ANALYSIS OF MANIFESTATIONS IN MENOPAUSE AND HERBAL MANAGEMENT OF POSTMENOPAUSAL BONE LOSS

A THESIS

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **ANALYSIS OF MANIFESTATIONS IN MENOPAUSE AND HERBAL MANAGEMENT OF POSTMENOPAUSAL BONE LOSS** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2004 to February 2009 under the supervision of Dr. A. K. Sharma and Dr. Pravindra Kumar, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

The matter embodied in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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ABSTRACT

The present investigation was undertaken to fulfill two aims. First, to investigate the menopausal experience of women residing in Uttarakhand state which has evaded attention so far and second to explore a new medicinal herb for preventing postmenopausal bone loss for which available therapies are limited by several side effects generated.

Interview based cross-sectional survey to reveal age at menopause, frequency and severity of symptoms and associated factors was conducted among healthy women (n= 129, 35-60 years) in Haridwar district of Uttarakhand. The study included only premenopause, early postmenopause and late postmenopause women. The recalled mean age was 45.02 ± 4.35 years and age computed by probit analysis was 46.82 years. Socio-demographic factors like socio-economic status and lifestyles significantly affect the onset of menopause. The Greene Climacteric Scale was used to assess the frequency and severity of the menopausal symptoms. Muscle and joint pain was the most prevalent and severe symptom in the whole population as well in the sample of postmenopausal women. This study is the first of its kind from the aforementioned state and an effort to fill the abandoned gap.

In an independent project, both acute and chronic treatment studies were conducted to investigate the potential of *Tinospora cordifolia* (TC), an herbal medicine to prevent bone loss in ovariectomized rat model. For the study done acutely, female Sprague Dawley rats (n=7) were either sham operated (sham ovx) or ovariectomized (ovx) and treated with vehicle (benzyl benzoate: castor oil; 1:4), E2 (1µg/day) or TC stem extract (10, 50, 100 mg/kg b.wt.) subcutaneously for 4 weeks. At necropsy, Bone Mineral Density (BMD) measured by pQCT in the metaphysis of tibia was lower in ovx than ovx+E2 or sham ovx group. TC administration to ovx rats at the dose level 10 mg/kg b.wt. provided bone sparing effects by improving BMD. This could be explained by a trend towards decreased bone resorption since a significant positive shift was observed in the serum markers for bone turnover. Administration of TC also

induced normalization of the increased levels of lipoproteins. Uterus and mammary gland showed no signs of proliferation after acute treatment with TC extract.

Chronic studies were performed with TC (10 mg/kg b.wt.) and E2 (1 µg/d/animal) for over 3 months. Sham ovx or ovx rats were subcutaneously treated with respective test substances. Administration of TC extract and E2 for 12 weeks prevented the ovariectomy induced bone loss in rats. Histology and scanning electron microscopy studies on decalcified bone samples revealed that TC prevented estrogen deficiency induced decrease in trabecular thickness and restored the increase in trabecular separation. Efficacy of TC extract was further ascertained by analyzing the changes occurring in the stability of bone matrix using thermal analysis. Representative curves so obtained from thermogravimetry, differential thermal analysis and differential thermo gravimetry record weight changes and associated endothermic and exothermic heat effects in bone as a function of temperature (50 to 1500°C). Results showed greater bone mass loss; particularly in the organic phase of matrix in ovx controls (69.4%) compared to TC treated animals (56.13%). Additionally, TC extract improved the energy absorption pattern of tibiae which in turn improve the integrity, structure and hence compactness of bone.

In search for active phytochemicals that may be responsible for the antiosteoporotic action of TC, 20- β hydroxyecdysone (Ecd) was identified as the possible candidate. To test the bone sparing effect of Ecd, ovx rats were orally treated over 3 months with 18 mg, 57 mg and 121 mg Ecd/day/animal. E2 served as the positive control. BMD of the metaphysis of tibia was determined before and after the treatment. Results revealed that BMD was reduced by more than 50% in the control but not in E2 animals. In the Ecd animals, BMD was dose dependently higher than in the control. Ovariectomy induced elevation in serum bone turnover markers was also modulated by E2 and Ecd. While cross-laps were lowered in Ecd and E2, osteocalcin was normalized only by E2. Ecd administration also lack uterotrophic effects. The findings derived

from bone mineral density and biochemical parameters indicate that the antiosteoporotic effect may be attributed at least in part to the presence of Ecd in the extract.

TC has been extensively used as an anti-inflammatory, immunomodulatory, anti-arthritic and anti-diabetic agent in the ayurvedic preparations. Present study is the first attempt to evaluate its anti-osteoporotic potential. Findings assessed show that TC can prevent ovariectomy induced bone loss without influences on reproductive organs. If these findings can be approved in human they may provide a useful alternative to HRT for the treatment of postmenopausal osteoporosis.

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"Journey is easier when you travel together
Interdependence is certainly more valuable than independence"

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PRIYA KAPUR

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ABBREVIATIONS

E2	17-β Estradiol
ALP	Alkaline phosphatase
ANOVA	Analysis of Variance
BMI	Body Mass Index
b.wt.	Body weight
BMD	Bone Mineral Density
cm	Centimeter
CT	Computer Tomography
Cross-laps	C-terminal telopeptides
°	Degree
°C	Degree Celsius
DTA	Differential Thermal Analysis
DTG	Differential Thermogravimetry
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ER β	Estrogen receptor beta
FSH	Follicle Stimulating Hormone
g	Grams
HPLC	High Performance Liquid Chromatography
HRT	Hormone Replacement Therapy
hr	Hour
IGF	Insulin like growth factor
IL	Interleukin
IU	International units
kD	Kilodalton
kg	Kilograms
LH	Leutinizing Hormone
m	meter
µg	Microgram
µm	Micrometer
mg	Milligrams

mm	millimeter
min	minute
nM	Nanomolar
OC	Osteocalcin
ovx	Ovariectomized
PTH	Parathyroid hormone
/	Per
qCT	Quantitative Computed Tomography
SD	Standard Deviation
SEM	Standard Error of Means
i.e.	That is
TG	Thermogravimetry
TC	Tinospora cordifolia
v/v	Volume per volume
w/v	Weight per volume
w.r.t	with respect to
Yrs	Years
Ecd	20-ß hydroxyecdysone
µl	Microlitre
ER-LBA	Estrogen Receptor Ligand Binding Assay
OECD	Organization for Economic Cooperation and Development
Vs.	versus
Avg.	Average
CO_2	Carbon dioxide
nm	Nanometer
M	Molar
ng	Nanogram
pg	Picogram
Cm	centimeter
SERM	Selective Estrogen Receptor Modulator

RESEARCH PUBLICATIONS

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CHAPTER 1

INTRODUCTION

Menopause, a natural event is defined as the permanent cessation of the menstrual cycle. It occurs during middle age and signals the end of a woman's child bearing years. It is usually diagnosed when a woman has gone a full year without menstruation in the absence of other obvious cause. It is not a single event but a process occurring over many years. The events that take place during the period can be best described in four stages, Premenopause: the very beginning of menopause; Perimenopause: the period leading to menopause; Menopause: the actual cessation and Postmenopause: the life after menopause. Menopause is best characterized as "inverse of puberty"- that is a time of reduced functioning of ovaries due to aging, resulting in the lower levels of estrogen and other hormones.

Estrogens that chiefly regulate the menstrual cycle are the group of chemically similar substances which belong to gonadocorticoides, one of the classes of steroid hormones. They are the most ubiquitous and important hormones in the female body. These steroids interact with multiple organ systems and play a pivotal role in the physiologic events that occur during woman's life. A dramatic change in levels of these regulating hormones during menopause creates disturbances in the normal homeostasis of the body and triggers a variety of somatic, vasomotor, sexual and psychological symptoms. These symptoms continue to intensify in the years leading up to menopause, after which they usually stabilize or lie down. Each woman experiences a different menopause. Most women have transient symptoms that are manageable with self-care approaches but some women ask health providers for help to manage symptoms that interfere with their healthy living. Variations in the experience of menopause by women from different cultures and even in women of same culture appears to depend on a combination of physical changes, cultural influences, individual perception and expectations. In addition, other variables such as socio-demography, parity, body mass index, dietary practices, physical

activity, environmental exposures, education level, employment and access to health care could also affect the woman's quality of life during menopause.

Signs and effects of menopause normally begin when women are in their mid to late 40's. Worldwide, the occurrence of menopause has been established between 45 and 60 years of age. The age recorded in well nourished populations of industrialized nations is higher than the developing nations. Population studies suggest that the variation in the age at onset of menopause across nations may be due to geographical, environmental, nutritional, cultural, and genetic factors or due to different data collection and statistical methodologies used. Although, a lot variation is observed in the occurrence of menopause but for the past few centuries the median age at onset is 51 years. This has not changed much over times, what has changed however, is the life expectancy. A century ago, most women died in their midlife but now women generally live until almost 80. This increasing life span means that women will now spend one third or perhaps half of their lives postmenopause and are expected to experience not only the physical effects of aging but also the hormone disturbances responsible for menopause. Owing to this fact, a need therefore arises to pay special attention to the health problems that will follow during postmenopause years. Various debilitating health conditions that postmenopause women are prone to include cardiovascular diseases, diabetes, malignancies (ovarian and endometrial) and osteoporosis.

Postmenopausal osteoporosis is a heterogeneous disorder characterized by progressive loss of bone mass and deterioration in the micro architecture of bone tissue that increases its fragility and susceptibility to fractures. Although low peak bone mass and age related loss may be the contributing factors, a hormone dependent increase in bone resorption and accelerated loss of bone mass in the first 5-10 years after the menopause appears to be the main pathogenetic factor. During the past decade, considerable evidence has accumulated that suggests that estrogen regulates bone homeostasis through regulatory effects on the immune system and on oxidative stress and direct effects on bone cells. They keep the osteoclasts (bone

resorbing cells) in check allowing osteoblasts (bone forming cells) to build more bone. Many of these observations derive from studies with ovariectomized (ovx) rat. It represents an optimal model to investigate the effects of estrogen deficiency and depicts that unless the estrogen that is lost is being restored the bones become thin and brittle quite rapidly.

Estrogen replacement therapy (ERT) has been used as the most common treatment for preventing postmenopausal bone loss. However, only 35-40% of women even start ERT and many do not continue it. Use and compliance are limited due to its numerous undesirable side effects such as uterine and breast cancer. Other pharmacological agents that have been approved by U.S. Food and Drug administration (FDA) for the management of osteoporosis include bisphosphonate alendronate, Teriparatide, calcitonin, selective estrogen receptor modulators and recombinant human parathyroid hormone. These agents treat either by decreasing bone resorption to produce secondary gains in bone mass or are anabolic and produce direct increase in the bone mass. They are efficacious but not all patients are willing to initiate them as treatment. This is true because their use is associated with certain risks and side effects like vaginal discharges, nausea, weight gain and development of cancers. Compliance is also a major obstacle among patients who cannot afford follow up care or are in conflict with taking any medication option for prolonged time.

Taking deep account of the risks and side effects associated with available therapies, health care providers across the world have now directed their attention to search some alternative therapies for postmenopausal osteoporosis. Tremendous global efforts are ongoing to develop alternatives that will yield benefits by strengthening the female body, encouraging it to balance, regulate and normalize itself naturally with minimal associated inconveniences. Recent findings suggest that dietary, nutritional and life style changes supplemented by therapies such as homeopathy, mineral and vitamin supplements, exercise and medicinal herbs could be the attractive alternatives. All of them help to halt the progression of disease but postmenopausal women are more inclined to use herbal alternatives.

Ayurveda, an ancient system of Indian medicine mentions several plants that are useful in the correction of bone metabolic disorders such as osteoporosis. They appear to act holistically to improve symptoms, slow disease progression, correct imbalance and adjust the immune system to restore bone health. They also have a reputation of being safe for long term use compared with chemically synthesized medicines. *Cimicifuga racemosa, Salvia Miltiorrhiza, Trillium erectum, Artemisia vulgaris, Glycine max, Trifolium pratense, Achillea millifolium, Withania somnifera, Onobrychis ebenoides Cissus quandrangularis and Asparagus officinalis* are some of the medicinal herbs that have been exploited commercially. They maintain bone health by either decreasing the rate of resorption or promoting the rate of bone formation and sometimes both. Appliance of single herb as well as the herbal formulas (combination of several herbs) for preventing osteoporosis has been mentioned in Chinese, Japanese and Indian culture. While the primary action of the herb is important it is the synergy of the plants various actions that make these herbs successful in providing reliefs. As more and more plants are being searched and screened, the beneficial effects of phytochemicals that have potential for treating postmenopausal bone loss will be known. Efforts to seek multiple characteristic actions of traditional medicines may lead us to new opportunities for developing ubiquitous drugs with moderate but specified actions.

AIMS AND SCOPE OF THE PRESENT STUDY

Across India, several studies have been done to determine the age at natural menopause and the factors affecting its onset. However, no such data has been generated from the northern state of India, known as Uttarakhand. Complete lack of information on menopause from the region prompted us to conduct a survey study with an aim to find age at menopause, the nature and prevalence of symptoms and identify socio-demographic, physical or other factors that may influence its onset. Any information obtained would be the first of its kind and might enable health care professionals and associated bodies to devise suitable health care strategies and programs for improving the conditions and quality of women living in this region.

A second major aim of this study has been to explore the vast diversity of medicinal herbs and continue search for biologically active antiosteoporotic agents that has eluded identification so far.

Taking the above points into consideration, the objectives of the study were framed as follows:

I. To establish the menopausal symptoms, age at natural menopause and factors affecting its onset in Uttarakhand population using a standard scale.

II. To develop a rat model of estrogen deficiency bone loss as occurs in postmenopausal women and evaluate the selected plant extract for its potential antiosteoporotic activity.

III. To investigate the salutary effects of plant extract in acute as well as chronic dose response studies.

IV. To assess the safety of the most effective dose by employing uterotrophic assays.

V. To identify the active phytochemical contributing towards the antiosteoporotic action of the plant extract.

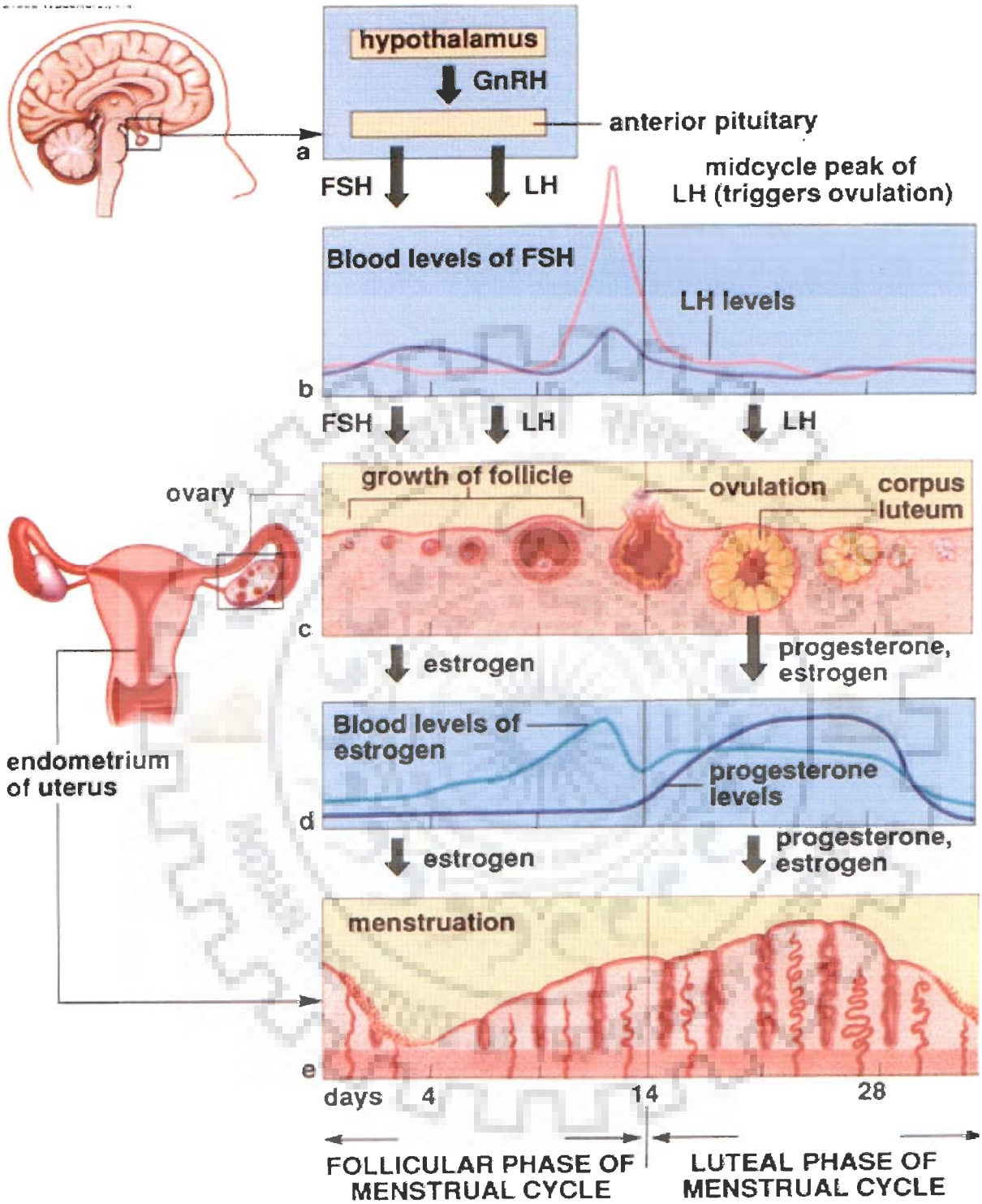
Details of the investigations on the above defined prospects have been enumerated in the following chapters of the present investigation.

CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL

Female reproductive system consists of ovaries (organs that produce the eggs or ovum), fallopian tubes, uterus, vagina, accessory glands and the external genital organs. Each month in reproductive age females, these parts are involved in the recurring cycle of physiologic changes known as menstrual cycle. It either results in pregnancy or menstruation. This cyclic event comprises of two main phases: the follicular phase and the luteal phase. During the follicular phase, the pituitary under the influence of gonadotrophins releasing factors (GnRH) by hypothalamus secretes gonadotrophins; FSH (follicle stimulating hormone) and LH (leutinizing hormone). High amounts of FSH secretions promote the development and maturation of ovarian follicle and surge of LH at the start of luteal phase causes the matured follicle to release egg in fallopian tube, the phenomenon termed as ovulation. Each month during ovulation, the ovaries not only release the egg but also produce the hormone estrogen which prompts the uterus to build up a lining that will allow the egg (if fertilized) to implant and grow. End of ovulation marks the start of luteal phase. In this phase the ruptured follicle is developed into corpus luteum; the structure which secretes increased amounts of progesterone. Progesterone causes the uterine lining to thicken further and prepare to support the embryonic development. If the egg is fertilized, it will be implanted in the uterus and fetus will develop. If the fertilization fails to occur within the specified time, the corpus luteum dies and progesterone and estrogen levels drop significantly. The hormone drop causes the washing away of the unfertilized egg along with the degeneration of uterine lining, the process known as menstruation. The relationship between hypothalamus, pituitary and ovaries in integrating the neural and hormonal signals into a physiological rhythm is shown in Fig. 2.1.



Changing hormone levels during the menstrual cycle.

Figure 2.1: MENSTRUAL CYCLE (www. sunnydutchess.edu/scala/bio102/)

At birth, woman has about two million eggs in her ovaries (Baker, 1986). This is a fixed number and no more are added in the lifetime. When a young woman reaches puberty, out of total only 300,000-500,000 eggs remain. After puberty, these eggs start to mature one at a time, leave the ovary, either get fertilized or pass out of the body with menstruation. Under normal conditions, the cycle works uninterruptedly for many years but in mid 30's, the supply of eggs start declining steadily. In late 40's and in early 50's the rate of loss speeds up. As the number of eggs gets reduced the cycle becomes erratic. Not only the numbers but the quality of follicles is also reduced. As the transition progresses, the egg supply exhausts (Richardson et al., 1987). The ovaries cease to make estrogen and progesterone (Longcope et al., 1986). Cycles are missed and ultimately stop. After the absence of menstrual cycle (amenorrhoea) for 12 consecutive months, the woman is said to be in menopause (WHO, 1981).

2.2 MENOPAUSE

2.2.1 Definition

Menopause is defined as the irreversible cessation of reproductive cycles in woman. It marks the permanent end of fertility. It is derived from two Greek words, *Mens* (Month) and *Pausis* (to stop). It is usually not referred to one day but to the whole of the menopause transition years. It is also referred to as the 'change of life' or 'climacteric'.

2.2.2. Types of menopause

There are two types of menopause; natural and surgical menopause. Natural or physiological menopause is the spontaneous ending of menstruation that occurs as a part of woman's normal aging process. Functioning of the ovaries is reduced causing a gradual decline in the estrogen level (to one tenth the normal). Following the reduction of ovarian hormone, pituitary gland reacts and an increase in the circulating gonadotrophins is observed. Since, the primary ovarian follicles are depleted and the remaining follicles have become less responsive to these hormones and produce estrogen; gonadotrophins level remain high and serve as one of

the measurable signs of menopause in woman. Menopause induced by medical interventions like surgery or cancer treatments is called as 'surgical menopause'. Sudden removal of ovaries cause immediate end of the cycles and hence sudden and complete drop of estrogen in the body. Hysterectomy (removal of uterus) does not cause menopause if the ovaries are left in place. However, it will bring an end to the estrogen production 2 or 3 years earlier than normal by disturbing the blood supply to ovaries. Cancer treatments cause severe ovarian damage and cycles may end immediately or several months later. Induced menopause can occur any time between first and the last menstrual cycle.

2.2.3 Stages of reproductive aging

Transition towards menopause is not an event but is experienced as a process by individual women (Leidy, 1994). The events that take place during the period are best described in the model system developed at the Stages of Reproductive Aging Workshop (STRAW) (Fig. 2.2). The staging system explained seven stages (-5 to +2) of reproductive senescence (Soules et al., 2001). The anchor for the model system is the final menstrual cycle. The seven stages are divided into five (-5 to -1) that precede and two (+1 to +2) that follow the final menstrual cycle (0). When subdivided further, stages -5 to -3 encompasses reproductive interval, -2 to -1 the menopausal transition and +1 to +2 belonged to postmenopause phase. Reproductive interval or premenopause is characterized by regular menstrual cycles and normal FSH. Menopause transition stage depicts variable menstrual cycles and high FSH. Postmenopause phase represents high FSH levels, beginning with the final menstrual cycle and lasting till the end of woman's life. Early postmenopause (+1) illustrated as five years since final menstrual cycle is subdivided into; a) the first 12 months of amenorrhoea after final menstrual cycle and b) the next 4 years for dampening of the ovarian function to a permanent level with no further change. Perimenopause marks the beginning when the body transitions into menopause. It can last six or more years before and ends one year after the last cycle.

