Female Rats.

MJ Jayo DVM, PhD[1], TC Register PhD[1], CL Hughes MD[2], PhD, U Blas-Machado DVM[1], PhD, E Sulistiawati DVM[1], PW Louderback BS[1], SE Rankin BA[1]. [1]Pathology/Comparative Medicine, Wake Forest University, Winston-Salem, NC and [2]Center for Women's Health, Cedars-Sinai Medical Center, Los Angeles, CA.

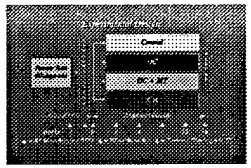
APPENDIX B

Introduction

Oral contraceptives (OC) significantly inhibit normal bone acquisition in intact young adult female monkeys (1). The OC effect on bone mineral accrual may be due to hypoandrogenemia, a well-known side effect caused by OC use. This experiment was designed to test if androgen supplementation during OC use may prevent the inhibition of bone mass acquisition in young subjects.

Materials and Methods

Seventy-day-old intact virgin female Sprague-Dawley rats were randomized to four groups based on body weight (BW) and lumbar spine bone mineral density (BMD g/cm^2) by DEXA. At 70 days of age (227 g), groups were treated with or without drugs mixed in their diet for 15 weeks: (1) ***Control***, (2) ***OC*** (Levlen®, levonorgestrel + ethinyl- estradiol at 0.0310 mg and 0.00619 mg per 100 g of diet, respectively); (3) OC + Androl® (***OC+MT***, ICN Pharmaceuticals, Inc, methyltestosterone at 0.516 mg/100 g), and (4) Casodex® (Zeneca Pharmaceuticals, ***Cas***, an antiandrogen at 10.33 mg/100 g). These doses were prepared to mimic a woman's contraception (0.5 mg LGN+0.03 mg EE) and postmenopausal hormone replacement (2.5 mg MT), and a man's anticancer (50 mg Cas) daily dose based on 1800 cal/day. Food consumption and BW were measured daily. ***OC*** and ***OC+MT*** groups received steroids for 3 consecutive days and base diet on the 4th day (similar to a woman's four week estrous and pill cycle). Animals in the ***Cas*** group received the antiandrogen-containing diet every day. Animals in the ***OC*** and ***OC+MT*** groups were sampled and killed on days 2 or 3 of the drug cycle.

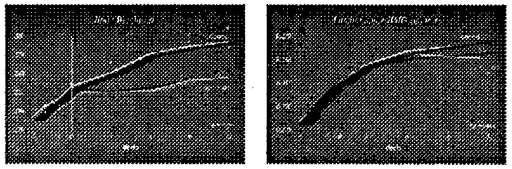


Serum osteocalcin and total alkaline phosphatase (ALP) were measured at times –3, –1, 3, 6, 9, and 12 weeks. Alanine aminotransferase (ALT), a liver enzyme, was measured at times 3, 6, 9, and 12 weeks. Spinal BMD was measured at –3, –1, 3, 6, and 12 weeks. Animals were necropsied after 14 weeks of treatment. Serum levels of 17-beta estradiol (17-B), ethinyl estradiol (EE), and testosterone (T) were measured at necropsy. Tibias were collected, their length measured, and kept frozen at -20EC until scanned using peripheral quantitative computed tomography (pQCT) with the Norland Stratec XCT960 pQCT Bone Densitometer (Ft. Atkinson, WI). pQCT scans were taken for proximal tibia at a constant 5 mm distance from the knee joint. Total bone mineral content (TBMC), area (Tarea), and mineral density (TBMD; trabecular BMD (Tb.BMD); cortical BMD (Ct.BMD) and cortical thickness (Ct.Th.); polar moment of inertia (P.M.I.) and moment of resistence (P.M.R.) were measured. Analysis of variance and post-hoc Tukey's were conducted at each time point. Longitudinal data were analyzed by analysis of covariance correcting for baseline values, and non-parametric rank (Kruskal-Wallis) and median (Chi-square) statistics were conducted on hormone analyses

RESULTS

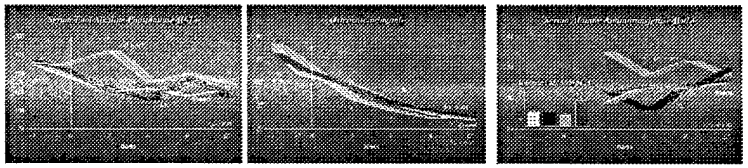
Body weight and Bone Density

Based on dietary consumption records, the actual dose was lower than estimated by approximately 30% in the ***OC*** and ***OC+MT*** groups and by 17% in the ***Cas*** group. All the animals were growing before and during the experiment. All groups gained significant ($p<0.05$) BW and spinal BMD through time. ***Control*** and ***Cas*** animals gained more BW and BMD than ***OC*** and ***OC+MT*** groups ($p<0.05$).