However, its actual duration in any individual women cannot be predicted in advance or during the transition. The model system made so forth is remarkable and help to describe the general progression of events leading to menopause but the described patterns are not universally followed. Most normal women progress from one stage to the next, but there are individuals who move back and forth between stages or skip a stage all together (Mansfield et al., 2004).

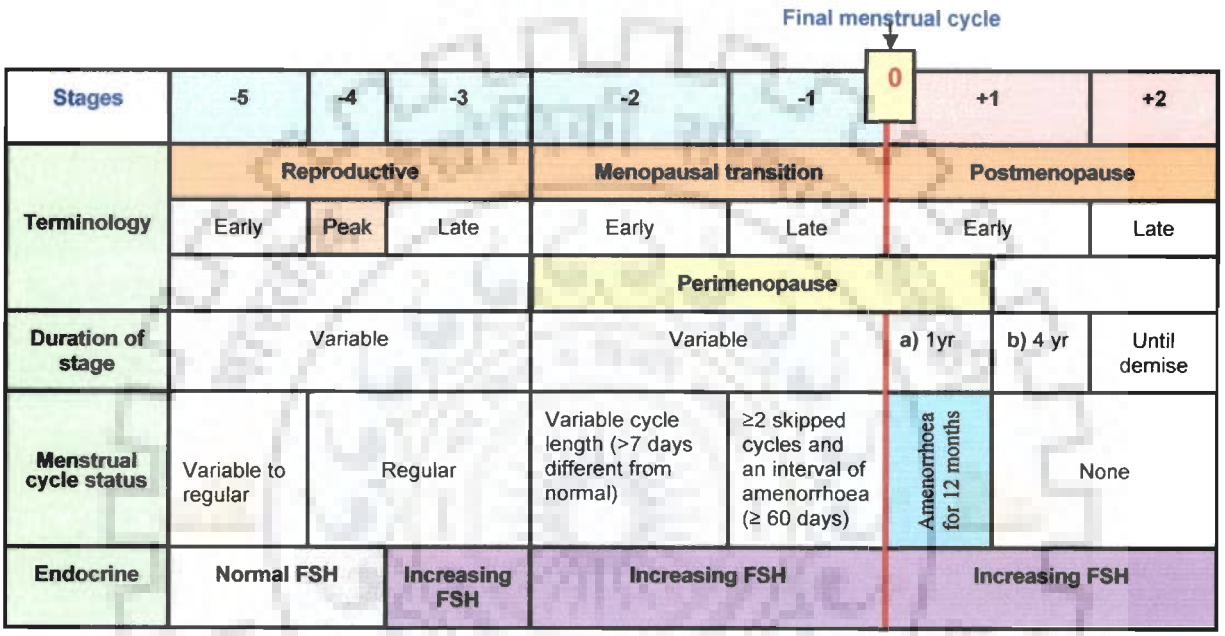


Figure 2.2: Stages of normal reproductive aging in women (Adapted from Soules et al, 2001)

2.2.4 Age at Menopause

Menopausal transition usually begins when women are in their mid to late 40s, and can last several years (Nelson, 2008). Worldwide, the occurrence of menopause has been established between 40 and 58 years (Society, 2007(a)). However, because of genetics, illness, or medical procedures, some women go through menopause before the age of 40. Menopause that occurs earlier than 40, whether natural or induced is known as "premature" menopause. Age at the onset varies across large surveys done in different countries. Cross-sectional, retrospective, cohort or prospective studies have placed the age at menopause between 49-51 years in well nourished populations of industrialized societies (WHO, 1981). For the

developing nations, the reported age is much earlier (Table 2.1). The difference in the age has been attributed to biological, social, nutritional, environmental factors or due to variation in data collection and statistical methods used. Most studies conducted in industrialized countries have used Probit/logit, Life table and Kaplan Meier methods to estimate the age at menopause. On the other hand, studies carried out in developing nations have typically relied on women's recall of age at menopause or assistance by reference to dates of important events like the date of last confinement, age of last child and information provided by husband or close relatives (Ayatollahi et al., 2003). Simple means or medians calculated by such methods always tend to underestimate the age at menopause. In developing countries, where appropriate statistical methods have been used, most estimates of age at the onset of menopause falls within the range estimated for industrialized countries (Table 2.1).Various socio-demographic factors have also been studied in relation to their affect on the age at natural menopause. Early onset has been related to smoking (1-2 yr earlier in smokers than non-smokers), low level of education, low socio-economic status, nulliparity, separation from partner, high strain jobs, mother's early age at menopause. Late onset is related to factors like use of oral contraceptives, parity (>5), high education, high BMI, late menarche, low altitude living and intake of nutritious diet rich in calcium etc. Most of the factors may affect the hypothalamic-pituitary-gonadal axis, alter the number of oocytes and hence the timing of the menopause. Table 2.1 shows the comparison of estimated age at menopause across different nations.

Table 2.1: Comparison of the estimated age at natural menopause across nations

PLACE	POPULATION	AGE (yrs) REPORTED	METHOD USED	FACTORS AFFECTING THE AGE AT ONSET OF MENOPAUSE	REFERENCE
INDIA					
Amritsar, Punjab	Cross-sectional	47.54	Probit analysis	Not reported	(Sidhu et al., 2005)
Delhi	Cross-sectional	48	Recall basis	No associations found	(Kriplani and Banerjee, 2005)
Jammu	Cross-sectional	47.35	Recall basis	Not reported	(Sharma et al., 2007)
Mohali, Punjab	Cohort	48.7	Recall basis	**A:** Working women **B:** Chronological age, higher education	(Kakkar et al., 2007)
Himachal	Cross-sectional	43.55	Recall basis	**B:** High socio-economic status, low altitude living	(Randhawa et al., 1987)
Assam	Cross-sectional	42.95	Recall basis	Not reported	(Sengupta and Gogoi, 1993)
Arunachal Pradesh	Cross-sectional	43.65	Recall basis	Not reported	(Kar and Mahanta, 1975)
Maharashtra	Cross-sectional	45.84	Recall basis	Not reported	(Rakshit, 1962)
North west	Cross-sectional	47.68	Recall basis	Not reported	(Mastana, 1996)
Mumbai	Cross-sectional	44.7	Recall basis	Not reported	(Shah et al., 2004)
North India	Cross-sectional	45.03	Recall basis	Not reported	(Bharadwaj et al., 1983)
EUROPE					
Finland	Cross-sectional	51	Kaplan Meier	**A:** Smoking & Nulliparity	(Luoto et al., 1994)

Italy	Cross- sectional	51.2	Recall basis	**A:** Smoking, late menarche, nulliparity **B:** High level of education, High BMI	(Parazzini, 2007)
Poland	Cross-sectional	51.25	Kaplan Meier	**A:** Early menarche, Smoking, low education, short menstrual cycle length **B:** Parity, use of oral contraceptives	(Kaczmarek, 2007)
Sweden	Cross-sectional	50	Recall basis	Not reported	(Bengtsson et al., 1981)
France	Cross-sectional	52	Kaplan Meier	**A:** Smoking, high strain job **B:** High education	(Cassou et al., 2007)
AMERICA					
SWAN study	Cross-sectional	51.4	Adjustments done for factor	**A:** Smoking, Low level education, Unemployment, separated/widowed/divorced **B:** Oral contraceptive use and Parity	(Gold et al., 2001)
New York	Prospective	51.3	Cox-proportional hazard model	**A:** Smoking and nulliparity **B:** BMI	(Kato et al., 1998)
USA	Cohort	51.1	Life table analysis	**A:** Low socio-economic status, low education, nulliparity	(Stanford et al., 1987)
USA	Cross-sectional	50	Recall basis	Not reported	(MacMahon and Worcester, 1966)
USA	Prospective cohort	51	Recall basis	A: Smoking	(McKinlay et al., 1992)
Other ASIAN Countries					
Japan	Cross-sectional	Not reported	Recall basis	**A:** Smoking, **B:** Parity, High calcium diet	(Nagata et al., 1998)
Singapore	Cross-sectional	49.1	Recall basis	**A:** High level of education	(Chim et al., 2002)

Malaysia	Cross-sectional	49.4	Recall basis	Not reported	(Dhillon et al., 2006)
Tibet	Cross-sectional	46.8	Status quo	Not reported	(Beall, 1983)
Malaysia	Cross-sectional	50.7	Recall basis	Not reported	(Ismael, 1994)
Japan	Cross-sectional/ Retrospective	50.54, 49.74	Probit, recall basis	**B:** Fibroma uteri	(Tamada and Iwasaki, 1995)
Korea	Cross-sectional	48.29	Recall basis	Not reported	(Kim et al., 2003)
Thailand	Cross-sectional	49.5	Status quo method	**B:** Parity	(Chompootweep et al., 1993)
Taiwan	Cross-sectional	51.09	Logistic model	Ethnic background	(Boulet et al., 1994)
Pakistan	Cross-sectional	47	Recall basis	Not reported	(Wasti et al., 1993)
The Philippines	Cross-sectional	44	Recall basis	Not reported	(Goodman et al., 1985)
AUSTRALIA					
Chinese in Sydney	Cross-sectional	50.3	Recall basis	Not reported	(Liu and Eden, 2007)
Twin study	Cohort	51	Kaplan Meier	**A:** Earlier birth year, late menarche, nulliparity, Smoking	(Do et al., 1998)
TURKEY					
West Anatolia	Cross-sectional	44.38	Recall or last digit preference	**A:** low sun exposure, familial, high education **B:** Parity > 2	(Discigil et al., 2006)

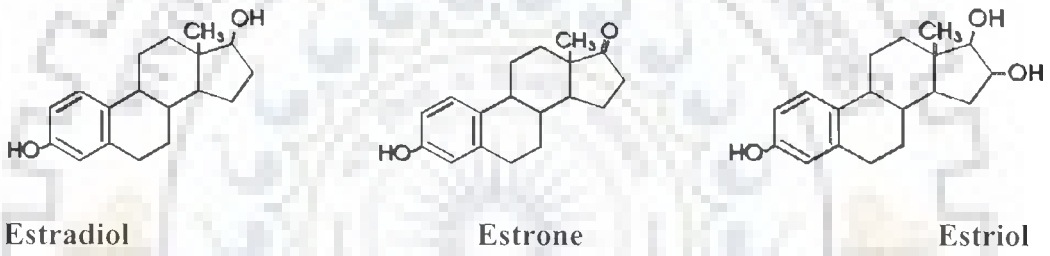
Turkish women	Retrospective	47.8	Recall basis	**B:** High parity and BMI	(Neslihan Carda et al., 1998)
ARABIC					
UAE	Cross-sectional	48	Recall basis	**B:** Familial, Parity >5, Oral contraceptive >1yr	(Rizk et al., 1998)
Iran	Cross-sectional	50.4	Cumulative distribution	**A:** Low socio-economic status **B:** High socio-economic status and education	(Mohammad et al., 2004)
Iran	Cross-sectional	48.3	Recall basis or Relation to events	**A:** Nulliparity	(Ayatollahi, 2003)
MEXICO	Cross-sectional	46.7, 49.6, 50	Recall, Probit, Kaplan Meier	**A:** Smoking, chronological age, Nulliparity	(Sievert and Hautaniemi, 2003)
AFRICA					
Morocco	Cross-sectional	48.4	Logit analysis	**A:** Early menarche **B:** No use of oral contraceptives	(Reynolds and Obermeyer, 2003)
Ghana	Prospective	48.05	Recall basis	Not reported	(Kwawukume et al., 1993)
Nigeria	Cross-sectional	48.4	Recall basis	No association found	(Okonofua et al., 1990)
African American	Cross-sectional	49.6	Recall basis	**A:** Smoking	(Palmer et al., 2003)
SOUTH AMERICA					
Peru	Cross-sectional	47.1	Kaplan Meier	**A:** Early menarche, less parity, low socio-economic status	(Gonzales et al., 1997)
Bolivia,Native America	Retrospective	42.3	Mean calculated	Not reported	(Castelo-Branco et al., 2005)

A: Factors causing the early onset of natural menopause; B: Factors causing the late onset of natural menopause

2.3 CLINICAL MANIFESTATIONS IN MENOPAUSE

2.3.1 Cause

Estrogen, that chiefly regulates the periodic cycle is not a single hormone but refers to a group of chemically similar substances that belong to gonadocorticoids, one of the classes of steroid hormones (Gruber et al., 2002). The three forms of endogenous estrogens are β-estradiol, estrone, and estriol (Fig. 2.3). The predominant female hormone before menopause is β-Estradiol (E2) made primarily in the ovaries.



Estradiol **Estrone** **Estriol**

Figure 2.3: Forms of endogenous estrogens

Estrogens have wide spread biological effects on mammalian tissues. Traditionally, they have been connected with female reproduction but their importance in non-reproductive processes such as the cardiovascular health, urinary tract, skin, hair, brain, mucous membranes, pelvic muscles, gastrointestinal tract, immune system and bone health has now been established (Fig. 2.4). The complicated interrelationships in the body for production, action and effects of estrogen make it difficult to view the biological importance of estrogens in isolation. The integration is such that, a tremendous decrease in the circulating estrogen levels during menopause profusely disturbs the normal balance and results in several physiological and psychological symptoms.

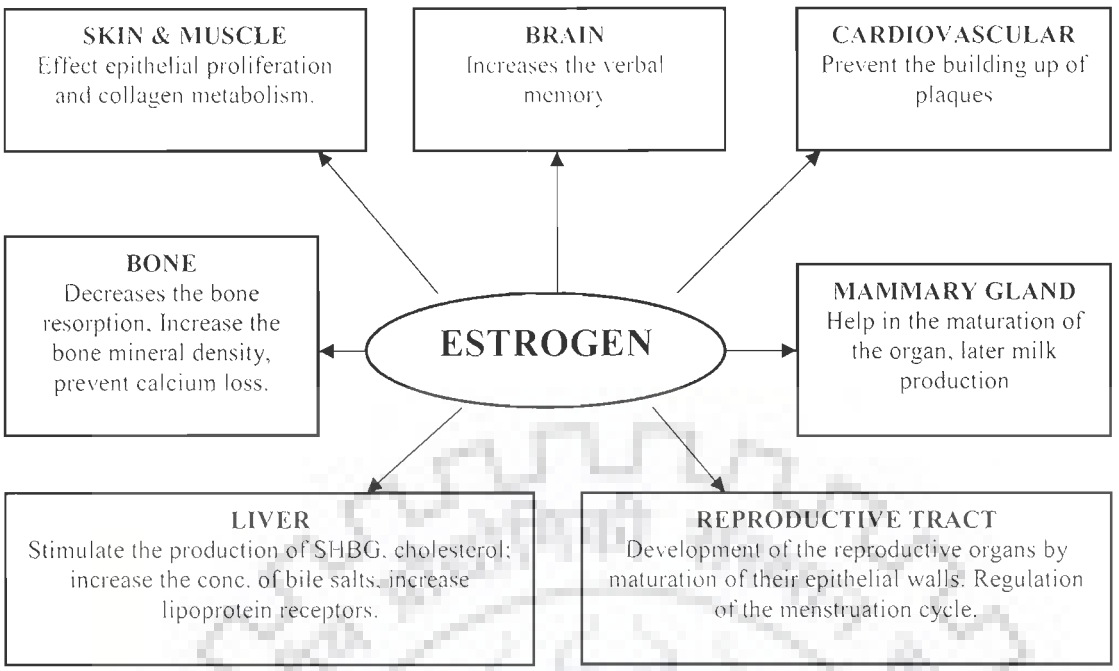


Figure 2.4: Biological functions of estrogen

2.3.2 Signs and Symptoms

The clinical signs and symptoms observed during menopausal transition are outlined below:

2.3.2.1 Changes in menstrual period

During the menopausal transition, changes in both menstrual cycle and frequency are common. Few women simply stop menstruating whereas others report about irregular, light or heavy period (Society, 2007(b)). Long or short cycles with periods occurring more often than usual are also reported. Although any menstrual pattern is possible, each woman will know that for her, a change has occurred.

2.3.2.2 Vasomotor symptoms

Hot flashes, the feeling of sudden sensation of flushing and extreme warmth often followed by profuse sweating and sometimes shaking or tremor is the hallmark symptom of menopause (Thompson et al., 1973). They are more frequent and severe in women whose menopause follows oopherectomy (Bachmann, 1999). Although, the pathophysiology is

debatable, they appear to arise as a result of change in the hypothalamus, the part of brain which regulates the body temperature. One of the proposed mechanisms states that the declining estrogen state causes decreased endorphin concentrations in hypothalamus, which in turn increases the release of nor-epinephrine and serotonin. The release of neurotransmitters lowers the set point in the thermoregulatory nucleus, and trigger inappropriate heat loss via vasodilation (Casper and Yen, 1985; Freedman et al., 1995). An increased pulse rate and a sensation of rapid heart beating may also occur. They cause serious discomfort and sleepless nights for women. If a woman perspires heavily, the condition is called night sweats.

2.3.2.3 Psychological changes

Central nervous system changes include headache, changes in memory and concentration, mood swings, depression and anxiety. Various factors that provoke headaches include certain foods, fasting, emotional changes, environmental changes and hormones. Women suffering from hormonal headaches during perimenopause are those who had history of headaches around their menstrual cycles. Hormonal headaches typically stop when menopause is reached and hormone levels are consistently low. Memory and other cognitive abilities change throughout life. Aging is generally associated with a trend of declining performance. However, disturbing memory lapses, difficulty concentrating, disorientation and mental confusion are common complaints during perimenopause and years after menopause (Brown, 1976). Erratic hormonal ups and down during the menopausal transition leads to loss of control and mood symptoms. Sleep deprivations associated with night sweats also results in fatigue, irritability and moodiness. Depressed moods are often associated with history of depressed mood earlier in life, a longer menopausal transition or severe symptoms like hot flashes. Other causes of mood disturbances include medication side effects and life stresses. Women begin to contemplate their own mortality and become introspective about the meaning or purpose of their lives. They may experience attacks of panic because of how they react to the changes during perimenopause or other midlife stresses. Symptoms of anxiety include

shortness of breath, chest pain, dizziness and heart palpitations. Psychological problems are not actually caused by menopause but they can arise or get worse during this time.

2.3.2.4 Urogenital changes

Nearly one third of all the women experience troubling symptoms of urogenital tract. These symptoms range in severity from mildly annoying to debilitating and includes vaginal discharge, burning, pain and shrinkage of vulval & vaginal walls (Barlow et al., 1997; Raz and Stamm, 1993). Estrogen decline causes the walls to become thin, dry (reduced blood flow), less elastic (reduced secretions) and vulnerable to infections (change in pH from acidic to alkaline) -- the condition known as atrophy. Changes in urinary tract often includes problem of incontinence i.e. involuntary loss of urine (Brown et al., 2002). Up to 30% of midlife women have urinary problems. During menopause and in the years that follow, lack of estrogen causes the lining of urethra to become thin and weakens the pelvic muscles thereby exposing women to increased risk of incontinence.

2.3.2.5 Changes in sexual function

Reduced ovarian production of estrogen influences sexual function at menopause and beyond (Dennerstein et al., 2002). Generation of hot flashes and night sweats make women restless and reduce her interest in having sex. Falling hormone levels also result in vaginal dryness, making intercourse uncomfortable. Fatigue, stress, anxiety, depression and social changes can also dampen the sexual desire.

2.3.2.6 Other potential changes

2.3.2.6.1 Weight gain

Midlife weight gain appears to be mostly related to aging and lifestyle than menopause. With aging the muscle mass often decreases and fat increases. Loss of muscle mass decreases the metabolic rate and lowers the calorie need, which can lead to weight gain.

2.3.2.6.2 Skin changes

Both the diminished levels of estrogen at menopause and aging contribute to the skin symptoms. Breakdown of collagen, decrease in the blood vessel supply and lesser sebum production damage the skin.

2.3.2.6.3 Hair changes

Menopause related shift in the balance between androgen and estrogen can result in either hair growth or loss. Excessive hair growth may occur in areas of the body where hair follicles are especially androgen sensitive such as chin, upper lip and cheeks. Hair loss is observed in about 50% of the menopausal women. It can be associated with stress, dietary changes and medications used for menopause.

2.3.2.6.4 Eye changes

Various eye changes may occur during the times of fluctuating hormone levels. Eye sight and shape may change. After menopause, some women report about condition known as chronic dry eye. Symptoms include scratchy eyes, light sensitivity, blurred vision, increased tearing and swollen or reddened eyelids. Estrogen control the secretions from tear glands so, any drop in the level of estrogen (like in case of postmenopausal women) translates into reduced tear production and hence increased symptomatology of ocular diseases (Mathers et al., 1998; Metka et al., 1991).

2.3.2.6.5 Mouth & Dental Changes

Increased dental problems include tooth loss, need for dentures, gum recession, higher risk of gum tissue injury, burning mouth and tongue, cold and hot mouth sensitivity and decreased bone mineral density in jawbone. Primary health care is advised because tooth loss can be a sign of underlying bone disease, osteoporosis.

2.3.2.6.6 Musculoskeletal pains

Midlife often bring musculoskeletal pains. Significant increase during menopause and years after phase has been attributed to factors like lack of physical activity and increased BMI due to gain in the body weight (obesity). Some studies relate pains to the levels of hormones like dihydroepiandrosterone and prolactin (Finset et al., 2004). Dihydroepiandrosterone was negatively and prolactin was positively associated with the said symptom. Estrogen decline during postmenopause has also been included in the list of factors because its loss leads to degeneration of the cartilage (osteoarthritis) in places like hand, hip and knee joints and, therefore generation of pains (Birchfield, 2001).

2.3.3 Methods used to access menopausal symptoms

Menopausal women experience the effects of ongoing aging process as well as the hormonal ups and downs during menopause. The relation is such that, distinction between the clinical signs related specifically to the menopausal transition, and those related to aging in general, is difficult. Many methods have been developed to carefully interpret the symptoms but only few are standardized, valid and reliable. Some methods are based on self reports of the presence, severity and frequency of individual symptoms such as hot flashes. Others use cumulative scores, based on the lists or scales of the symptoms that are thought to be associated with menopause such as changes of mood, cognition, quality of life, sexual function and somatic symptoms. The Greene climacteric scale (Greene, 1998), Kupperman index (Blatt et al., 1953), Menopause specific quality of life questionnaire (Hilditch et al., 1996), and Menopause Rating Scale (MRS) (Heinemann et al., 2003) are the examples of commonly used scales for menopausal symptoms.