Bone Biomarkers

Osteocalcin and ALP significantly decreased ($p<0.05$) with time in all four groups, consistent with an age dependent decline in these markers. ***OC+MT*** had higher levels of ALP and ALT at intermediate time points (liver effects) and lower levels of osteocalcin (bone effects) than ***Control*** and ***OC*** groups.

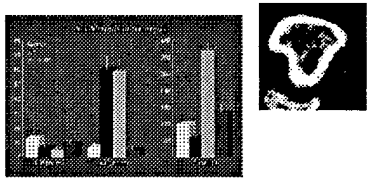


Sex Steroids

At necropsy, the ***OC*** and ***OC+MT*** groups had significantly ($p<0.05$) lower serum levels of endogenous 17-B ($p<0.05$). EE levels were significantly higher in the ***OC*** and ***OC+MT***, and levels of T were significantly lower in ***OC*** and higher in ***OC+MT*** groups when compared to ***Controls***.

Tibia length and Proximal pQCT Scan Data

Tibia lengths were significantly ($p<0.05$) shorter in ***OC+MT*** and tended to be shorter in ***OC*** ($p<0.10$) compared to the ***Cas*** group. pQCT measurements indicated significant bone deficit in the ***OC*** and ***OC+MT*** groups (table below, letter indicates post hoc differences between groups).



	Control	OC	OC+MT	Cas	ANOVA
N	14	14	14	13	Sig-value
Length (mm)	38.82 ± 0.21[ab]	38.26 ± 0.17[bc]	38.17 ± 0.19[c]	39.09 ± 0.18[a]	0.002
TBMC (mg/mm)	13.90 ± 0.34[a]	11.98 ± 0.32[b]	10.77 ± 0.30[c]	15.51 ± 0.38[d]	0.000
Tarea (mm^2)	20.24 ± 0.55[a]	17.82 ± 0.52[b]	16.03 ± 0.39[c]	20.09 ± 0.64[a]	0.000
TBMD (mg/mL)	684 ± 11.55[a]	624 ± 10.56[b]	596 ± 14.01[b]	675 ± 12.47[a]	0.000
TbBMD (mg/mL)	308 ± 9.04[a]	270 ± 8.77[b]	254 ± 10.82[b]	305 ± 8.47[a]	0.000
CtBMD (mg/mL)	922 ± 13.31	920 ± 9.56	917 ± 10.67	909 ± 19.78	NS
CtTh (mm)	0.78 ± 0.03[a]	0.65 ± 0.01[b]	0.63 ± 0.02[b]	0.77 ± 0.02[a]	0.000
PMI (mm^4)	36.7 ± 1.29[a]	27.6 ± 1.26[b]	27.1 ± 1.02[b]	35.8 ± 1.31[a]	0.000
PMR (mm^3)	11.3 ± 0.34[a]	8.90 ± 0.36[b]	8.78 ± 0.33[b]	11.1 ± 0.41[a]	0.000

Discussion

Since androgens, natural or synthetic, are not part of any OC therapy available to women, to our knowledge, this is the first time that anyone has tried to evaluate the effects of a low-estradiol containing OCs with or without androgens on bone tissues of skeletally immature and reproductively sound rats. As in our previous monkey study, we provided the hormones admixed in the diet. However, in contrast to the monkey study, the rats in this study did not eat equivalently the diets containing the hormones. Manoharan, et al (2) also used diet as the method for OC delivery which lead to less food consumption and lower BW. Interestingly, Liu and Lin (3) found that SQ injections for delivery of OC also led to lower BW. Regardless as to cause, lack of appetite and/or food aversion, BW were significantly reduced in the OC and OC+MT groups. Unfortunately, it is not possible to determine the absolute role that alterations in BW play in the effects of the OC and OC+MT treatment on the skeleton. BW at scan time 12 weeks correlated positively and significantly ($p<0.05$) with spine BMD and pQCT TBMD at the same time point ($r=0.566$ and 0.429, respectively). However, the amount of diet and drug consumed was sufficient to provide for measurable differences in circulating sex hormones, and liver and bone biomarkers.