2.3.4 Symptom experience across populations

No woman will experience every one of the listed changes. Prevalence rate of symptoms vary greatly among individuals. Some notice little physical change in their bodies

and mood while others complain about the change as bothersome and disruptive. Robinson (Robinson, 1996) concluded that the differences in the experience of menopause by women from different cultures and even in women of same culture appear to depend on a combination of physical changes, cultural influences, individual perception and expectations. Also, the variations in methodology like; the way data is used to access the menopausal status, which stages are compared, when and how frequently the symptoms are measured, how the terms for reproductive stages are used can generate differences (Nelson, 2008). In addition, survey studies suggest that other variables such as socio-demography, parity, body mass index, dietary practices, physical activity, environmental exposures, education level, employment and access to health care could also influence the menopausal woman's quality of life (Dhillon, 2006; Gold et al., 2000; Kakkar, 2007; Melby et al., 2005). Table 2.2 shows the comparison of symptom experience across few nations. Most studies report cross-sectional data and compare results for premenopausal, perimenopausal and postmenopausal groups of women, whereas others provide serial measures from individuals as they progress through stages. Reproductive stages in the epidemiological studies are often defined by terms existing in STRAW model system (explained above, Fig. 2.2). Trend of increase in the percentage occurrence and severity of symptoms with transition towards menopause is observed. Population studies show that Asian women suffer less from vasomotor than psychological symptoms in comparison to the women in the west.

2.4 POSTMENOPAUSAL HEALTH CONCERNS

Natural menopause occurs at a median age of 51 years, which has not changed much over the past few centuries. What has changed, however, is life expectancy. A century ago, most people died around 50. Now women generally live until almost 80. This increasing life span means that women will spend a third (perhaps even a half) of their lives after menopause (Finn, 2000). Like the issues related to perimenopause and menopause, the health problems in the years that follow-during postmenopause are equally important. Decisions made around

menopause affect a woman's health for the rest of her life. In the postmenopause years, women are prone to number of debilitating health conditions, such as cardiovascular diseases, diabetes, malignancies (ovarian and endometrial) and osteoporosis (Haney, 1986; Saltiki and Alevizaki, 2007). Menopause presents an opportunity for a woman to undergo a personal risk evaluation. Determining the risk factors as early as possible allows women to employ preventive strategies (Society, 2007 (c)).

2.4.1 Heart disease

Coronary heart disease (CHD) is a multifactorial disease. Its expression is influenced by the interaction of genetic and environmental risk factors. Estrogen deprivation may play an important role in the early appearance of CHD in women (Barrett-Connor and Bush, 1991). Several epidemiological studies indicate a higher incidence of the disease in postmenopausal women when compared to women of reproductive age (Colditz et al., 1987; Matthews et al., 1989). In addition, postmenopausal women with CHD have more advanced coronary artery stenosis in comparison to premenopausal women (Gurevitz et al., 2000). At a younger age women are at lower risk for CHD than men. However this disparity tends to disappear after menopause (Barrett-Connor, 2003). Estrogens protect the vasculature at different levels. Through their direct genomic actions, estrogens influence the vasodilation by increasing the production of endothelial cells and smooth muscle cells of vessels and myocardium. They also protect from the formation and calcification of atherosclerotic plaques. Direct non genomic actions include rapid vasodilation through increased production and modulation of ion channels.

Indirect protective actions involve effects on the metabolism of lipids (increase HDL, decrease LDL and oxidized LDL), effects on coagulation system (affect fibrinogen and factors V, VII, IX X and TFPI, decrease the levels of anti-thrombinIII, protein S and PAI-1), changes in proinflammatory factors and antioxidative effects (Saltiki, 2007).

PLACE	POPULATION	METHOD USED	FREQUENTLY OBSERVED SYMPTOMS & FACTORS AFFECTING	REFERENCE
ASIA				
Amritsar INDIA	Postmenopausal women 40-50 yrs	Structured Questionnaire, face to face interviews	Hot flashes & Night sweats (55%), Insomnia (53%), Body ache (38%), Fatigue (42%), Irritability (35%)	(Sidhu, 2005)
Varanasi INDIA	Pre, Peri and Post menopausal women (45-55 yrs)	Checklist prepared from Neugarten & Kraines (Neugarten and Kraines, 1965) Face to face interviews	Hot flashes more in postmenopausal than pre and perimenopausal women. Perimenopausal suffered most	(Sharma and Saxena, 1981)
Bangalore INDIA	45-55 yrs women	Greene climacteric scale face to face interviews	Psychological symptoms reported more than somatic and vasomotor	(Chattha et al., 2008)
North India	Menopausal , 40 above	Checklist prepared from Neugarten & Kraines & Greene climacteric scale	Lack of energy (73%), Headache (56%), Hot flashes (53%). More mean duration since menopause more psychological and rheumatic symptoms	(Sharma, 2007)
North India	Perimenopausal and early Postmenopausal women (35-65 yrs)	Menopausal rating Scale (MRS)	Perimenopausal > psychological Postmenopausal > somatic and urogenital Working women > psychological, Non working > somatic symptoms	(Kakkar, 2007)
Singapore	Pre, Peri and Post menopausal women (45-60yrs)	Questionnaire, face to face interviews	Perimenopausal suffered more than postmen. Backaches (51%), Vaginal dryness (20%), Hot flashes (18%), night sweats (8%)	(Chim, 2002)

Malaysia	Early Menopausal (5 yrs since final period) & Late menopausal (after 5 yrs till death) 40-70 yrs	Questionnaire, face to face interviews	Tiredness, Muscle &bone pains, concentration loss (67-79%) > hot flashes (45%)	(Dhillon, 2006)
Thailand	Pre, Peri, and post menopausal women (45-59 yrs)	Questionnaire, face to face interviews	Perimenopausal suffered most Sexual desire loss (82%) and Hot flashes (73%)	(Chompootweep, 1993)
LATIN AMERICA				
Ecuador	Pre, Peri and post menopausal women, > 40 yrs	Menopause Rating Scale (MRS) Face to face interviews	Muscle & Joint pains (77%), Depression (75%), Sexual problem (70%), Hot flashes (65%). Low education, sexually inactive & low socio-economic status women suffered more from symptoms.	(Chedraui et al., 2007)
Ecuador	Premenopausal, Perimenopausal and Postmenopausal women > 50 yrs	Greene climacteric scale face to face interviews	Postmenopausal score greater than pre. Difficulty in concentrating > feeling unhappy > headaches > hot flashes. Age > 47, Parity > 4, Education < 12 yrs suffered the most	(Sierra et al., 2005)
ARABS				
Turkey	Premenopausal, Perimenopausal and Postmenopausal women (40-65 yrs)	Questionnaire, face to face interviews	Perimenopaual > hot flashes Postmenopausal > Incontinence, decreased libido, poor memory and lack of energy	(Discigil, 2006)
UAE	Menopausal women: , > 40 years , six month amenorrhoea	Structured and pre tested questionnaire, face to face interviews	Hot flashes (45%), Urinary incontinence	(Rizk, 1998)

USA				
USA	Premenopausal, Perimenopausal and Postmenopausal women (40-55 yrs)	Structured Questionnaire, face to face, telephonic interviews	Hot flashes highest (Perimenopausal and Premenopausal). High BMI, Low socio-economic status, smoking and sedentary life style causes more symptomatology	(Gold, 2000)
USA	45-60 yrs women, Perimenopausal and Postmenopausal	Menopause specific quality of life MENQOL questionaire & Short form survey (SF-36)	Hot flashes (53%) and Psychosocial (29%). Employed, highly educated, high income report less symptoms	(Brzyski et al., 2001)
NATIVE AMERICA	Pre, Perimenopausal and Postmenopausal women 35-54yrs	Questionnaire, face to face interviews	Loss of libido (51%), hot flushes (45%), Itching (41%)	(Castelo-Branco, 2005)
AUSTRALIA **Chinese in Sydney**	Pre, Peri and Postmenopausal women (45-65 yrs)	Menopause specific quality of life scale, personal & telephonic interviews	Hot flushes (34%), Muscle & joint pains (68%), poor memory (76%), dry skin (69%) Overweight, working women Unmarried, Less educated, Parity < 2 reported more symptoms	(Liu, 2007)
EUROPE				
Netherlands	Pre, Peri and Postmenopausal women (45-65 yrs)	Greene climacteric scale face to face interviews	Scores highest in perimenopausal than postmenopausal women	(Barentsen et al., 2001)
Italy	Premenopausal, Perimenopausal and Postmenopausal women (45-55 yrs)	Women health questionnaire (WHQ) face to face interviews	Postmenopausal women symptoms more than Pre and Perimenopausal Lifestyle and past history of mood instability suffered more depressive symptoms	(Amore et al., 2004)

2.4.2 Diabetes

Diabetes is one of the most common chronic diseases in the world. Type 2 diabetes mellitus is more common than type 1 and is most frequent in obese individuals over the age of 40 years (Khoo and Perera, 2005). It increases the risk of cardiovascular diseases, vision loss, kidney failure, uterine cancer, gallstones and nerve damage. Estrogen therapy in postmenopausal women appears to decrease the incidence of type 2 diabetes mellitus and improve the glycaemic control. Diet, exercise and weight control are especially important for the woman who is at high risk or has the disease (Khoo, 2005).

2.4.3 Malignancies

Menopause is not associated with increased cancer risk. However, some cancer rates typically increase with age and so postmenopausal women may be affected (Parker et al., 1996). Also, some of the therapies used to treat menopausal symptoms are associated with an increased risk for certain types of cancer. The most common types of cancer are presented herewith.

2.4.3.1 Breast cancer

It is the most frequently diagnosed cancer in women. Advancing age, personal history of cancer, genetics, long menstrual history, nulliparity, high breast tissue density, late menopause (after 55 yrs), obesity, more than one alcoholic drink per day, lack of exercise, diet low in vegetables and fruits, chemotherapy, never having breast fed and use of hormone therapy are the factors that increase breast cancer risk.

2.4.3.2 Endometrial cancer

Cancer can develop on the inside lining of uterus called as endometrium (Parazzini et al., 1991). Fewer than 3% will develop endometrial cancer and far fewer die from this. Risk factors for developing endometrial cancer includes use of estrogen without progesterone, use of tamoxifen (breast cancer therapy), menarche earlier than age 12, late menopause, irregular

menstrual cycles during reproductive years, never being pregnant, obesity, diabetes, gall bladder disease and perhaps the high blood pressure and hereditary colon cancer.

2.4.3.3 Ovarian cancer

It is not primarily affected by menopause but the risk does increase with age, particularly in women without children or in those with family history of the breast or ovarian cancer.

2.4.4 Osteoporosis

Osteoporosis is a skeletal disorder in which bone strength has weakened to a point where the bone is fragile and at higher risk for fractures. Bone fractures resulting from osteoporosis can lead to pain, height loss, difficulty in locomotion, deformation of backbone and possibly permanent disability. Although, some decline in bone strength can be attributed to aging, lower estrogen levels are the major cause.

2.5 BONE LOSS

2.5.1 Background information

The skeleton provides the mechanical support to the body and a reservoir for normal mineral metabolism. In both capacities, bone is an active tissue constantly being remodeled and changing metabolically (Calvo et al., 1996). Bone modeling is important for the growth, repair, maintenance of normal bone strength and preserving calcium homeostasis (Putnam et al., 2007). The two major types of bones are cortical and trabecular bone. Cortical (compact) bone is so-called due to its minimal gaps and spaces. This hard outer layer gives bones their smooth, white, and solid appearance. It accounts for 80% of the total bone mass of an adult skeleton. Trabecular (cancellous) bone is lighter and less dense than compact bone. It is composed of a network of rod and plate-like elements (trabeculae) that make the overall organ lighter and allowing room for blood vessels and marrow. It is the site for bone forming cells and a reservoir for minerals such as calcium, phosphorus and magnesium. It fills the interior and

accounts for the remaining 20% of total bone mass. It has nearly ten times the surface area of compact bone (Fig. 2.5).

2.5.2 Bone cells

There are three main cell types of bone: osteoblasts, osteoclasts and osteocytes. Each has an important part to play in bone turnover and the remodeling process (Putnam. 2007).

Osteoblasts are derived from mesenchymal stem cells. They cluster along the bone surface, where they synthesize organic matrix and regulate mineralization by extruding collagen to form osteoid, followed by deposition of calcium in amorphous masses of calcium phosphate. After the synthesis of matrix a large proportion of osteoblasts become redundant and are removed. Some remain and line the bone surface or become embedded in the mineralized tissue as osteocytes. Osteoblasts are critical in the regulation of osteoclasts function through the production of stimulatory RANK-L (ligand for receptor activator of nuclear factor κB) and inhibitory factor osteoprotegerin. Osteocytes are derived from osteoblasts. They no longer move about the bone surface or divide. They remain embedded within the osteoid matrix and are in limited contact with the blood supply and extracellular fluids.

Osteoclasts are derived from the haemopoietic stem cells of the macrophage/monocyte lineage. They are multinucleated cells which line the bone forming surface of the bone tissue. They resorb bone by producing hydrogen ions which serve, firstly to dissolve the mineral crystals within the matrix and secondly provide the optimum pH for proteolytic enzymes to work and hydrolyze the organic portion of matrix. Their action can be stimulated by various factors like parathyroid hormone, vitamin D, prostaglandins, interleukins and tumor necrosis factors. It is inhibited by estrogen, androgen, calcitonin, transforming growth factor β and nitric oxide.

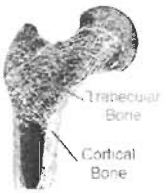


Figure 2.5: Types of bone tissue

2.5.3 Bone remodelling

Throughout life the skeleton undergoes continuous turnover of bone in which there is removal of old bone and replacement with newly formed bone. Bone remodelling occurs in small packets of cells called as basic multicellular units (BMUs). These units turn over the bone at multiple surfaces (Frost, 1991). Bone turnover is always initiated by osteoclasts. Osteoclasts erode the mineralized surface of an old worn or damaged part of the bone structure by producing hydrogen ions and proteolytic enzymes in the localized environment leaving a cavity. This process is followed by the recruitment of successive teams of osteoblasts to the outer edge of the erosion cavity. Firstly, the osteoblasts lay down a protein matrix structure. This provides the scaffolding of the bone and allows for a certain amount of flexibility. Secondly, in a well regulated manner, the minerals such as calcium, magnesium, phosphorus and zinc are deposited into this protein matrix. This gives the bone the strength and rigidity. The sequence of events in the normal remodelling cycle is always the same as osteoclastic bone resorption, a reversal phase, followed by osteoblastic bone formation (Fig. 2.6 A). This process is necessary for the skeletal system to periodically grow and repair its structural damages (Calvo, 1996). Both the systemic hormones and local factors influence bone growth and turnover. Parathyroid hormone and calcitriol stimulate bone resorption while calcitonin inhibits it (Vaananen, 1993). Sex steroids also help protect the bone loss (Compston, 2001). Growth factors and cytokines, of which the interleukins IL-1, IL-6, IL-11, transforming growth factor-β, platelet derived growth factors, fibroblast growth factor and tumor necrosis factor-α seems to be involved most closely in the regulation of bone turnover (Hill, 1998).

In the steady state, the coupling between bone formation and resorption maintains the bone mass. Abnormalities occur, when this balance is disturbed. In postmenopause, due to loss of sex steroids, bone resorption rate exceeds the rate of formation resulting in the condition known as osteoporosis (Fig. 2.6 B).

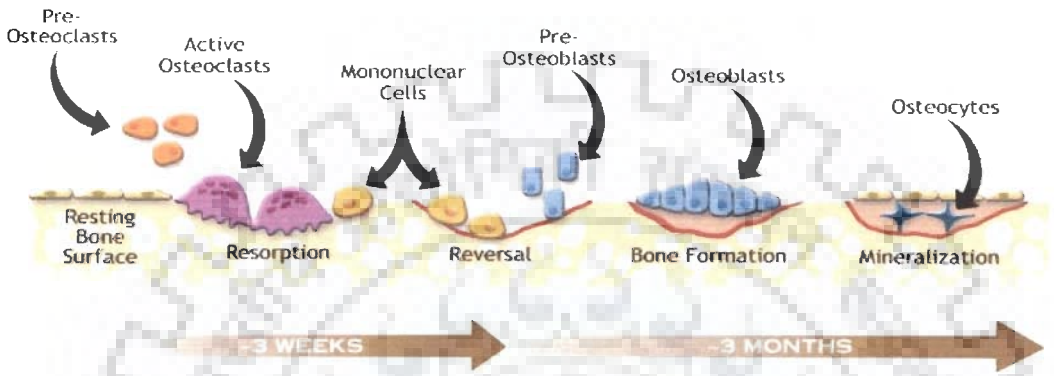


Figure 2.6 (A): Bone remodelling cycle (www.umich.edu/news/Releases/2005/Feb05/bone.html)

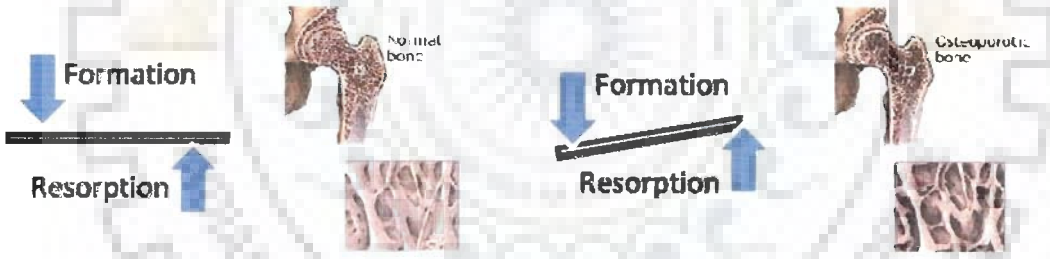
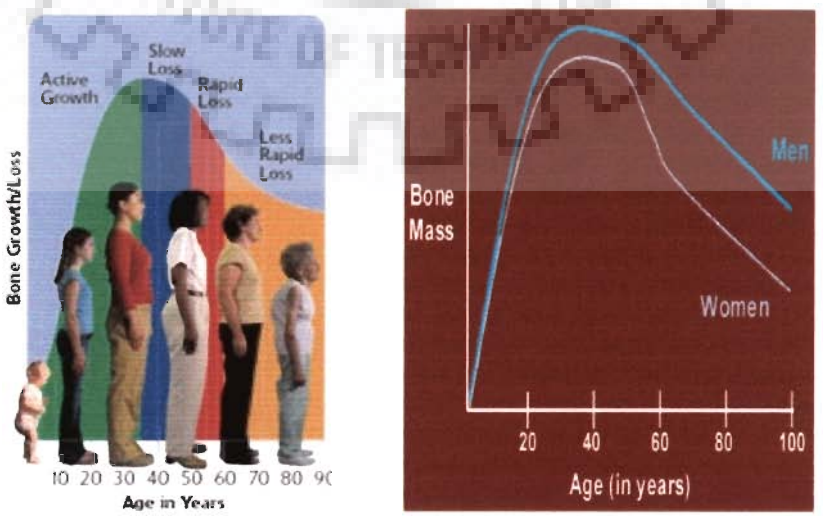


Figure 2.6 (B): Osteoporosis

2.5.4 Osteoporosis

It is defined by WHO as "A progressive skeletal disease characterized by low bone mass and micro architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (WHO, 1994). It is the most common and widespread metabolic disorders affecting one in three women and one in twelve men at some point in their lives. Trabecular bone has larger surface: volume ratio and is more metabolically active. Owing to this cancellous bone mass is lost much earlier than cortical bone mass (Edelson and Kleerekoper, 1996). The osteoporosis continuum has been picturised in Fig. 2.7(A). As seen, the bodies gain bone mass throughout childhood and adolescence until the peak mass is

achieved in the third decade (formation>resorption). After the peak mass has been obtained, orderly remodeling ensures that the amount of bone resorbed by osteoclasts is balanced by the amount of new bone formed by osteoblasts until the age of 40. A net loss in bone mass occurs when changes in bone turnover result in increased bone resorption or decreased bone formation (Hurley and Khosla, 1997). With aging, a slow phase of bone loss occurs both in men and women (Type II osteoporosis). It is due to a relative deficiency of bone formation while the rate of bone resorption remains normal. Type I osteoporosis occurs in postmenopausal women because of the estrogen deficiency. In this subset of osteoporotic women, bone resorption and bone formation are both occurring rapidly in a coupled fashion; however, there is a greater increase in bone resorption as compared to bone formation. The greatest loss in bone with Type I osteoporosis is characterized by increased incidence of vertebral and wrist fractures (Riggs and Melton, 1983). For most women entering menopause, trabecular loss is approximately 1% per year. Some women however, may have accelerated loss of up to 5% per year beginning at menopause (Fig. 2.7(B), continuing for 15-20 years. In contrast for men, the rate of loss of bone mass is 0.25-1% per year thus, women have an increased predisposition to osteoporosis with aging, not only because of lower peak mass than men but also because of the menopause related accelerated bone loss (Riggs and Melton, 1986).



(A) (B)

Figure 2.7: The Osteoporosis Continuum (www.osteofoundation.org/facts.htm)

2.5.5 Risk factors

Several risk factors associated with the development of osteoporosis have been identified and categorized as biological (or non-modifiable), medical (modifiable) and lifestyle (modifiable), as indicated in Fig. 2.8. The relative contribution of the individual risk factors in predicting fracture due to osteoporosis is greatly influenced by the age at which they are expressed (Owens). Studies demonstrate that genetic factors play an important role in regulating bone density, skeletal geometry and bone turnover as well as contributing to the pathogenesis of osteoporotic fracture itself (Sutcliffe, 2005). Racial differences are too observed in skeletal size, with black people having larger, heavier bones and lower fracture risk whereas white and Asian women at highest risk (Melton, 1991).

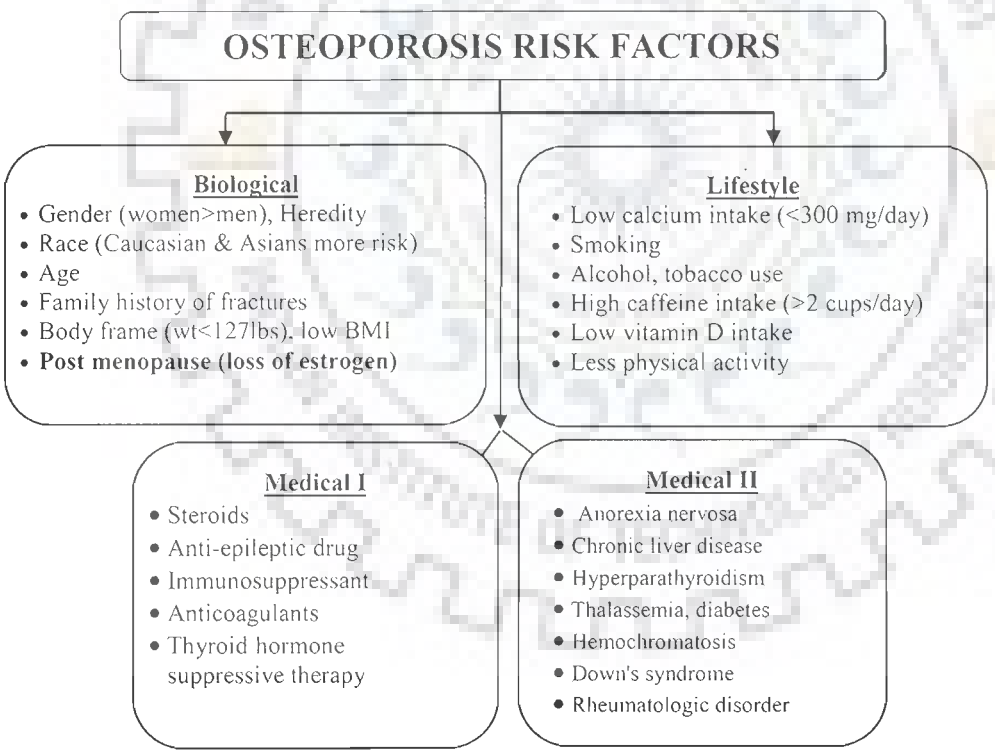


Figure 2.8: Osteoporosis risk factors (www.mayoclinic.com/health/osteoporosis/DS00128)

Sex steroids are also essential for skeletal development and the maintenance of bone health throughout adult life. Estrogen deficiency at menopause is a major pathogenetic factor in the development of osteoporosis in postmenopausal women (Compston, 2001). The age at which females begin menstruation and menopause is also the factor that influences the

development of osteoporosis. The shorter the period between menarche and menopause, the higher the subject's risk for development of osteoporosis (Kenny and Raisz, 2002).

Evidence suggests that calcium intake is important during skeletal growth and peak bone mass development and calcium supplementation may be effective in reducing bone loss in postmenopausal women, particularly in those with low habitual dietary calcium intake (Heaney, 2000). Vitamin D is necessary for the optimal absorption of calcium and its deficiency contributes to osteoporosis and fractures through its effects on bone fragility and impaired muscle strength (Reid, 2003). Reduced sunlight exposure in certain ethnic race females due to strict dress codes with full body covered also exposes them towards osteoporosis. High caffeine intake has been associated with decreased bone mineral density in postmenopausal women who have low calcium intake (Massey, 1991). Smoking increases the risk, but effect weans slowly after a person stop smoking (Kanis et al., 2005). Physical activity stimulates bone formation, while immobilization leads to rapid bone loss (Snow et al., 1996). Number of clinical disorders that affect bone density includes endocrine abnormalities, immobilization, and adverse effect of medications, renal disease, cancers and disorders of gastrointestinal tract. It is estimated that 20 to 30 percent of postmenopausal women may suffer from these secondary causes (Caplan et al., 1994).

2.5.6 Diagnosis of osteoporosis

2.5.6.1 Bone mineral density measurements

Bone mineral density (BMD) test is the best way to determine bone health. BMD tests can identify osteoporosis, determine the risk for fractures and measure the response to osteoporosis treatment. DXA (dual-energy x-ray absorptiometry) and quantitative computer tomography (qCT) are the most widely recognized bone mineral density tests.

DXA is an extremely effective procedure for diagnosing the bone mass changes. The fundamental principal behind the machine is measurement of the transmission through the body

of X-rays of two different photon energies. It measures BMD at the specific vulnerable sites of spine and hip and may also be used to perform total body and forearm measurements (Sutcliffe, 2005). Measurements of BMD are generally given as the concentrations of mineral content in areas that are scanned. Clinical benefits of DXA include low radiation dose, usefulness in evaluating multiple sites, reliability, good precision, short scanning time and ease of use (Kenny, 2002). BMD measurements are expressed as statistical scores (T & Z) measured in SD or standard deviation units. T-score is calculated by taking difference between an individual's BMD and the mean value for normal young adult and Z score is relative to the mean value for control subjects of the same age. According to WHO, osteoporosis is defined on the basis of T score. The lower the score, the lesser is the bone present and higher is the risk for fractures. T-score more than -1 SD is normal. Value between -1.0 to -2.5 SD defines the state of osteopenia, where BMD is below normal and may lead to osteoporosis. Score < -2.5 SD defines the osteoporotic bone site (Fig. 2.9). The test is highly recommended by the expert groups for all the women above 65 years, woman who suffered any fracture and postmenopausal women under 65 years with one or more risk factors for osteoporosis.

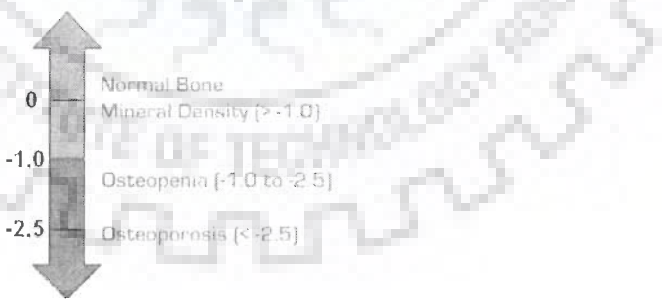


Figure 2.9: WHO criteria for Osteoporosis (www.osteofoundation.org/facts.htm)

Quantitative computed tomography (qCT) uses computer tomography (CT) scanning to measure bone mineral density. A phantom with known concentration of hydroxyapatite is scanned simultaneous to the subject. Attenuation numbers for specific regions of interest from the CT scan of the subject is compared to this control (Owens). The region of interest of bone mineral measurement with qCT is a well defined volume and can allow for measurement of

trabecular bone without including measurement of cortical bone. qCT measures bone mineral as a true volume density (mg/cm3) and is therefore less influenced by the size of the bone (Shore and Poznanski, 1999). qCT gives a true bone density measurement (mass/unit volume) but it is more costly than DXA and fewer normative data are available for reference (Gertner, 1999). Ultrasound techniques are also used. They measure bone density in the heels, fingers and leg bones. Advanced ultrasound techniques such as quantitative ultrasound (QUS) are promising for improving accuracy in predicting fractures when used with DXA. It is less expensive and uses no radiation. Furthermore, in the laboratory setting one of the methods used to measure BMD based on Archimedes principle had also been established (Kalu, 1991).

2.5.6.2 Biochemical markers

Bone remodelling is a continuous process involving bone resorption and formation. In the recent years, the isolation and characterization of cellular and extracellular components of the skeletal matrix have resulted in the development of various serum and urinary markers. These markers specifically reflect the activity of processes involved in remodelling and are therefore useful in the assessment and differential diagnosis of osteoporosis (Seibel, 2006). They are non invasive and comparatively inexpensive. Table 2.3 shows the summary of bone turnover markers including both cellular derived enzymes and non enzymatic peptides.

2.5.6.2.1 Markers of bone formation

Osteoblasts synthesize and secrete number of proteins which can be measured in serum as markers of their activity and therefore of bone formation. The most commonly used markers of bone formation (Table 2.3) are alkaline phosphatase, osteocalcin and propeptide of type I procollagen (Shields and Chesnut, 2001; Swaminathan, 2001).

Table 2.3: Summary of biochemical markers

Marker	Specimen	Analytical method
Bone formation markers		
Serum osteocalcin (OC)	Serum	RIA, ELISA,IRMA
Serum bone specific alkaline phosphatase (BAP)	Serum	Electrophoresis, precipitation , IRMA
Serum procollagen type I c-terminal propeptide (PICP)	Serum	RIA, ELISA
Serum procollagen type I n-terminal propeptide (PINP)	Serum	RIA, ELISA
Bone resorption markers		
Collagen related		
Serum n- & c- telopeptides (NTx, CTx)	Urine, serum	ELISA, RIA
Urinary total & free pyridinoline & deoxypyridinoline	Urine, serum	HPLC, ELISA
Non collagen related		
Bone sialoprotein (BSP)	Serum	RIA, ELISA
Osteoclasts enzymes		
Serum tartrate resistant acid phosphatase (TRAP)	Plasma serum	Calorimetry, RIA, ELISA

(modified from (Seibel, 2006)

Alkaline phosphatases (ALP)

Alkaline phosphatases are the plasma membrane enzymes. Total ALP in serum includes several isoforms. Bone alkaline phosphatase is the bone specific isoenzyme where it is thought to have a specific function involved in mineralization (Whyte, 1994). It may increase local concentrations of inorganic phosphate, or act as calcium binding protein or ATPase but no definitive function has been established. The major factors that modify ALP activity are age, sex and hormonal status. Activity is generally found to be higher in postmenopausal women than in premenopausal women (Schiele et al., 1983).

Osteocalcin (OC)

OC is one of the most extensively studied biological markers of bone formation. It is a small protein (5800kDa) synthesized by mature osteoblasts, odontoblasts and hypertrophic chondrocytes. It is the most abundant non-collagenous protein in bone. It contains three residues of gamma carboxyglutamic acid (γ-gla) residues and is sometimes referred to as bone gla protein or BGP. The gla residues cause a specific conformational change in protein in the presence of ionic calcium, which in turn causes the binding of OC to bone mineral and

accumulation in bone matrix (Hauschka et al., 1989). While OC is primarily deposited in the extracellular matrix of bone, a small fragment is released into circulation during bone resorption. Therefore, at any point in the serum, concentration of osteocalcin should be regarded as measure of bone formation in particular and bone resorption in general. Serum OC is greater in infants and children than in adults, with peak values occurring at puberty. Several studies in women show a rise with menopause which is correlated with an increase in bone turnover rate as assessed by histomorphometry and calcium kinetics (Brown et al., 1984).

Procollagen I extension peptides

Type I collagen, which makes up to 90% of the organic matrix of bone, is synthesized as a procollagen precursor molecule. Before the collagen incorporates into the bone matrix, specific endopeptidases cleave the procollagen molecule at precise sites. First cleavage occurs at amino terminus and then at carboxy terminus to produce type I collagen propeptide; PICP and PINP. The propeptides are produced not only by bone but also by tissues that synthesize type I collagen like skin, gingival, cornea, dentin, tendon and fibro cartilage. None of the assays eliminate potential contribution to circulating propeptide from soft tissues but because the rate of turnover of collagen in bone is faster than in other tissues, the changes in propeptides are assumed to reflect changes primarily in bone collagen synthesis (Calvo, 1996).

2.5.6.2.2 Markers of bone resorption

The most commonly used markers of bone resorption (Table 2.3) are tartrate resistant acid phosphatase, serum and urinary n- & c- telopeptides (NTx, CTx) and urinary total & free pyridinoline & deoxypyridinoline (Calvo, 1996; Swaminathan, 2001).

Acid phosphatases

Acid phosphatases are the heterogeneous group of enzymes. There are at least six isoforms. Two isoforms have been demonstrated in osteoclasts. Two types include a large isoenzyme, which is sensitive to tartrate and a small isoenzyme which is resistant to tartrate i.e.

TRAP (Tartrate resistant acid phosphatase). TRAP also known as type 5 acid phosphatase is present in large quantities in the ruffled border of osteoclasts and is released during resorption. Serum TRAP can be measured by immunoassays based on antibodies (Nakasato et al., 1999). TRAP activity was inversely correlated to bone mineral density in postmenopausal women with osteoporosis (de la Piedra et al., 1989).

Pyridinoline & deoxypyridinoline (PYD, DPD)

Collagen is stabilized by the formation of covalent cross-links between the end of one collagen molecule and the helical portion of the adjacent collagen molecule. Two major types of cross-links are pyridinoline (PYD) and deoxypyridinoline (DPD) (Swaminathan, 2001). Cross-links are formed extracellularly after the deposition of collagen molecule into the matrix. They are released from bone only during bone resorption or collagen breakdown. PYD is widely distributed in cartilage and DPD is present in bone, dentine and ligaments. When bone matrix is degraded by osteoclasts, these molecules are released from mature collagen into circulation and excreted into urine. The excretion of cross-links is increased in osteoporosis and in situations where bone resorption is increased such as hyperthyroidism and hyperparathyroidism. PYD and DPD can be measured by HPLC after hydrolysis to measure total or without hydrolysis to measure free forms. Immunoassays sensitivity has allowed for measurement of free pyridinoline fractions and hence a method of analyzing bones degradation (Urena et al., 1995).

Cross-linked telopeptides of collagen I (NTx, CTx)

During bone resorption, only about 40% of the cross-links are released as free pyridinium cross-links. The remaining 60% are in form of peptide attached cross-links. Two cross-links forming sites exist in type I collagen. One site is in carboxy terminal and another in the amino terminal of the molecule. Antibodies have been raised against both the telopeptides

and immunoassays have been described (Garnero and Delmas, 1998) to measure the N-terminal (NTx) and C-terminal telopeptides (CTx, Cross laps) in urine and serum and urine respectively.

2.5.7 Treatment and Prevention

Osteoporosis affects considerable portion of female population. Once a woman goes through menopause and rapid bone depletion occurs, the line between prevention and treatment blurs. Management of osteoporosis involves both pharmacologic and nonpharmacologic treatments.

2.5.7.1 Pharmacologic approach

In general, pharmacological agents treat either by decreasing bone resorption to produce secondary gains in bone mass or are anabolic and produce direct increase in bone mass As the turnover of bone is slow, the time between starting treatment and assessing its effect on bone mass or fracture takes several years (Akesson, 2003). Anti resorptive treatments include hormone therapy, bisphosphonates, calcitonin and SERM (selective estrogen receptor modulators). Teriparatide acts in an anabolic way. Strontium ranelate is the new class of dual acting bone agents (DABAs) which increases bone formation and reduces bone resorption (Sutcliffe, 2005).

2.5.7.1.1 Hormone replacement therapy

Maintaining adequate hormone (estrogen) levels remains the most important way of maintaining adequate bone density in women (South-Paul, 2001). This therapy comprises treatment with estrogen with the addition of cyclical or continuously administered progesterone in women with a uterus (Sutcliffe, 2005). Prospective cohort studies and large randomized clinical trials have demonstrated its efficacy in terms of prevention of postmenopausal bone loss (Akesson, 2003). Findings from the Women's Health Initiative (WHI), a series of large randomized clinical trials performed in healthy women aged 50-79 yrs in US have shown beneficial effects of continuous combined estrogen and progesterone therapy on fracture

outcomes (Rossouw et al., 2002). However, this study was stopped after 5 years due to the development of breast cancer and the long term absence of benefits for cardiovascular events. Based on the study, the therapy is now curtailed in older women but in young postmenopausal woman HRT remains effective in preventing fracture and climacteric symptoms if any (Randell et al., 2002). Women who start HRT within five years of menopause had a decreased risk of fractures compared to those who never used estrogen. Long term users who initiated therapy five years after menopause had no significant reduction in risk for fractures, despite an average use of 16 years. Therefore, early initiation with respect to menopause is more important than the total duration of use (South-Paul, 2001).

2.5.7.1.2 Bisphosphonates

They are the most commonly used anti-resorptive agents. They are derived chemically from pyrophosphates compound that inhibits precipitation of calcium carbonate. The P-O-P structure of pyrophosphate is replaced by P-C-P in bisphosphonates thus reducing the bone turnover by inhibiting recruitment and promoting apoptosis of osteoclasts (Delmas, 1996). The plasma half life of bisphosphonates is very short but the half life of bisphosphonates deposited in bone is probably up to 10 years or could be longer. Alendronate and risedronate are the readily available forms of bisphosphonates. Most studies for these two have been performed in post-menopausal women but Gonnelli et al, 2003 also demonstrated favorable long term effects of alendronate in men (Gonnelli et al., 2003). They are also the most effective agents for managing glucocorticoid induced osteoporosis (Amin et al., 2002). Alendronate and risedronate increase BMD at relevant skeletal sites and significantly reduce the risk of vertebral and non vertebral fractures (Cummings et al., 1998; Harris et al., 1999). Bisphosphonates are absorbed poorly and therefore may cause mild gastrointestinal problems. Poor absorption complicates the oral administration and has prompted the development of new dosing regimes as well as new compounds through side chain substitutions. Intravenous injections of aminobisphosphonate, ibandronate, administered as bolus (every 3 months) in postmenopausal

women led to increase of 3 – 5.2% after 12 months when compared with patients who received placebo (Thiebaud et al., 1997). Long term risks involve development of stomach ulcers.

2.5.7.1.3 Calcitonin

Calcitonin has rapid but short lived effect on the osteoclasts. It is given subcutaneously or intranasally with variation in dose and administration (Sutcliffe, 2005). Formulations are developed from salmon calcitonin, which is about 10 times more potent than naturally produced human calcitonin. In a randomized trial, in postmenopausal women, vertebral fractures were reduced by 33% after salmon calcitonin was given at a dose of 200 IU daily via nasal spray (Chesnut et al., 2000). It is very useful in the acute management of vertebral fractures where it appears to confer analgesic properties, leading to reduction in pain within two weeks with subsequent improvement in mobility (Maksymowych, 1998). Side effects include flushing, vomiting, diarrhea, headache, dizziness and local irritation when injected and nasal crusting or secretion when taken intranasally. Long term use involves the risk of developing allergic reactions or resistance to this protein.

2.5.7.1.4 SERM (Selective Estrogen Receptor Modulator)

Selective estrogen receptor modulators are the class of medications which act on estrogen receptors. Raloxifene and tamoxifen belongs to this class of substances.

Raloxifen, induces conformational changes in the estrogen receptor and therefore has opposite activities in specific tissues. It has agonist actions on skeleton (anti resorptive agent) and antagonist effect on breast tissue, where it can lead to a reduction in estrogen responsive tumors (Cauley et al., 2001). In postmenopausal women treated with raloxifene vertebral fractures were reduced by 30% but no effect was observed in nonvertebral sites (Ettinger et al., 1999). Side effects include the risk of developing thromboembolism and generation of menopausal symptoms.

Tamoxifen has been known to antagonize bone resorption, uterine growth and reduces the number and size of osteoclasts (Turner et al., 1988). Tamoxifen acting as a partial estrogen agonist seems to have beneficial effects on the conservation of bone mass in postmenopausal women, while in premenopausal women it acts as an estrogen antagonist (Diez, 2000).

2.5.7.1.5 Teriparatide

Teriparatide is the portion of human parathyroid hormone (PTH). It represents amino acid sequence 1-34 of the complete molecule of 84 amino acids. Endogenous PTH is the primary regulator of extracellular calcium levels. Daily injection of teriparatide, owing to its anabolic mode of action helps to increase bone formation and improves bone strength, bone volume and trabecular architecture. It has been reported to increase the spine BMD within three month of administration and reduces the risk of all vertebral fractures by 65% (Neer et al., 2001). Although well known, the high cost of drug limits its use to either those with severe osteoporosis or those who are unresponsive to other agents or those who are prohibited to use other agents because of the side effects generated (Akesson, 2003). The most common side effects reported are nausea, limb pains and dizziness.

2.5.7.1.6 Strontium ranelate

Compound of ranelic acid and strontium is a tasteless water soluble powder. It is the first drug in new class of therapies that has dual action in both stimulating bone formation and decreasing bone resorption. This action leads to overall increase in the bone strength due to increased bone mass and BMD (Sutcliffe, 2005). Randomized trials have shown increase in both spine and hip BMD with a reduction in vertebral, hip and other non-vertebral fractures (Reginster et al., 2003). In randomized trials, in postmenopausal women with osteoporosis, strontium ranelate increased BMD in a dose dependent manner by 2-3% per year compared to placebo (Meunier et al., 2002). Mild side effects include nausea and diarrhea.

2.5.7.2 Non-pharmacologic approach

Although pharmacologic approaches represent the cornerstone of treatment, some patients cannot, or will not, comply with medication regimens. This is particularly true for regimens involving drugs with potential adverse effects, but also occurs in those who cannot afford certain medication options or are in conflict with taking any medication options for prolonged time (Lin and Lane, 2008). Non-pharmacological approaches such as use of orthoses, exercise programs, calcium and vitamin D supplementation, fall prevention and kyphoplasty complement the traditional pharmacologic treatment of osteoporosis and can have a significant role in minimizing and preventing the fracture risk.

2.5.7.2.1 Orthoses

Orthoses, such as thoracolumbar braces, are often prescribed for osteoporotic patients with vertebral compression fractures. Jewett and cruciform anterior spinal hyperextension (CASH) braces are rigid hyperextension braces traditionally used in patients with acute fractures. Some find these braces constricting so they opt for other types. Potential alternatives include the posture training support brace and vest, both with the weights as well as spinomed and hip protectors. Posture training support has been found to result in pain reduction in patients with acute compression fractures in one pilot study (Kaplan and Sinaki, 1993). Spinomed is a newer brace that consists of a back pad and a strap system with fabric hook and loop fasteners (Pfeifer et al., 2004). It has been shown that patients who wore this brace for 6 months experienced increased trunk muscle strength and posture as well as improved quality of life secondary to pain reduction. Hip protectors are composed of garments with padding over the trochanters. These orthoses help to absorb the impact of a fall and therefore reduce the risk of hip fractures. (**see** also (Lin, 2008).

2.5.7.2.2 Exercise programs

Exercise programs can do wonders for osteoporotic patients. It can provide overall increase in strength, flexibility and balance with diminished risk of falling. It has also been shown that supervised exercise programs increase BMD with larger changes noted in patients undergoing exercise and pharmacologic treatment than in those undergoing pharmacologic treatment alone (Villareal et al., 2003). Additional effects included decreased weight, decreased fat mass and improved muscle strength. Weigh bearing exercises like walking, running and impact exercises applies tension to muscle and bone and thus encourages body to compensate for the added stress by stimulating osteoblasts to increase the bone density. In menopausal women such exercises are very protective (Kohrt et al., 1995). Thoracic stabilization exercises, particularly which strengthen the back extensor can help to improve posture and might reduce the risk of falls (Sinaki et al., 1996). Osteoporosis fitness classes are often coordinated by physical therapists. A comprehensive program should include strengthening exercises, balance training, body mechanics and home exercise program.