Addition of MT to OC caused liver effects (ALP and ALT) and bone effects (osteocalcin).The liver effects were not seen grossly (see liver weight bar graph) or histologically (not presented). Peak circulating levels of osteocalcin in the rat are found at about 21 days of age and rapidly and significantly decrease to a nadir by 16 weeks of age (4). Our findings were similar to those previously reported. Interestingly, addition of MT to OC resulted in significantly suppressed osteocalcin levels. Young women who take OCs suppress endogenous formation and serum levels of bioavailable sex hormones, by direct and central negative feedback and by indirectly affecting the circulating levels of SHBG. Consequently, the level of bioavailable androgen and estrogen at the tissue level may be modulated with OCs. Effects at other hormone sensitive organs (endometrium and mammary gland) are underway. In the OC-treated rats, serum ALP and osteocalcin levels (which have been significantly suppressed in OC-treated monkeys) were not affected, suggesting species differences in the response to OC or a dose-dependent effect since comparatively the rats here received 30% less than the human dose (based on consumption).

Although interpretation is complicated by the BW effects, our findings support the previous finding that OC use by young individuals appears to prevent proper bone accrual and maximal peak bone mass (PBM) (1,5,6). OCs, at the dose and route given, negatively affected acquisition of PBM and skeletal integrity in young rats. Supplementation of OCs with androgens, in the dose and form of MT, failed to prevent the OC-induced bone effect. Use of the anti-androgen Casodex®, at the dose provided, did not cause adverse skeletal effects, but affected liver weight's (see bar graph). Bone deficits have been reported in rats at a Casodex® dose of 25 mg/kg (7), which was approximately 3 times higher to ours. Future studies to examine effects of androgen supplementation may require reevaluation in a primate.

Bibliography

1. Register TC, Jayo MJ, Jerome CP. Osteoporosis Intl. 7(4):348-53, 1997.
2. Manoharan K, Yang MG, Mickelsen O. PSEBM. 133(3):774-9, 1970
3. Liu FTY, Lin HS. PSEBM. 133(4):1354-7, 1970
4. Modrowoski D, del Pozo E, Miravet L. Horm Metab Res 24(10):474-477, 1992.
5. Polatti F, Perotti F, Filippa N, et al. Contraception. 51(4):221-4, 1995.
6. Kreipe RE, Hicks DG, Rosier RN, et al. J Adolescent Health. 14(4):319-24, 1993.
7. Lee, C Kendall N, Flanagan AM. Calcif Tissue Int 58:268-72, 1996.

Acknoledgement
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Address Changes
Dr. Jayo's present address is Pathology Associates International, 119 Highway 801 S, Advance, NC 27006, USA. Dr. Blas-Machado's present address is the Department of Pathology, OSU, Stillwater, OK. Dr. Sulistiawati's present address is Primate Research Center, Institut Pertanian Bogor, Indonesia

APPENDIX C

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September 27, 1999

Dr. Thomas C. Register
Section on Comparative Medicine (CMCRC)
Department of Pathology
Medical Center Blvd.
Winston-Salem, NC 27157-1040

Subject: Progress Report for bones from experiment DAMD117-98-1-8514

Dear Dr. Register:

The purpose of this letter is to inform you of the status on the bones submitted to PAI from the above-mentioned project for histology, histomophometry, and pathology. The tibias and vertebrae were submitted to us fixed in alcohol. We measured each tibia's length with a caliper prior to cutting them with a slow speed diamond blade saw. As per the original request, we sectioned each tibia 1.0 mm proximal to the fibular junction to provide for cortical bone samples. The proximal tibia, the cortical tibia's cross-sectional sample, and the vertebrae were then embedded in methyl methacrylate to produce three blocks from each of 54 samples submitted for a total of 161 blocks (one vertebrae was missing from necropsy). Three slides were produced by sectioning from each the vertebrae and proximal tibias (one unstained, and two stained with Von-Kossa Tetrachrome and Toluidine blue). Two slides were produced by grinding from the cortical cross-sectional tibia samples (one unstained and one stained with Von-Kossa Tetrachrome).

Concurring with the initial gross pathology at necropsy, no histopathological lesions are present in any of the stained slides. We are now in the process of establishing the histomorphometric standards and tailoring the templates of our new BIOQUANT™ histomorphometric equipment to your project. We will report our results to you using the established nomenclature (ASBMR, 1987). For cortical samples we will provide you with: Tb.BV, Ct.Th, Ct.BV, periosteal and endosteal surfaces (SL, DL, NL), and a calculated Moment of Inertia. For the cancellous bone samples in the vertebrae and proximal tibia we will provide you with: TV, BV, BS, OV, OS, and surface perimeters (SL, DL, NL, resorptive, eroded, quiescent). Derived calculations will be measured and reported.

Sincerely,

Manuel J. Jayo, DVM, PhD, DACVP
Senior Pathologist

119 Highway 801 South, Suite A-300 • Advance, North Carolina 27006 • (336) 998-5077 • (336) 998-3021 FAX