2.5.7.2.3 Calcium and Vitamin D

Both calcium and vitamin D are essential in the treatment of osteoporosis. Calcium is available in multiple formulations, the most common being calcium nitrate and calcium carbonate. Vitamin D is essential for calcium absorption and therefore has a key role in bone health. This is really important in older age because calcium absorption and vitamin D production are affected by normal biological age changes at the organ, cellular and molecular level affecting the absorption, transport, synthesis and depositing mechanisms. Recommended intakes of calcium and vitamin D for the prevention or treatment of osteoporosis are as follows:

- Children: 800-1200 mg /day
- Adolescent girls: 1200-1500 mg/day
- Premenopausal women (19-50yrs): 1000 mg/day
- Older adults (51-70yrs): 1200-1500 mg/day
- Older adults (51-70yrs): 400 IU vitamin D /day and > 71 yrs 600 IU/day.

Postmenopausal women receiving supplemental calcium over a three year period in a placebo controlled, randomized clinical trial had stable total body calcium levels and BMD in lumbar spine, femoral neck compared with women in placebo group (Aloia et al., 1994).

2.5.7.2.4 Kyphoplasty

Kyphoplasty is the minimally invasive spine procedure that involves the infiltration of bone cement into a fractured vertebral body after fracture reduction using a balloon tamp. It can result in diminished pain and reduce kyphosis. Study by Garfin et al, 2006 demonstrated that balloon kyphoplasty resulted in rapid, significant and sustained improvements in back pain, back function and quality of life (Garfin et al., 2006).

2.5.7.2.5 Lifestyle changes

Smoking and heavy alcohol consumption speeds up the bone loss and thus significantly increases the risk of fractures. Avoidance of both helps sustain the BMD and hence will improve the bone strength.

2.5.7.2.6 Natural products

The phytoestrogenic herbs e.g. *Cimicifuga racemosa, Glycine max, Dong Quai, Ginseng, Gingko* contain estrogenic components produced by plants. Phytoestrogens is a general term used to define this wider class of polyphenolic compounds found in all plants (Duncan et al., 2003; Knight and Eden, 1995). They are non-steroidal and are either of plant origin or derived from the *in-vivo* metabolism of precursors present in several plants eaten by human beings. They have a chemical structure very similar to the mammalian estrogen, estradiol, and therefore it comes as no surprise when they possess estrogenic properties. However, their estrogenic activity is generally 1/500 to 1/1000 of the activity of human estradiol. As far as their metabolism is concerned these compounds after entering the body undergo hydrolysis yielding aglycones. These aglycones are either excreted in the urine or bile

or absorbed from the gut. On absorption they undergo conjugation in the liver with glucuronic acids or sulphate and follow the pathways of further metabolism.

Phytoestrogens forms the actual components that make herbs useful as alternative for the hormone replacement therapy. Phytoestrogens interact with numerous molecules, carrier proteins, enzymes, membrane and nuclear receptors directly or indirectly involved in the transfer of estrogen signals (Benassayag et al., 2002). Phytoestrogens are broadly classified into Isoflavones, Lignans, Stilbenes and Coumestanes. Isoflavones and Lignans are the most extensively studied. Genistein, Daidzein, Biochanin and Formononetin are some of the main types of isoflavones that has been characterized. They are found almost exclusively in legumes including soy, lentils and beans, the soybean being the most abundant source. Foods containing soya has been known to be beneficial to bone. Several in vitro and in vivo studies have shown the beneficial effects of soy proteins during osteoporosis (Mundy, 2001; Yamaguchi and Gao, 1998). It is an excellent source of phytoestrogens, the major type being isoflavones genistein and daidzein. Arjmandi et al showed that in ovariectomized rats the bone sparing effect of soy is related to its isoflavones (Arjmandi et al., 1998). Several other studies have corroborated the studied effect of Arjmandi and co-workers (Blair et al., 1996; Harrison et al., 1998). Study in the Japanese women also suggests that high consumption of soya in their diet (30-60 mg/day) may contribute to the low prevalence of osteoporosis in Japan (Muhlbauer et al., 2003). There have been few studies in patients but Baum et al has showed in a 6 month study of 66 postmenopausal women that the women who were receiving a diet with larger doses of isoflavones had beneficial effects on bone mineral content and density in the lumbar spine (Baum et al., 1998). Ipriflavone, a synthetic isoflavone derivative, has been studied extensively with regard to the prevention of osteoporosis. It appears to inhibit bone resorption slowing the bone loss, thereby improving bone density. Daidzein is one of the main metabolites of ipriflavone. Phytoestrogens also reduce menopausal symptoms like hot flashes and other climacteric symptoms.

2.5.8 Animal models for study of osteoporosis

Animal model of postmenopausal bone loss can be defined as a living animal in which spontaneous or induced bone loss due to ovarian hormone deficiency can be studied, and in which the characteristics of the bone loss and its sequalae resemble those found in postmenopausal women (Kalu, 1991). Several small and large animals like rats, sheep and rabbit have been used to study osteoporotic condition (Miller et al., 1995). Of these animals, the mature ovariectomized (ovx) rat, 3 month old has been validated to represent the most important clinical features of cancellous bone loss induced as a result of estrogen deficiency as in case of postmenopausal women. It has been extensively used for testing the potential of new drugs to prevent and reverse bone loss (Namkung-Matthai et al., 2001; Thompson et al., 1995).

Characteristics which rat model shares with postmenopausal bone loss includes:

- Increased bone turnover that led to greater resorption than formation of cancellous bone.
- An initial rapid phase of loss followed by a much slower phase.
- Significant decrease in metaphysial cancellous bone than cortical bone.
- Increased sensitivity of long bones to loss than vertebras.
- Increase in serum and urinary biochemical markers of bone turnover.
- Decreased intestinal calcium absorption, protective effect of obesity against bone loss
- Similar response to therapies like estrogen, bisphosphonates, parathyroid hormone, calcitonin, tamoxifen, exercise, vitamin D and its analogs. for detailed references for all characteristics see (Kalu, 1991).

It also offers other advantages like; easy handling and maintenance, cost effective, easy availability and purchase due to less of social pressures. The most important advantage is the ease to use this model system in monitoring systems like peripheral micro-computed tomography (qCT) compared to other animals like sheep, rabbits, dogs and non human

primates. From the above available research it is evident that ovariectomized rat is the appropriate and relevant model to study postmenopausal bone loss.

2.5.9 Plant extracts – --As Anti-osteoporotic agents

Nowadays much of the focus has been driven towards seeking regimes like medicinal herbs. This involves little or no risk and can be considered as effective and safest way for treating osteoporosis. Several studies have revealed that administration of plant extracts (via food, subcutaneous, orals, i.g. or po) is associated with greater mineral density in the animal model system or human trial studies of osteoporosis. They either decrease the rate of resorption, promote the rate of bone formation and sometimes both thereby protecting the model system against bone loss. As the single herb extracts are tested, Chinese, Japanese and Indian culture also uses some traditional formulae containing combination of herbs in the treatment of osteoporosis. Table 2.4 shows some of the many reported plants and formulations that have been useful in the management of osteoporosis. The mode of treatment includes type of extract, model tested and duration of application of plant dose.

2.5.10 Safety and efficacy of plant extracts

The herbal drugs are believed to act holistically to improve symptoms, slow disease progression, correct imbalance and adjust the immune system to restore health and quality of life. They also have reputation of being safe and efficacious (Nagareddy and Lakshmana, 2006). Current means available to evaluate the safety and efficacy of plant extracts in the model system of osteoporosis include checking their proliferative effect in reproductive organs, histomorphometric measurements (cellular activity during remodelling), bone quality tests (predict risk of fractures), physicochemical studies (gross structural differences) and biochemical analysis (balance between formation and resorption). Table 2.5 shows the detailed techniques as per categories and the selected references to look for complete protocols.

Table 2.4: Review of plant extracts as antiosteoporotic agents

PLANT / FORMULATIONS	MODE OF TREATMENT	REFERENCE
Decreased Bone resorption		
Abelmoschus manihot	Via food, 3 months	(Puel et al., 2005)
Curculigo orchioides	Ethanolic extract, ig, 3 months	(Cao et al., 2008)
Salvia miltiorrhiza	Tashinone extract, 10 weeks	(Cui et al., 2004)
Shu Di Shan Zha	Postmenopausal women, 4 months	(Putnam, 2007)
Erythrina variegate	Ethanolic extract, oral, 14 weeks	(Zhang et al., 2007)
OST-6 herbomineral prep.	Orally , 16 weeks	(Prabhakara Reddy and Lakshmana, 2003)
Withania somnifera	Water extract, oral, 16 weeks	(Nagareddy, 2006)
Increased bone formation		
Anemarrhena asphodeloides	Ethanolic extract, po, 3 months	(Nian et al., 2006a)
Anoectochilus formosanus	Water extract, oral, 3 months	(Shih et al., 2001)
Cimicifuga racemosa	Extract BNO1055, food, 3 months	(Seidlova-Wuttke et al., 2003b)
DBWC (Dae-Bo-Won-Chun)	Water extract, 7 weeks	(Chae et al., 2001)
Lepidium meyenii	Ethanol extract, oral, 28 weeks	(Zhang et al., 2006)
Onobrychis ebenoides	MeOH extract, water, 3&6 months	(Dontas et al., 2006)
Effecting both formation and resorption		
Epimedii sagittatum	Water extract, ig, 11 weeks	(Nian et al., 2006c)
Cissus quadrangularis	Ethanol extract, oral, 3 months	(Shirwaikar et al., 2003)
Shen Gu	Human trial , 6 month	(Mingyue et al., 2005)
Dioscorea spongiosa	MeOH and water extract, oral, 6wk	(Yin et al., 2004)
Er xian decoction (EXD)	Water extract, oral, 3 months	(Nian et al., 2006b)
Herpa epidemii	Water extract, oral, 3 months	(Xie et al., 2005)
Wedelia calendulacea	Ethanolic extract, 3 months	(Annie et al., 2006)

Table 2.5 Evaluation means for the safety and efficacy of the plant extracts

<table>
<tr><td colspan="2">Safety evaluation</td></tr>
<tr><td colspan="2"><u>Reproductive organs examination</u>
• Uterine and vagina weight (if increased, usage as medication is concerned).
• Histological examination of the change in thickness of uterine layers and lobules (I & II) and ducts in mamma
• Gene expression studies (IGF-1, C3, ERα, ERβ) (Seidlova-Wuttke et al., 2003a).</td></tr>
<tr><td colspan="2">Efficacy evaluation</td></tr>
<tr><td colspan="2"><u>Histomorphometry</u>
• Processing and sectioning of the undecalcified bone specimens and assessing the activity going on during turnover using histochemical staining (H&E, goldner staining, tetracycline labeling).
• Static and dynamic measurements include bone volume, trabecular (trab) bone volume, trab separation, trab number, trab thickness, osteoclasts and osteoblasts number, % of labeled perimeter (Chae, 2001; Cui, 2004; Xie, 2005).</td></tr>
<tr><td><u>Physicochemical studies</u>
• Bone mineral density measurements (DEXA, qCT, Archimedes)
• Scanning electron microscopy (Prabhakara Reddy, 2003)
• Thermal analysis (DTA, DTG) (Okamoto et al., 1998).
• X-ray diffraction patterns, Infra red spectroscopy (Bigi et al., 1997).</td><td><u>Biochemical analysis</u>
• Bone formation and resorption markers (discussed earlier)</td></tr>
<tr><td><u>Bone quality tests</u>
• Length and width of bone
• Bone ash measurements (bone mineral content) (Prabhakara Reddy, 2003).
• Mechanical strength tests (bending, compression & loading tests (Nian, 2006b).</td><td><u>Additional benefits</u>
• Body weight normalization
• Reduction in the body fat (Seidlova-Wuttke, 2003b).</td></tr>
</table>

CHAPTER 3

MEASURING CLIMATERIC SYMPTOMS AND AGE AT NATURAL MENOPAUSE IN UTTARAKHAND POPULATION USING THE GREENE CLIMACTERIC SCALE

3.1 INTRODUCTION

Natural menopause is caused by aging of the ovaries, which leads to a decline in the production of the hormones (estrogen and progesterone) that control the overall function of a female's body. Hormone deficiency elicits various somatic, vasomotor, sexual and psychological symptoms that affect the overall quality of women's life. The nature and severity of symptoms are such that they vary not only among individuals of same population but also among different cultures, ethnicities and countries. The occurrence of menopause between 45-60 yrs of age has been established worldwide. In the Indian subcontinent, the reported age is between 40-49 yrs (Sharma, 2007) and is younger than that of other developed countries with onset at more than 50 yrs (Gold, 2001; Obermeyer, 2000). Across India, several studies have been done to determine the age at natural menopause and the factors affecting its onset. However, no such data has been generated from the northern state of India, known as Uttarakhand. The present study was planned with an aim to find the age at onset of menopause and the nature and prevalence of menopausal symptoms and to identify any socio-demographic, physical or other factors that may influence its onset.

3.2 MATERIALS AND METHODS

3.2.1 Participants

Women residents of the Haridwar district of Uttarakhand state were considered for the survey. The sample size comprised of 200 women in the age group of 30-65 yrs. The randomly selected sample population included participants from different locations, such as women attending the gynecological departments of various hospitals, private nursing homes and ladies clubs; those employed in schools and colleges; and also women living in any other household

or street in the district. Out of the total 200 women who gave their consent, only 129 healthy women were selected in the survey. Those participants who a) had surgical menopause, b) had serious illness like hypertension, fibroids, thyroidism, migraines, diabetes and spondilitis, c) were users of any type of medication for menopause, d) were unable to understand the questionnaire and e) returned forms with missing information were excluded from the survey.

3.2.2 Classification of participants

The selected participants were divided into three categories: premenopause (n=70), early postmenopause (1-5 yrs after last menstrual cycle; n=33) and late postmenopause (>5 yrs after last menstrual cycle; n=26). Menopausal status of the participants was defined using World Health Organization (WHO) criteria. Women who had regular menstruation cycles during the last three months were in the category of premenopause and those who had no cycle in the previous 12 months were considered in the postmenopausal phase. Early and late postmenopause status was defined using the Stages of Reproductive Aging Workshop (STRAW) staging system (Soules, 2001). The staging system has seven stages; five precede and two follow the final menstrual cycle. Stages -5 to -3 encompass the reproductive interval; -2 to -1, the menopausal transition; and +1 to +2, the postmenopause interval. In the staging system, early postmenopause is 1 to 5 yrs after the final menstrual period was considered relevant because it encompasses dampening of the ovarian function to a permanent level with no further change. Late postmenopause duration was considered variable, as it ends with woman's death.

3.2.3 Collection of information

Personal interviews were conducted using a well structured questionnaire based on a four point scale. The questionnaire collected information about the age, religion, marital status, education, socio-economic status, employment, use of oral contraceptives, lifestyles, dietary habits, exercise regime, and parity (Fig. 3.1, PART A). Height and weight were also recorded

to calculate body mass index (BMI; weight [kg]/ height [m^2]). On the basis of WHO guidelines, (WHO, 1996) participants were classified as underweight (BMI <18.5 kg/m^2), normal (BMI 18.5-25 kg/m^2), overweight (BMI > 25 kg/m^2) and obese (BMI > 30 kg/m^2). The socio-economic status of the participants was broadly divided into two groups. Illiterate nonworking or labourers, who find it hard to pay for their basic amenities, were grouped in the category of poor and participants who were literate as well as working and can easily pay for their basic amenities but strive hard to enjoy luxuries, were considered in the category of middle class. For the classification of lifestyles, participants were considered sedentary when they were unemployed, do less of physical activity and for recreation only watch television; Active when they were employed (< 6 hr) for example, in schools, do housework and for recreation, do activities like walking. Participants were included in the category of hectic lifestyle when they were employed (> 6 hr), for example, a full time job, do housework and for recreation do walking and play sports like swimming. Women's perception of menopause as a positive or negative change in life was also recorded. The Greene Climacteric scale was used to assess the nature and severity of occurrence of climacteric symptoms among the selected participants (Fig. 3.1, PART B).

3.2.4 Greene Climacteric scale

The Greene climacteric scale is a standard scale for the measurement of climacteric symptoms (Greene, 1998). It independently measures 21 symptoms as psychological (further divided into anxiety and depression, symptoms 1-11), somatic (symptoms 12-18), vasomotor (symptoms 19 & 20) and sexual dysfunction (symptom 21). Four symptoms (22-25), namely, skin dryness, eye problems, high blood pressure and low blood pressure, were not the part of Greene Climacteric scale but were taken in the analysis because many women reported them during the interview. Each symptom is self rated by the participating women according to its current severity using a four point rating scale: not at all (0), a little (1), quite a bit (2) and extremely (3). Total score for a given participant is the sum of all the scores.

Figure 3.1: Format of the Questionnaire used to collect information during survey

PART A

Number:	**Age:**	**Date:**	
Height :		**Weight :**	
BMI :		**Religion :**	
Number of Children:		**Age at start of menopause/ last cycle :**	
		Age at Menarche or start of cycle:	
Menopause: Natural or surgical : If surgical which surgery has been done:			
Tick the appropriate in the following			
Marital status		**Education**	
Divorced		Primary	
Married		Secondary	
Widow		High school	
Single		University level	
Socio-economic status		**Life style**	
Poor		Active	
Middle class		Sedentary	
Rich		Hectic	
Diet		**Employment status**	
Vegetarian		Yes	
Non vegetarian		If yes where	
Mixed		No	
Tea			
Coffee		**Exercise**	
Tobacco		Yes , what kind	
Alcohol		No	
Oral contraceptive use		**Any serious illness**	
Yes		Yes	
No		No	
If yes how long before		If yes which one	
Presently taking			
HOW YOU RATE MENOPAUSE			
As a positive change			
A burden for life			

PART B GREENE CLIMACTERIC SCALE

Symptom	Not at all (0)	A little (1)	Quite a bit (2)	Extremely (3)	Score (0-3)
Heart beating quickly or					
Feeling tense or nervous					
Difficulty in sleeping					
Excitable					
Attacks of panic					
Difficulty in concentrating					
Feeling tired or lacking in					
Loss of interest in most things					
Feeling unhappy or depressed					
Crying spells					
Irritability					
Feeling dizzy or faint					
Pressure or tightness in head					
Parts of body feel numb or					
Headaches					
Muscle and joint pains					
Loss of feeling in hands or					
Breathing difficulties					
Hot flushes					
Sweating at night					
Loss of interest in sex					
Skin dryness					
Eye problems					
Blood pressure high					
Blood pressure low					

(modified from (Greene, 1998)

3.2.5 Statistics used for analyzing data

Data were analyzed by using SPSS 16 (Statistical Package for Social Science software, SPSS, Chicago, IL) and Microsoft Excel for windows. One way analysis of variance (ANOVA) was used to analyze the significant effect of various demographic factors on the onset of menopause. Fischer's exact test was used to analyze the prevalence of symptoms distributed on the basis of menopausal status. One way analysis of variance followed by Bonferroni's multiple comparison tests was applied for analyzing the significance in the total scores and scores for clusters and sub-clusters. All the values for p are two tailed. p-values < 0.05 were considered statistically significant. Significance level $p < 0.05$ remained the same after the Bonferroni's correction.

3.3 RESULTS

3.3.1 Sample population

Table 3.1 shows the demographic characteristics of the population studied. Out of the total 200 women who participated in the survey, only 129 were finally selected and analyzed. Mean ± S.D. age of the population was 51.83 ± 5.8 yrs, and recalled mean ± S.D. age at menopause was 45.02 ± 4.35 yrs. The age at menopause calculated by probit analysis was 46.82 yrs. The entire sample was divided into three categories, with 54.26% in premenopause, 25.58% in early postmenopause (1-5 yrs after last menstrual cycle), and 20.15% in late postmenopause (>5 yrs after last menstrual cycle). Ninety-six percent of the women were Hindu and married, 67% had normal BMI, 73% belonged to middle class families and were housewives, 79% followed an active lifestyle and 82.9% were vegetarians. Parity equal to two was recorded with 44% of the population. An exercise routine was not followed much because 72.8% of the women did not mention it in the questionnaire. Menopause was rated as a positive change in life by 88.1% of the participating women.

3.3.2 Prevalence of symptoms

The standardized Greene Climacteric scale with additional miscellaneous group was used for recording the menopausal symptoms among the population studied. Table 3.2 shows the percentage of occurrence of all the individual symptoms for three categories representing different menopause status. A significant increase in the frequency of symptoms while transitioning from premenopause to early postmenopause and, subsequently stability or decline towards late postmenopause was observed. It is believed that the rise during early postmenopause is because of the substantial fall (about 10 times) in the estrogen levels in comparison to what was present during the cycling life. The significant difference among groups shows that the scale used for the purpose differentiates the categories well. Symptoms most prevalent among the women under study were muscle and joint pains (55.81%), feeling

tired or lack of energy (51.19%), eye problems (49.61%), headache (43.41%) and feeling unhappy or depressed (36.43%), hot flushes (33.33%), irritability (32.36), attacks of panic (31.78%), and feeling dizzy or faint (30.23%). Specifically for postmenopausal women, muscle and joint pains were again ranked at the top, with an occurrence of 22.03%, followed by attacks of panic, loss of interest in sex with values 20.34% and 18.64%, respectively. Mean scores for all the symptoms were calculated, and the total Greene score and individual scores for clusters and sub clusters were computed from these values and are represented in Table 3.3. The psychological cluster scored highest in all the categories and vasomotor scored the least.

3.3.3 Age at the onset of natural menopause

Relationship between various demographic factors like parity, education, BMI, socio-economic status, lifestyles, employment, diet, consumption of tea, exercise, duration of stay in the district, and age at the onset of natural menopause was computed. Table 3.4 lists the factors (socio-economic status and lifestyles) that significantly affect the onset of menopause. Mean ± S.D. age is presented in the table 3.4. Women who belonged to the middle class families were found to have significant late onset of menopause (45.47 yrs) in comparison with women of poor socio-economic status (42.13 yrs). Women who had active or hectic lifestyles achieved earlier menopause compared with women with a sedentary lifestyle. Apart from these two categories, no significant relation between other factors and age at the onset of menopause was observed. Marital status, religion and use of oral contraceptives were excluded from the analysis because more than 96% of the participants belonged to one category and inclusion of such data would have resulted in biased interpretation.

Table 3.1 Social demographic data of the region

Factor	Categories	N	%
Groups (yrs)	Premenopause	70	54.26
	Early post menopause (1-5 yrs)	33	25.58
	Late post menopause (>5 yrs)	26	20.15
Religion	Hindu	125	96.89
	Others	4	3.10
Children	None	12	9.3
	Less than 2	10	7.75
	Equal to 2	57	44.18
	More than 2	50	38.75
BMI	Underweight	6	4.65
	Normal	87	67.44
	Overweight	30	23.25
	Obese	6	4.65
Marital status	Single	2	1.55
	Married	122	94.57
	Widowed	5	3.87
Education	Primary	9	6.97
	Secondary	6	4.65
	High school	8	6.2
	University	56	43.41
	Illiterate	50	38.75
Years in district	≤ 10	11	8.52
	10 to 20	45	34.88
	20-30	52	40.31
	> 30	21	16.22
Socio-economic status	Poor	29	22.48
	Middle	100	77.5
Lifestyle	Active	103	79.84
	Sedentary	7	5.42
	Hectic	19	14.72
Diet	Vegetarian	107	82.94
	Mixed	22	17.05
Tea	≤ 2	62	48.06
	More than 2	67	51.93
Employment	Working	29	22.48
	Housewife	99	76.74
	Retired	1	0.78
Exercise	Yes	35	27.13
	No	94	72.86
Oral contraceptive use	Yes	2	1.55
	No	127	98.44
Menopause rating	Positive	52	88.13
	Burden	7	11.86

Table 3.2 Prevalence percentage of the menopausal symptoms in different groups

	Groups ⟶	Premenopause (n=70)	Postmenopause	
			Early 1-5 yrs (n=33)	Late >5 yrs (n=26)
No.	Symptoms ↓			
1	Heart beating quickly or strongly	8.57	39.39[a]	34.62[a]
2	Feeling tense or nervous	17.14	39.39[a]	26.92
3	Difficulty in sleeping	20.00	36.36	30.77
4	Excitable	20	36.36	26.92
5	Attacks of panic	22.86	48.48[a]	34.62
6	Difficulty in concentrating	18.57	48.48[a]	26.92
7	Feeling tired or lacking in energy	38.57	72.73[a]	57.69
8	Loss of interest in most things	15.71	30.30	34.62[a]
9	Feeling unhappy or depressed	27.14	51.52[a]	42.31
10	Crying spells	10.00	45.45[a]	34.62[a]
11	Irritability	21.43	63.64[a]	23.08[b]
12	Feeling dizzy or faint	18.57	51.52[a]	34.62
13	Pressure or tightness in head or body	15.71	36.36[a]	30.77
14	Parts of body feel numb or tingling	10.00	33.33[a]	7.69 [b]
15	Headaches	42.9	45.45	42.31
16	Muscle and joint pains	42.86	69.70[a]	73.08[a]
17	Loss of feeling in hands or feet	8.58	18.18	15.38
18	Breathing difficulties	11.43	24.24	23.08
19	Hot flushes	17.14	57.58[a]	46.15[a]
20	Sweating at night	8.57	30.30[a]	30.77[a]
21	Loss of interest in sex	22.86	51.52[a]	26.92
22	Skin dryness	10.00	45.45[a]	50[a]
23	Eye problems	37.14	78.79[a]	46.15
24	High blood pressure	21.43	48.48[a]	42.31
25	Low blood pressure	4.29	12.12	15.38

[a] significant difference vs. premenopause;
[b] significant difference vs. early postmenopause (1-5 yrs) ; $p < 0.05$

Table 3.3 Cluster, sub cluster and total Greene score based on the menopausal transition

Clusters	Premenopause (n= 70)	Early postmenopause 1-5 yrs, (n= 33)	Late postmenopause >5 yrs, (n= 26)
Psychological cluster	2.64 ± 3.63	6.63 ± 5.01[a]	4.81 ± 4.72
• Anxiety	1.37 ± 2.02	3.27 ± 2.86[a]	2.58 ± 2.73
• Depression	1.27 ± 1.90	3.36 ± 2.52[a]	2.23 ± 2.19
Somatic cluster	1.74 ± 2.50	3.48 ± 2.75[a]	2.88 ± 2.68
Vasomotor cluster	0.30 ± 0.72	1.21 ± 1.51[a]	1.15 ± 1.56[a]
Sexual interest	0.32 ± 0.71	0.85 ± 1.00[a]	0.46 ± 0.90
Total	5.00 ± 6.29	12.17 ± 8.13[a]	9.3 ± 7.98[a]
Miscellaneous	0.77 ± 1.18	2.70 ± 2.20 [a]	2.08 ± 2.44[a]

One way ANOVA followed by Bonferroni's Multiple Comparison Test, $p<0,05$

Each value denotes mean ± S.D; [a] significant difference vs. premenopause, $p< 0.05$

Table 3.4 Relationship between the demographic factors and age at the onset of menopause

Category	Classes	Age at menopause	P value (ANOVA)	Status
Socio-economic status	Poor	42.13 ± 3.18	0.04	S
	Middle class	45.47 ± 4.35		
Lifestyle	Sedentary	50.50 ± 2.89		
	Active	44.65 ± 4.37	0.02	S
	Hectic	44.44 ± 3.25		

Each value denotes mean ± S.D; S= significant difference between groups

3.4 DISCUSSION

So far, there has been no generation or analysis of the data on menopause from the northern state of India, called Uttarakhand. The present study was performed with an aim of gathering data on menopausal symptoms from the aforementioned state of India. Calculated mean ± SD and median for the recalled age of natural menopause were 45.02 ± 4.35 and 45 yrs respectively. The reason for no significant difference between the values might be the exclusion

of participants who achieved menopause surgically, were seriously ill, or were taking medications for menopause. Recalled age for the onset of menopause correlates well with the study of (Bharadwaj, 1983) and is comparable to that of the study done on the women of north India (Kriplani, 2005). However, the calculated value is much higher than the lowest age, that is 40.32 yrs, but lesser than the highest age, that is 48.84 yrs, reported as the mean age at the onset of natural menopause in India (Sharma, 2007). The age at menopause recorded in developed countries is higher than the observed recalled mean age of the present study (Kato, 1998; Malacara et al., 2002; Rymer and Morris, 2000). Variation in the age at onset of menopause across nations may be due to geographical, environmental, nutritional, cultural or genetic differences. In addition, different statistical methodologies used for analyzing even the same set of data may generate different outcomes in the surveys. One such study was done in Mexico and demonstrated a variation of up to 5.3 yrs in the computed age of menopause when different methodologies were compared using the same available set of data (Sievert, 2003). A similar trend can be seen in the present study as well. When calculated by probit analysis the mean age rose by approximately two yrs, that is from 45.02 to 46.82 yrs, in comparison with the recalled mean age. This value is higher than most of the previously calculated mean ages of menopause in India. It is probable that if the earlier studies were analyzed with methods like probit, the mean ages would have increased because recalled mean ages are always lower than the means computed by better methods like probit analysis. When compared with the mean age calculated by probit in India, the computed age was found to be comparable (Sidhu, 2005).

Several tools have been designed to date for measuring climacteric symptoms (Blatt, 1953; Heinemann, 2003; Kupperman et al., 1953) but because of shortcomings like summing up of scores without being based on independent factors, they have now been reassessed. The Greene Climacteric scale was selected for the present study because it independently measures the psychological, somatic, vasomotor and sexual clusters and hence specifically identifies the problem area and guides a clinician to the therapy required. This scale was also used for a

different set of studies (Barentsen, 2001; Blumel et al., 2001; Derman et al., 1995; Sierra, 2005). In India, perimenopausal women have been analyzed with this scale (Chattha, 2008), but a comparative study with premenopausal or postmenopausal women was not done. This study is an effort to fill that gap.

The present study indicated an increase in the percent occurrence of symptoms during the transition from premenopause to early post menopause and, subsequently, stable symptoms or a decline towards late post menopause phase. This can be explained in terms of variations observed in the levels of estrogen. Circulating hormone levels show a similar decline from premenopause until a few yrs after the final menstrual period and then become stable or low until the late postmenopause phase (Burger et al., 1999). The pattern so obtained correlates well with the population studies done using such a staging system (Dhillon, 2006).

The observed average number of symptoms did not vary much across the three categories, but the percent occurrence did. Except for a few symptoms like loss of interest in most things, muscle & joint pains and skin dryness, all the other symptoms either declined or remained stable in late postmenopause. This depicts their correlation with fluctuating levels of estrogen in the blood. The rise in a few symptoms with phase transitions might be due to the aging process which is ongoing; independent of menopause. This fact suggests that the criterion that symptoms will subside after five yrs in all women is not fixed, but the non-homogenous sample population in terms of symptoms could also be one cause. To delineate the exact cause of symptoms to be either loss of estrogen or aging or both during postmenopause phase more detailed studies are needed. Symptom like irritability declined significantly in the late postmenopause. One reason may be the experience and maturity attained by the women at that age, which helped them cope more effectively with the biological change (Neugarten, 1965).

In the present population, all the symptoms showed a significant increase during transition towards early post menopause, but which specific category troubles women the most

is not clear. To determine this, mean scores for all the symptoms were calculated, and the values specifically for psychological, vasomotor, somatic, sexual and miscellaneous clusters were computed. Table 3.3 depicts total Greene Climacteric score and individual scores for various clusters and sub clusters. The early postmenopause group showed significantly high scores in comparison with the premenopause group. This finding correlates well with the studies done in other states of India, Hong Kong and China (Boulet, 1994; Flint and Samil, 1990; Ho et al., 1999). When ranked in the order of scores, the psychological cluster appeared at the top and the vasomotor cluster came last. The decline in the percent occurrence of vasomotor symptoms in Indian women is in agreement with a number of other studies reporting that Asian women suffer less from vasomotor symptoms because of the factors like genetics, lifestyle and dietary habits, which include intakes of foods rich in phytoestrogens (Chompootweep, 1993; Gold, 2000; Haines et al., 1994). Severity of psychological symptoms over other clusters in the present study also correlates well with other studies done with Indian women, demonstrating that concerns regarding growing children, social issues related to family and stresses of modernization contribute to the greater frequency of psychological symptoms (Gupta et al., 2006; Kakkar, 2007).

Muscle and joint pain was the most prevalent (55.81%) and severe (22.03%) symptom in whole as well as in the sample of postmenopausal women. Several other studies have also reported musculoskeletal pains as more frequently occurring symptom than others during the menopausal transition (Dugan et al., 2006; Sievert and Goode-Null, 2005). A significant increase during the postmenopause compared to premenopause phase has been attributed to factors like lack of physical activity and increased BMI due to gain in the body weight (obesity). Sometimes, the presence of higher parity and lack of education has also been linked to postmenopause women having more musculoskeletal pains. A study, done in Norway, related pains to the levels of hormones like dihydroepiandrosterone sulfate (DHEA-S) and prolactin (Finset, 2004). DHEA-S was negatively and prolactin was positively associated with

this symptom. An estrogen decline during postmenopause has also been included in the list of factors because its loss leads to degeneration of the cartilage (osteoarthritis) in places like the hand, hip and knee joints and, therefore generation of pains (Birchfield, 2001).

In the miscellaneous group of symptoms, eye problems and skin dryness showed a significant increase both in frequency and severity during the early menopause transition. Studies state that menopause causes dry eyes because of the loss of estrogen. Estrogen control the secretions from tear glands, so any drop in the level of estrogen (as in postmenopausal women) translates into reduced production of tears and, hence increased symptomatology of ocular diseases (Mathers, 1998; Metka, 1991). Estrogen supplementation via HRT has claimed to reduce the chance of the lens becoming cloudy and hence provide better eye health and a clear vision. Study by Nagar and Dave (2005) also showed a greater decrease in vision of postmenopausal women in comparison with premenopausal women (Nagar and Dave, 2005). The skin problems reported in the present sample population were increasing dryness and wrinkling. This can also be explained in part by the decline in estrogen levels during postmenopause. Estrogen loss causes the breakdown of collagen, a decrease in the blood vessel supply and lesser sebum production in the skin, thereby leading to increased pathogenesis of the skin symptoms. Moreover, estrogen receptors are abundant around the genital area, the face and the lower limbs; therefore, reduced amounts of circulating estrogen also render these areas vulnerable to skin problems. Study of Liu and Eden (2007) reported women complaining about skin dryness in genital areas, which in turn led to their declined sexual desires (Liu, 2007).

Undoubtedly, the occurrence of menopausal symptoms is attributed to the loss of gonadal hormones but various social and demographic factors also affect the age at onset of menopause as well as the nature and severity of the symptoms in an individual. The present study showed a significant correlation between socio-economic status, lifestyles and the onset of menopause. Women of high socio-economic status showed later onset of menopause than did poor women. This may be due to the high levels of stresses (due to difficulties with

personal and social responsibilities) accompanying low socio-economic status, which can affect the ovarian function (Gold, 2001). On the other hand, the high socio-economic status provides women with better living conditions, nutrition, education and hence, healthy living with increased reproductive span. The pattern observed is in agreement with other studies (Brzyski, 2001; Do, 1998). Lifestyle pattern affect women in the same way. Sedentary women reported later onset of menopause in comparison with the working women. This finding can be explained by the fact that stress originating from the family work as well as from the job may lead to the depletion of energy sources, affect neuro-endocrine activity and therefore cause a decline in the reproductive function of working women. Effect of factors like BMI, parity, education, diet, consumption of tea, employment, exercise and yrs in district was also evaluated, but none of them significantly correlated with the age at onset of menopause.

3.5 LIMITATIONS OF THE STUDY

Data collection was based on recall since women had to self-report their age at menopause, which may thus lead to a bias. No correlation between marital status, religion, use of oral contraceptives and age at onset of natural menopause could be analyzed because majority of the women belonged to one class. The relatively small sample size of the study also reduces the power of detecting associations.

CHAPTER 4

EVALUATION OF THE ANTIOSTEOPOROTIC POTENTIAL OF *Tinospora cordifolia* IN OVARIECTOMIZED RAT MODEL OF POSTMENOPAUSAL BONE LOSS

4.1 *Tinospora cordifolia*

Tinospora cordifolia (TC) has been widely used in ayurvedic preparations since ancient times as a tonic and vitalizer (Fig. 4.1). It is a deciduous climbing shrub indigenous to tropical Indian subcontinent, Sri Lanka and China. The plant belongs to Meninspermaceae family and is commonly known as Guduchi, Giloya, which in Hindu mythology refers to the heavenly elixir which has saved celestial beings from old age and kept them eternally young. In the Indian system of medicine, the biological properties of TC can be broadly classified as 'adaptogenic', with potential protection against environmental, drug, and diseased induced states. Insulin like action of its roots, leaves have made this plant applicable in treating diabetes mellitus (Stanely et al., 2000). It has been shown to exert significant hypoglycemic and anti-hyperglycemic effects in mild to moderate degree drug induced diabetic rats (Grover et al., 2000). Restoration of antioxidant defence by TC during diabetes has also been proved (Prince et al., 2004). The plant stem is one of the main constituent of herbal preparations that are being used in general debility, dyspepsia, fever, and urinary diseases. It is bitter, stomachic, diuretic, stimulates bile secretion, causes constipation, thirst, burning sensation, vomiting, enriches the blood, cures jaundice and is useful in the management of skin disorders (Ahmed et al., 2006). The bitter principles have been identified as columbin, chasmanthin and palmarin. It has also been tremendously used for the treatment of variety of inflammatory conditions (Pendse et al., 1977). Use of plant in the treatment of rheumatoid arthritis and differential regulation of the proinflammatory and proangiogenic cytokines like IL-1β, IL-6, TNF-α, granulocyte monocyte-colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF) elevation in angiogenesis induced animals support the anti-inflammatory action of TC (Leyon

and Kuttan, 2004). TC has been extensively reported as a general tonic, neuroprotective, antispasmodic, anti-arthritic, anti-allergic, chemopreventive, radioprotective, anti-stress, hepatoprotective and cardio-protective agent (Badar et al., 2005; Bishayi et al., 2002; Chaudhary et al., 2008; Goel et al., 2004; Mathew and Kuttan, 1997; Nayampalli et al., 1986; Rao et al., 2005). The plant is also well known for its immunomodulatory activity which includes neutrophils and macrophage activation, increased humoral and cell mediated immunity, antibody response and synthesis of interleukins (Singh et al., 2004). Immune stimulating activity of TC has been attributed to many isolated compounds like cordioside, syringin, cordifolioside A, B, cordial, α-D-glucan and arabinogalactan (Chintalwar et al., 1999; Kapil and Sharma, 1997; Nair et al., 2004). A study by Jagetia and coworkers found that TC killed HeLa cells effectively *in vitro* and thus indicates this plant to have anti-neoplastic activity as well (Jagetia et al., 1998).

A plethora of compounds have been isolated from the whole plant, stem, aerial part and roots of TC and their structures elucidated (Table 4.1). They belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides (Singh et al., 2003).

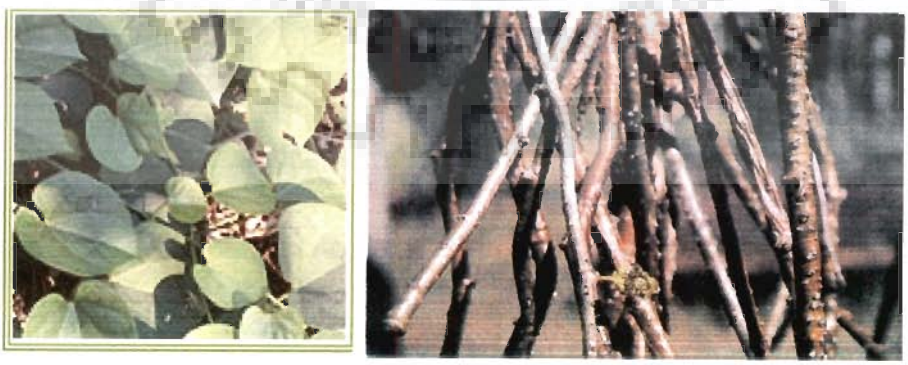


Figure 4.1: *Tinospora cordifolia*: Plantation and the stem pieces

Ahmed et.al, describes the separation and quantification of four compounds such as 20β-hydroxyecdysone, tinosporaside, cordioside and columbin which are being used as biomarkers

for the identification of TC species and which are also the important bioactive principles reported from this medicinal plant (Ahmed, 2006).

Table 4.1 Constituents isolated from *Tinospora cordifolia*

Type of chemical	Active principle	Part in which present
Alkaloids	Berberine (I), Palmatine, Choline (V),Tinosporin, Isocolumbin, Tetrahydropalmatine, Magnoflorine	Stem, Root
Glycosides	Tinocordiside (X), Tinocordifolioside (XI), Cordioside, Cordifolioside A,B, Cordifoliside C,D,E, Syringin, Palmatoside C,P, Furanoid diterpene glucoside	Stem
Diterpenoid Lactones	Furanolactone XIV, Clerodane derivatives, Columbin Tinosporon, Tinosporides XVIII, XIX, Jateorine,	Whole plant
Steroids	20 β- hydroxyecdysone, β- sitosterol, δ-sitosterol, Ecdysterone XXIV, Makisterone A, Giloinsterol	Aerial part, Stem
Sesquiterpenoid	Tinocordifolin	Stem
Aliphatic	Octacosanol, Heptacosanol, Nonacosan	Whole plant
Miscellaneous	Jatrorrhizine, Tinosporidine, Cordifol, Cordifelone, Giloin, Giloinin, Tinosporic acid	Root, Whole plant

Modified from (Singh, 2003)

Selection of plant

How estrogen deficiency after menopause causes increased bone resorption and accelerated loss of bone mass involves various complex and multifaceted mechanisms. Of the many, interrupted control of estrogen on immune system leading to rise of inflammation appear to be the major pathogenetic factor (Weitzmann and Pacifici, 2006). Owing to the profound use of TC in the ayurvedic preparations especially the anti-arthritic, immunomodulatory and anti-inflammatory medicines, we hypothesized this plant to exert protective effects on skeleton and therefore its antiosteoporotic potential was investigated.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Tinospora cordifolia, stem parts were collected from Roorkee, Haridwar district, Uttarakhand, India.

4.2.1.1 Authentication

Authentication of the plant material was done for both the whole plant and crude plant extract. The morphological characters of the whole plant were identified by Professor M. L. Sharma, Department of Botany, Panjab University, Chandigarh, India. The voucher specimen has been deposited in the herbarium of the department with a PAN number 19756.

4.2.1.2 Preparation of the extract

Oven dried, powdered stems of TC were exhaustively extracted with 95% ethanol under reflux in a soxhlet extractor. The total extract obtained was concentrated in a rotor *vacuo* to a syrupy consistency and finally lyophilized. The lyophilized material was preserved for future use.

4.2.2 Estrogen receptor ligand binding assay (ER-LBA)

ER-LBA was performed with cytosolic fraction from porcine uteri. This preparation contains ERα, ERβ and possibly other ER binding proteins. The porcine uterus was collected at the local slaughter house. With minor modifications, the method of (Jarry et al., 2003) was employed to prepare the cytosolic fraction from porcine uteri. Instead of tritium labelled tracer, 16α-^{125}I labelled estradiol (2200Ci/mmol) purchased from NEN, (Dreirich, Germany) was used in the assay. All other chemicals were purchased from Sigma (Deisenhofen, Germany).

4.2.3 Animals

4.2.3.1 Ethical approval of experiments

All experiments were performed according to the animal welfare regulations with approval from institutional ethical authorities.

4.2.3.2 Strain and husbandry

All experiments were performed with Sprague-Dawley rats. Female rats, 3 months old and weighing 250 ± 10g were purchased. The animals were housed as 5-6 animals per cage in the experimental animal division. Polycarbonate cages with wire tops and wood chip bedding were used. All were acclimatized to laboratory conditions of 25±2°C, 12h light/dark cycle and relative humidity (55 %) with free access to the soy free diet; V 1355-000 ssniff R-Z, 15 mm Phytoestrogenarm, Maus/Ratte, ssniff Spezialdiät GmbH, Soest, Germany (Fig. 4.2) and water *ad libitum*.

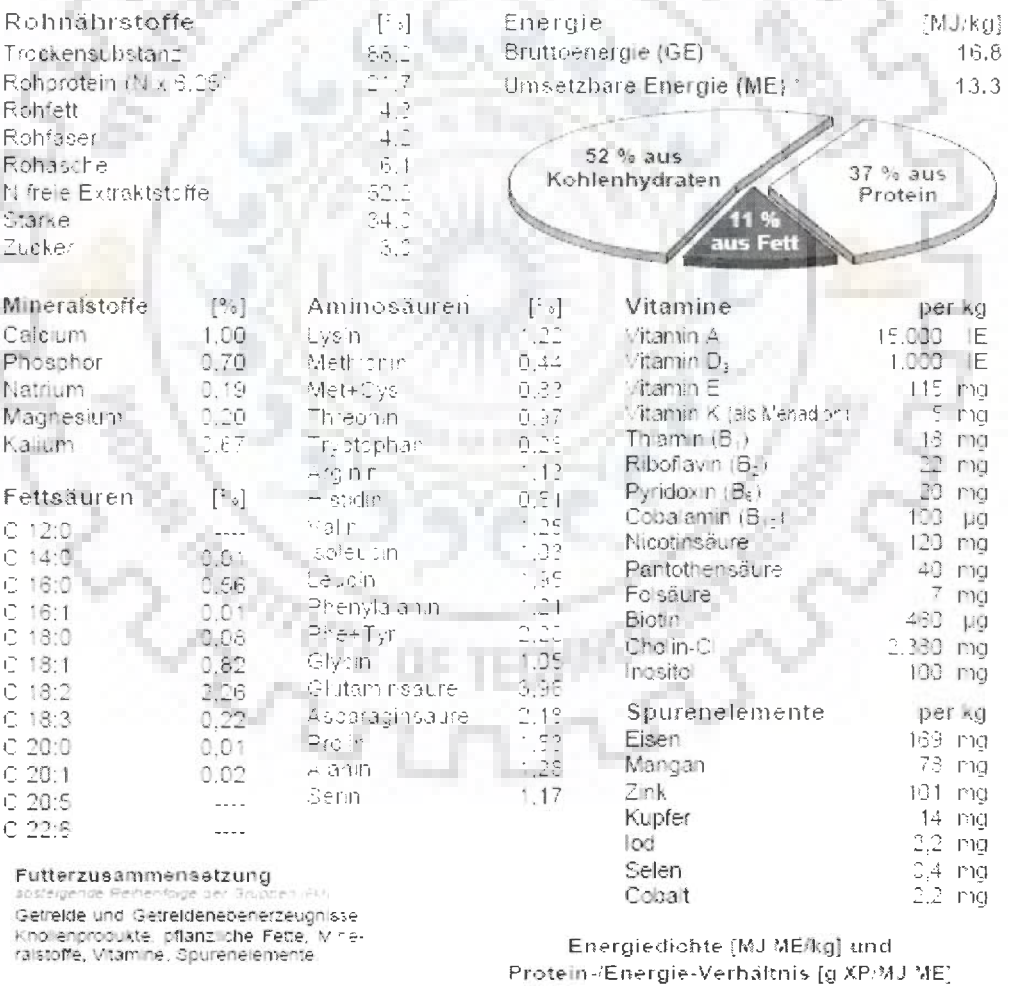


Figure 4.2: Soy free diet composition chart -- Rohprotein: crude protein; Rohfett: crude fat; Rohfaser: crude fiber; Rohasche: crude ash. This feeding stuff contains genetically modified corn, potato proteins and sugar beet pulp products.

4.2.3.3 Ovariectomy

For the experimental procedure the animals were bilaterally ovariectomized after the period of acclimatization. They were anaesthesized and dorsal surface of each animal was shaved and disinfected before the incision. Sterilized instruments and sterile technique were used during all surgical procedures. A small midline incision was made dorsally on each side and connective tissue was bluntly dissected to the muscle level. A small incision was made through the muscle layer approximately 1 cm lateral to midline and half way between the caudal most rib and the superior rim of the pelvis to expose each ovary. In subjects receiving a sham ovariectomy the ovaries were exposed, grossly examined for intactness and adequate blood flow and then replaced to their original location. The remaining subjects underwent an ovariectomy in which the ovaries were exposed, ligated beneath the tubes with sterile resorbable catgut (3 metric, Ethicon) and resected with a scalpel. After the completion of procedure repositioning of the uterus horn in the abdominal cavity was done. The muscle tissue was sutured with catgut and the skin closed with clamps. Animals were monitored until reawake.

4.2.4 Dose preparation

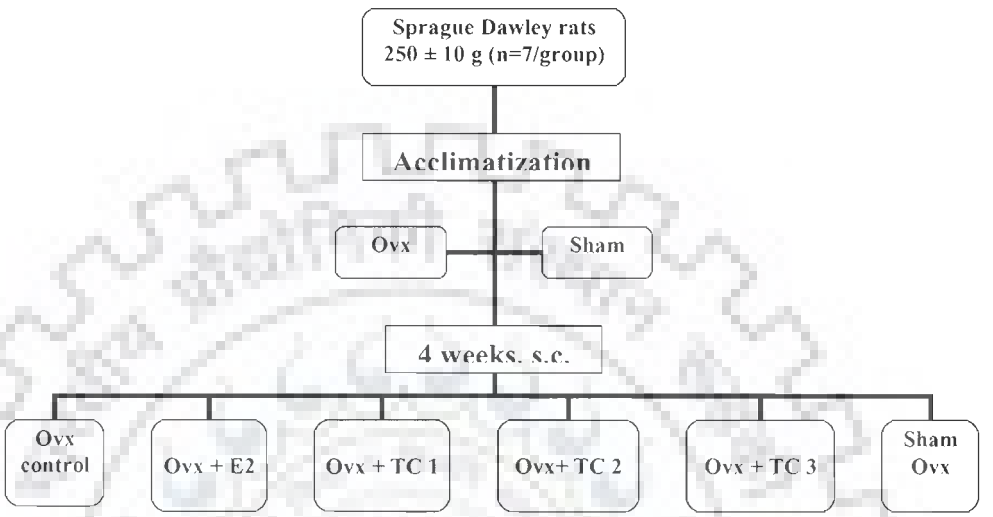
The solvent vehicle used for the preparation of the doses was benzyl benzoate: castor oil (1:4) emulsion. The lyophilized powder of the crude plant extract was dissolved in the vehicle to the dose concentration levels of 10mg/kg/b.wt (TC 1), 50mg/kg/b.wt (TC 2) and 100mg/kg/b.wt (TC 3). 17-β-Estradiol (E2) concentration was prepared to provide 1µg/animal/day for the experiment.

4.2.5 Acute treatment

4.2.5.1 Allocation and dosage

Two weeks after acclimatization, female rats divided by weight matched groups (n=7/group) were anaesthetised with Isofluran (Forene®, Abbott). Sham or the bilateral

ovariectomy (ovx) operations were performed. The ovx rats were separated into five groups. After 24 hours of recovery, subcutaneous treatment of the groups was started. To identify the individual animals, they received a subcutaneous implant of a transponder microchip after ovariectomy while still under anaesthesia.



The respective doses for the allocated groups are as follows:

Group 1: Sham ovariectomized animals (sham ovx, control) ---- vehicle, daily

Group 2: Ovariectomized (ovx control) ----- vehicle, daily

Group 3: Ovariectomized (ovx + E2) ----- E2 (1µg/animal), daily

Group 4: Ovariectomized (ovx + TC 1) -----10 mg/kg b.wt, alternately

Group 5: Ovariectomized (ovx + TC 2) -----50 mg/kg b.wt, alternately

Group 6: Ovariectomized (ovx + TC 3) -----100 mg/kg b.wt, alternately

The animals were maintained in these conditions for 4 weeks. At the end of the treatment the animals were sacrificed under CO_2 anaesthesia.

4.2.5.2 Evaluation parameters

4.2.5.2.1 Body weight development

Body weight measurements for all the group animals were done once each week and finally before the obduction.

4.2.5.2.2 Bone mineral density measurements

Bone mineral density was determined to record ovariectomy induced osteoporosis in rats. Peripheral quantitative computer tomography (qCT) was performed at the proximal metaphysis part of tibia using the STRATEC XCT 4.50 equipment (Stratec Inc., Pforzheim, Germany) (Helterbrand et al., 1997).

Animals were anaesthesized with isoflurane and placed in the tomograph (Fig. 4.3). The scanner was positioned at the epiphysis of tibia and a coronal computed radiograph (scout view) in distal direction was carried out. The scout view was used to position the scanner at the site of measurement. Along the leg, a reference line is placed through the most proximal site of the joint (Fig. 4.4). This is necessary to enable the exact positioning of repeated measurements. The actual measurement level through the metaphysis of the tibia lies in defined distance from the reference line down the bone. The transversal picture is obtained by 15 linear scans across the bone axis that moves around the leg and cover 180°. Two tomographic slices at a distance of 3.75 and 4.25 mm distal to reference line were used for cancellous bone parameters. A third slice was taken 15mm distal to the reference line for determination of cortical bone parameters.

Image acquisition, processing and calculation of the results were performed using the software package (XCT 5.40, Stratec Inc; Pforzheim, Germany). Each CT-measurement was evaluated by manually marking the region of interest, i.e. the metaphysis of tibia. Cancellous density was calculated with a lower and upper density threshold of 280 and 710 mg/cm^3 respectively. Tissue with a density above 710 mg/cm^3 was regarded as the cortical bone. From basal CT of all animals before treatment the mean was calculated and set as reference value (preovx). The individual values of the final CT after 1 month treatment on tibia were percentage related to the reference value and the resulting means of the control and treatment groups were compared statistically.

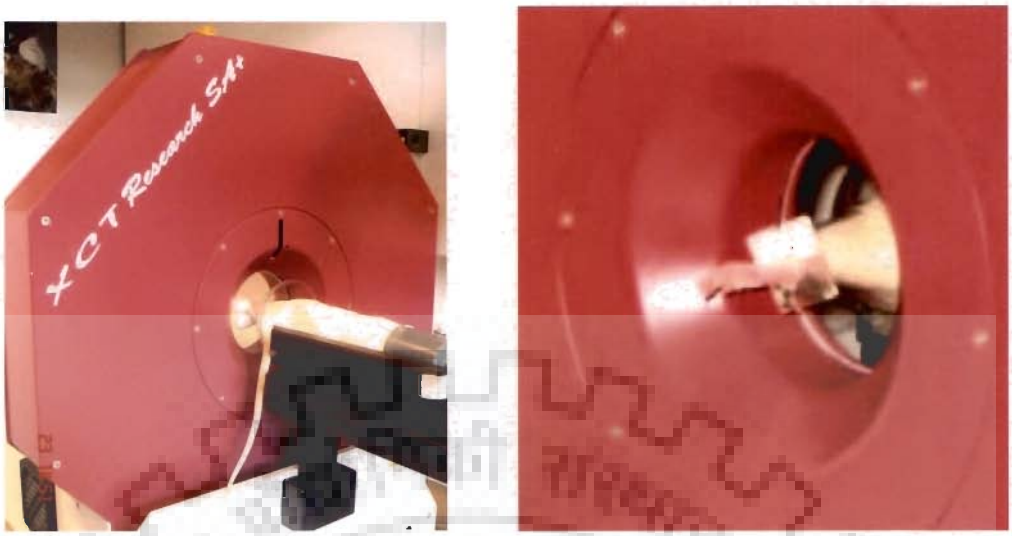


Figure 4.3: Position of the animal and its leg in the computer tomography for the tibia scan

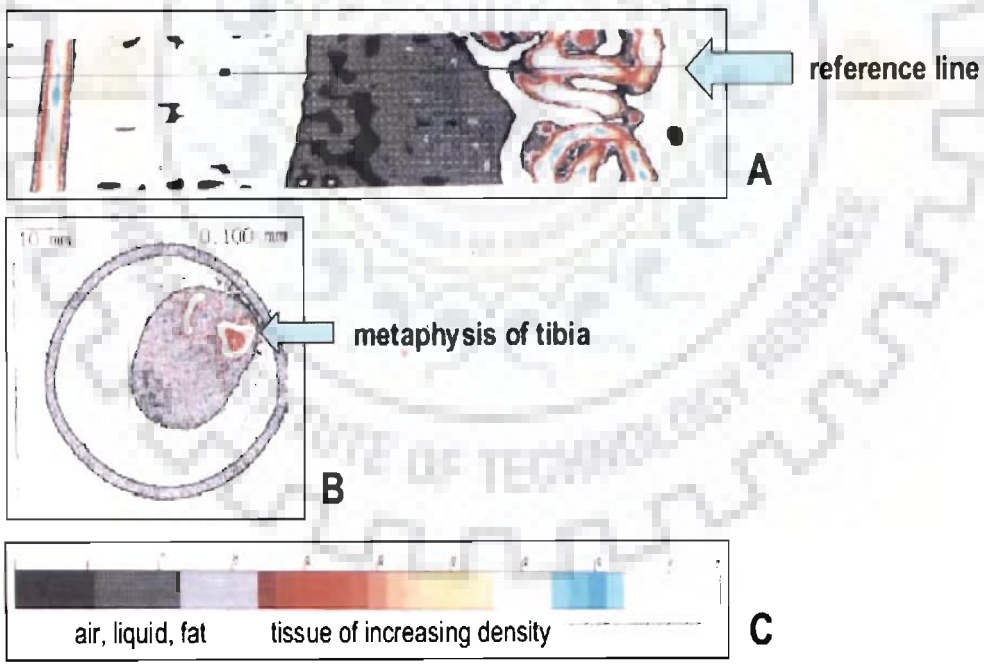


Figure 4.4:

A) Tomographic picture displayed after the scout view scan and illustration where the reference line was placed.

B) Example of the final transversal scan for density evaluation.

C) Color code of the tomography to discern varying density.

4.2.5.2.3 Biochemical evaluation

Trunk blood was collected after decapitation. Samples were centrifuged at 3000 g for 20 minutes. Clean serum samples were pipetted off and stored in vials at -20° C until ready for analysis.

The measurement of the indices of bone turnover was done in the serum samples. Levels of osteocalcin and β-cross-laps were measured with specific electrochemiluminescence immunoassay (Nordic Bioscience, Herlev, Denmark) utilizing commercially available kits for Elecsys 2010 analyzer system, Roche, Mannheim. Assessment of the activity of alkaline phosphatase and lipid profiling (Triglycerides, Cholesterol, LDL and HDL-lipoproteins) was also performed by using Hitachi 902 system, Roche, Mannheim. Details of the assays are described in the data sheets provided by the distributor.

4.2.5.2.4 Safety assessment

Some estrogenic effects of compounds supposed to replace HRT are undesired. ER-LBA as explained above (section 4.2.2) can be used to verify or deny binding properties of test compounds to two ERs. Similarly, so called uterotrophy assay is recommended by OCED to test for estrogenicity (Yamasaki et al., 2003) and utilizes the capacity of estrogenic compounds to stimulate uterine weights in ovx rats. For the safety issues regarding the use of TC as alternative to HRT, weights as well as histological measurements were done on uterus and mammary gland.

4.2.5.2.4.1 Weight measurements

During sacrifice, the tissues were dissected out and their weights recorded. The comparison of both the absolute (mg) and the relative weight (mg/100g b.wt) of the tissues was made between different treatment groups.

4.2.5.2.4.2 Histological analysis

Parts of tissues were kept in neutral buffered formalin immediately after the removal and fixed for 48h for histological analysis. The samples were dehydratized with a histokinet

(TP 1020, Leica) and later embedded with a paraffin dispenser (EG 1060, Leica) following the standard protocols. Three micrometer transversal sections of the tissue blocks for all the treatment groups were cut with a RM 2135 microtome (Leica) and placed on slides with permanent positive charge on the surface (Superfrost plus), which improves binding between formalin fixed sections and the glass. Later all the tissue sections were stained with standard Haematoxylin and Eosin (H&E) protocol and mounted. Hematoxylin (Haematoxylin Mayer, Merck) dilutions were pretested for staining strength. Eosin yellow (Merck) was used in a concentration of 0.25% in ethanol (80%).

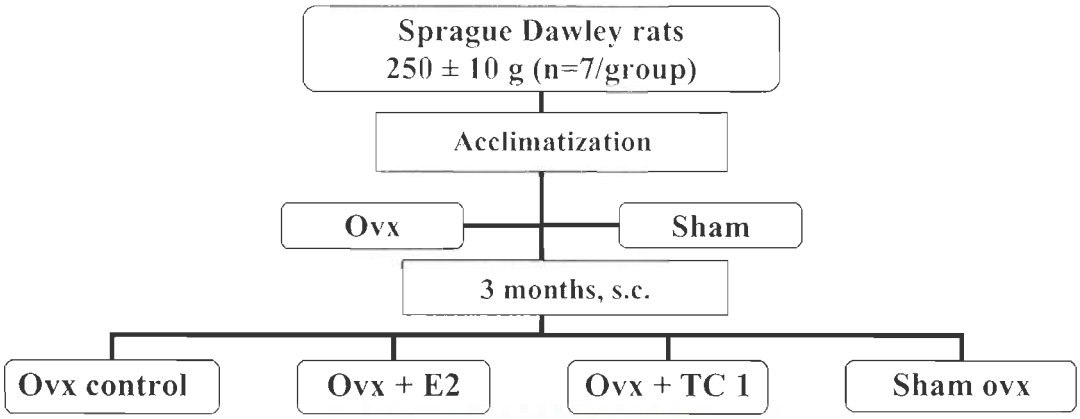
Estradiol induces morphological changes in uterus and vagina at the dose concentration level used in the present study. These changes are well known and allow comparison to those of any test substances used.

The thicknesses of epithelium, endometrium and myometrium layers were measured in the uteri. In the mammary gland number of ducts and lobuli were quantified per μm^2 area (Russo and Russo, 1978). All the measurements were made with an Axiophot microscope (Zeiss). Microscopic pictures were taken with a Color View 12 camera (SIS) and transferred to the computer by the aid of the computer assisted program, AnalySIS (Analysis Soft Imaging System, Münster, Germany) for histological quantification.

4.2.6 Chronic treatment

4.2.6.1 Allocation and dosage

After acclimatization, female rats raised on soy free diet were divided by weight matched groups (n=7/group). They were anaesthetised and sham or bilateral ovariectomy (ovx) operations were performed. The ovx rats were separated into three groups. After 24 hours of recovery, subcutaneous treatment of the groups was started. The respective doses for the assigned groups given alternately are as follows:



Group 1: Sham ovariectomized animals (sham ovx, control) ---- vehicle

Group 2: Ovariectomized (ovx control) ----- vehicle

Group 3: Ovariectomized (ovx + E2) ----- E2 (1µg/animal)

Group 4: Ovariectomized (ovx + TC 1) -----10 mg/kg b.wt.

The animals were maintained in these conditions for 3 months. At the end of the treatment the animals were sacrificed under CO_2 anaesthesia. The animals were sacrificed between 8 and 12 a.m. to ensure a negative feedback of estradiol or test samples on gonadotrophins secretion. To exclude any effect of time of death between the groups, they were evenly distributed over the whole time frame. Trunk blood and tibiae were collected. The uteri were also dissected out and weighed. Selected tissues were immediately fixed with 10% formalin solution for histological analysis.

4.2.6.2 Evaluation parameters

Body weight measurements for all the group animals were done once each week and finally before the obduction. After removal of the adherent soft tissues, the bone samples were dried and processed with respect to the analysis method involved.

4.2.6.2.1 Thermal analysis

Similar portion of the bone was cut and weighed to 0.1g for all the samples. Powders were prepared under liquid nitrogen and equal weight (10mg) was used for thermal analyses. TG (Thermo Gravimetry), DTG (Differential Thermo Gravimetry) and DTA (Differential

thermal analysis) were done using Perkin Elmer Pyris Diamond in an air atmosphere (200ml/min) at a heating rate 10°C/min from 50 to 1500°C.

4.2.6.2.2 Histology of decalcified bone samples

The bone samples were fixed in 10% neutral buffered formalin for days and then decalcification was done in 5% ethylene diamine tetra-acetic acid (EDTA, pH 7.4) for several days. Weight gain weight loss method was used as endpoint testing to examine complete decalcification. The samples were dehydrated through graded series of alcohol, embedded in paraffin and cut into sections of 5µm thickness. The sections so cut from the metaphysial region of tibia were processed and stained with haematoxylin and eosin (H & E) for observations.

4.2.6.2.3 Scanning electron microscopy (SEM)

SEM is a technique whereby both structural and analytical information can be obtained from bone. In contrast to transmission EM, in which only very small pieces of tissue can be examined, the sample size for SEM is much less restrictive. Using SEM, a wide range of magnifications (10- to 10,000-fold) can be employed to obtain good overviews as well as detailed images.

The detailed morphological studies of the bone samples were carried out using SEM. Samples were taken and cut along the metaphysis region of tibia. They were then fixed using 3% (v/v) glutaraldehyde- 2% (v/v) formaldehyde (4:1). After fixation, samples were treated with alcohol gradients of 30%, 50%, 70%, 80%, 90% and 100% for dehydration (24 hours in each grade), air dried, mounted on metal stubs and sputter coated (S-150 sputter coater) with thin layer of gold ions. Electron photomicrographs of the metaphysis of tibia were taken at desired magnifications using SEM, LEO435VP at 15-kV accelerating voltage.

4.2.7 Material for Future Studies

Due to the magnitude of information available from this study, a selected group of experiments were chosen for completion of this project. Due to the extensive time and resources invested in this study, we did not want to dispose of any materials that might be beneficial for further analyses. Therefore, various materials were preserved for future possible studies.

4.2.8 Statistical analysis

Mean, standard deviation (SD) and standard error of the mean (SEM) were calculated for all data. All the results are presented as mean ± SEM, if not stated otherwise. Statistical analysis of the data was done by analysis of variance (ANOVA) followed by Dunnett's multiple t-test comparison utilizing the Graph Pad Prism Software. Values of $p<0.05$ were considered to be statistically significant.

4.3 RESULTS

4.3.1 Acute treatment

4.3.1.1 Body weight measurements

None of the treatment groups had significant differences in mean initial body weights. Although all rats were fed the same diet for 4 weeks of treatment period, significant differences were observed in the final body weights. Effect of different treatments on whole body weight is shown in Fig. 4.5. The final recorded body weight for various groups was normalized with respect to preovx. Ovx control and ovx+TC treated animals showed significant gain in weight after 4 weeks of ovariectomy. In the sham ovx and ovx+E2 group the original body weight was retained.

4.3.1.2 Estrogen ligand binding assay

Figure 4.6 depicts the interaction of TC ethanolic stem extract with ER from cytosolic preparation of porcine uteri. As shown, there is little competition of the radio labelled estradiol

with compounds from the TC extract for the ER receptor. While E2 displaced the radio labelled ligand in the low nM range, TC has low potency for binding to the receptors.

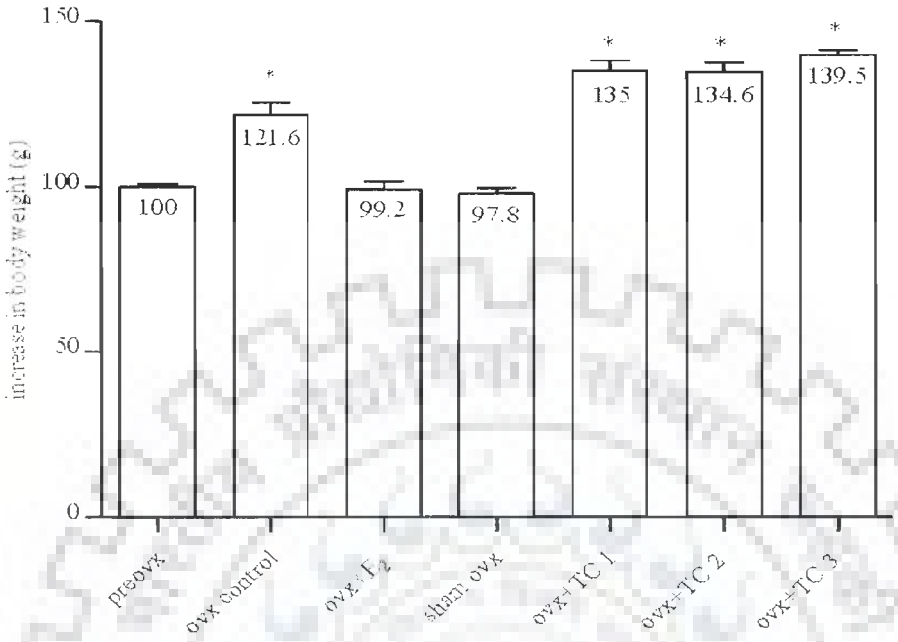


Figure 4.5: Comparison of body weight among various groups plotted as normalized value w.r.t preovx. Each value denotes mean ± S.E.M. (n=7/group). * p<0.05 vs. ovx control

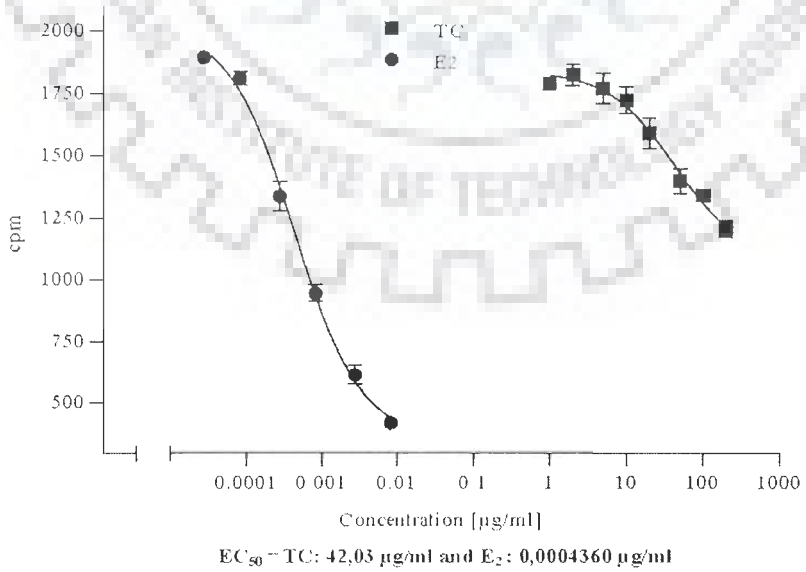


Figure 4.6: Displacement curve of estradiol and TC extract in an ER-LBA with porcine uterine cytosol. Constituents of TC extract causes a dose dependent competition with radiolabeled estradiol (n=3 at each concentration).

4.3.1.3 Bone mineral density measurements

Trabecular bone mineral density (BMD) of the metaphysis of tibia measured by qCT for all treatment groups is presented in Fig. 4.7. The normalized percent change in trabecular density (mg/cm^3) of various groups compared to preovx control is plotted. Ovx control rats showed 50% loss of the bone mineral density after 4 weeks of ovariectomy. Loss was significantly ($p<0.05$) prevented in ovx+E2 (1µg/day) treatment group. Sham ovx animals also did not lose bone mineral density. Ovx animals treated with TC extract at the dose level of 10mg/kg b.wt showed significant bone sparing effect compared to ovx control. No significant change in BMD was observed in animals treated with either 50mg/kg b.wt or 100mg/kg b.wt TC.

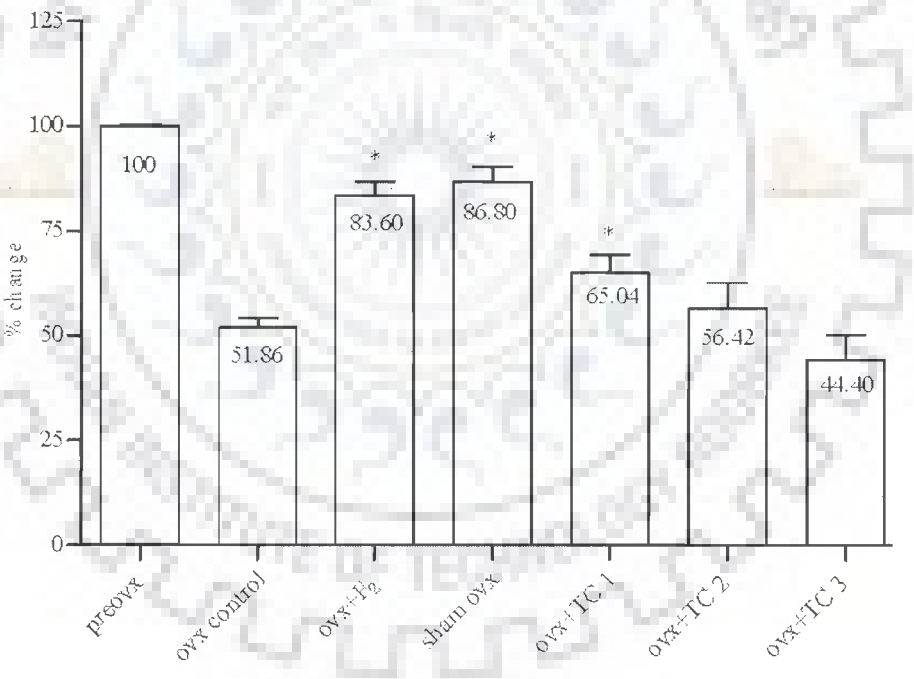


Figure 4.7: Trabecular density of the metaphysis of tibia for various groups plotted as normalized percent change compared to preovx. Absolute measurements (n=7/group) were made with quantitative computer tomography. * $p<0.05$ vs. ovx control

4.3.1.4 Biochemical evaluation

Serum osteocalcin concentration (Fig. 4.8) was significantly reduced in ovx+E2, sham ovx and ovx + TC (10, 50 and 100mg/kg b.wt) compared to ovx control.

Serum cross-laps levels (C-terminal telopeptides) are shown in Fig.4.9. This parameter was significantly reduced in ovx+E2, sham ovx, and ovx + TC (10, 50 and 100mg/kg b.wt) groups

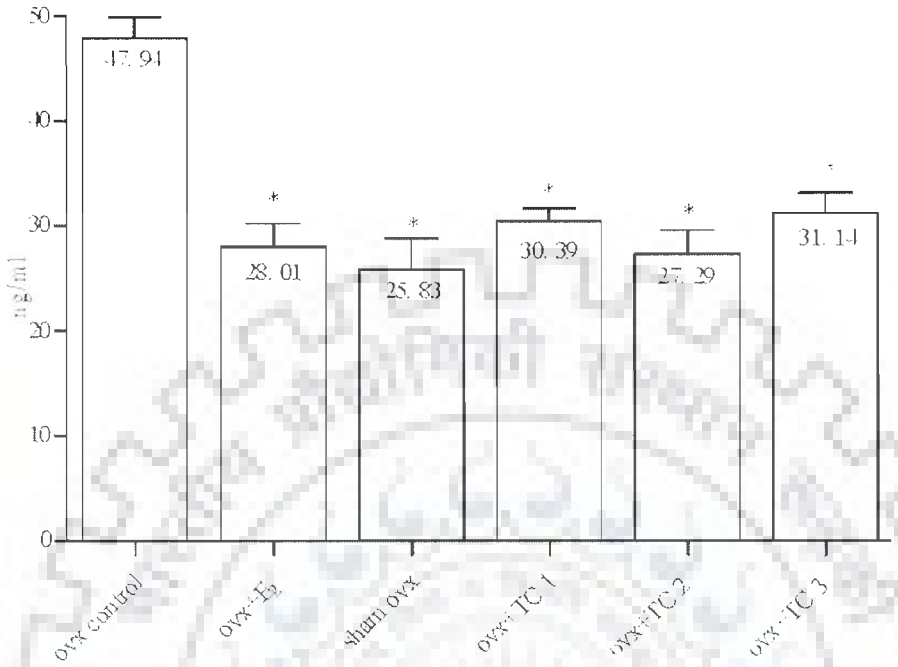


Figure 4.8: Effect of TC extract on serum osteocalcin level (ng/ml). Each value denotes mean ± S.E.M. (n=7/group). * p<0.05 vs. ovx control

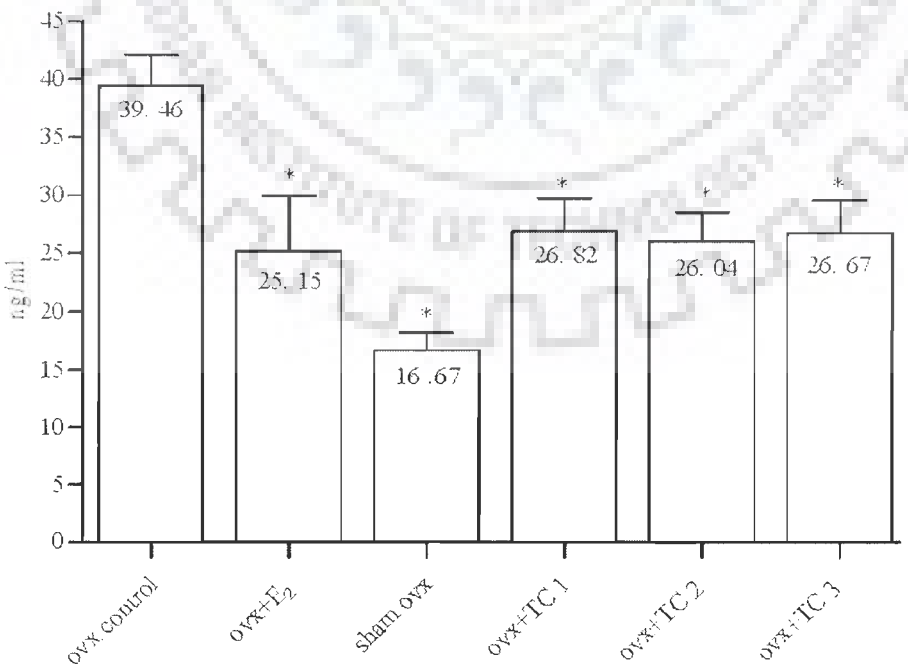


Figure 4.9: Effect of TC extract on serum cross-laps (C-terminal telopeptide) level (ng/ml). Each value denotes mean ± S.E.M. (n=7/group). * p<0.05 vs. ovx control

compared to ovx control animals. E2 treatment significantly reduced the activity of serum alkaline phosphatase in ovx rats. However the activity was substantially elevated in TC treated groups. The lipid profile pattern showed no significant change in the levels of cholesterol in all groups except ovx+TC 2 (50mg/kg b.wt). HDL (High density lipoprotein) was significantly reduced in sham ovx, ovx+E2 and ovx+TC 2 (50mg/kg b.wt) treated animals whereas LDL (Low density lipoprotein) was only significantly reduced in ovx+E2 animals (Table 4.2).

Table 4.2: Effect of the treatment with TC extract on biochemical parameters.

Parameter	ovx control	ovx+ E2 (1µg)	Sham ovx	ovx + TC 1 (10mg/kg b.wt)	ovx + TC 2 (50mg/kg b.wt)	ovx + TC 3 (100mg/kg b.wt)
Alkaline phosphatase (U/l)	164.5±8.88	113.4± 6.05*	131.6±31.77	209.5±20.75*	233.1±11.59*	197.9±14.79*
Total cholesterol (mg/dl)	139.8±5.72	136.8±4.46	126.7±6.0	135.2±8.69	122.7±3.61*	137.1±2.48
High density lipoprotein (mg/dl)	94.01±6.44	78.09±2.88*	67.78±5.4*	80.68±4.11	75.71±2.04*	89.47±2.67
Low density lipoprotein (mg/dl)	34.94±5.05	21.50±1.42*	23.50±2.8	30.33±1.76	26.29±1.29	28.43±1.04

Each value denotes mean ± S.E.M. (n=7/group). * p<0.05 vs. ovx control

4.3.1.5 Safety assessment

4.3.1.5.1 Weight measurements

No significant change in the relative weight of organs like heart and thymus in TC treated animals compared to vehicle control was found.

Changes in absolute weight of the uterus of animals used with experiment are consolidated in Fig.4.10. As expected uteri of sham ovx and ovx+E2 group was significantly heavier than those of ovx control. The weight of uterus in TC treated animals was not significantly different from ovx controls. The above pattern with results did not change even when the relative weights (mg/100g b.wt.) were compared.

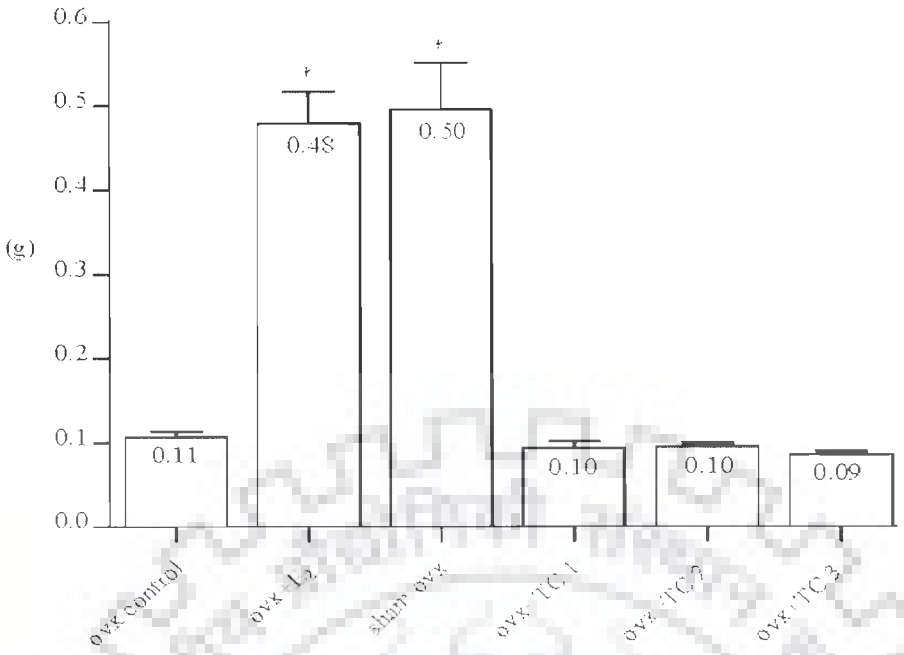


Figure 4.10: Comparison of absolute uterine weight among various groups. Each value denotes mean ± S.E.M. (n=7/group). * $p<0.05$ vs. ovx control

4.3.1.5.2 Histological analysis

Measurement of the thickness of uterine layers confirmed that TC extract had no uterotrophic effect. The thickness of the uterine layers on the basis of Fig. 4.11 is presented in Table 4.3. It is abundantly clear from the data that E2 treatment was able to sustain the thickness of the uterine layers very close to sham operated animals whereas TC treated groups are in line with ovx control (Fig. 4.12 and Fig. 4.13).

A similar pattern was observed in mammary gland of sham ovx and ovx +E2 group. Ducts were often observed with secretion confirming the proliferative effect (Fig. 4.14). The quantified number of ducts and lobuli were also increased in sham ovx and ovx +E2 compared to ovx control (Fig. 4.15). No such proliferative effects were observed in TC ethanolic extract treated groups.

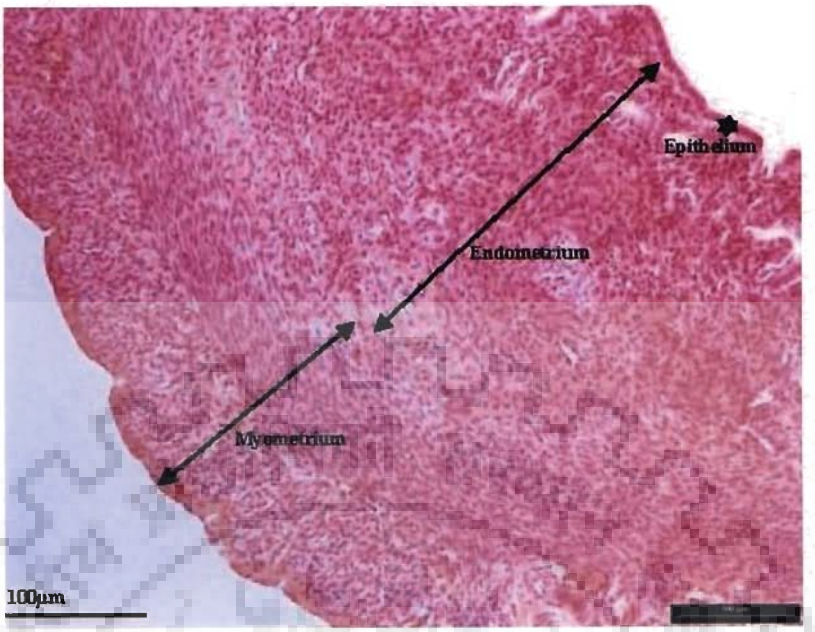


Figure 4.11: Model picture to depict how the areas were selected for the measurements of the thickness of the uterine layers i.e. myometrium, endometrium and epithelium.

Table 4.3: Effect of treatment with TC extract on the thickness of uterine layers. Each value denotes mean ± S.E.M. (n=7/group). * $p<0.05$ vs. ovx control

Parameter	ovx control	ovx+ E2 (1µg)	Sham ovx	ovx +TC 1 (10mg/kg b.wt)	ovx +TC 2 (50mg/kg b.wt)	ovx+TC 3 (100mg/kg b.wt)
Endometrium thickness	329.0 ± 13.9	663.3 ± 33.9*	756.9 ± 54.8*	355.4 ± 26.9	306.0 ± 12.4	333.6 ± 15.2
Myometrium thickness	257.4 ± 20.6	432.4 ± 23.5*	406.9 ± 31.4*	258.2 ± 12.8	240.2 ± 16.2	247.3 ± 11.3
Epithelium thickness	8.03 ± 0.50	36.39 ± 3.7*	27.35 ± 2.9*	8.33 ± 0.54	7.77 ± 0.44	7.12 ± 0.53

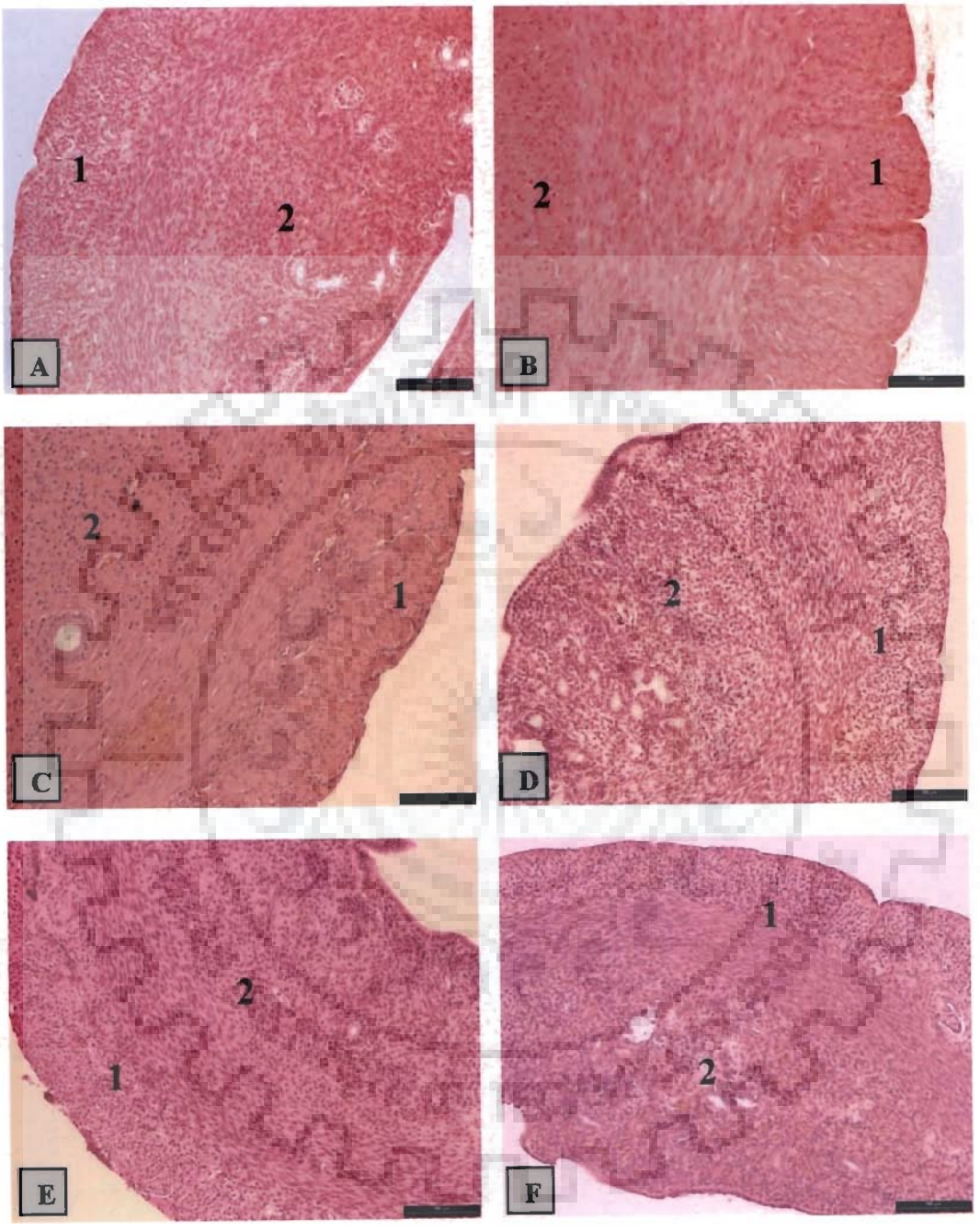


Figure 4.12: Photomicrographs of the transverse sections of uterine myometrium (1) and endometrium (2) layers. All slides stained with H & E and viewed at 12,5X.

A: Ovx control group, B: Ovx + E2, C: Sham ovx, D: ovx+TC 1, E: ovx+TC 2 and F: ovx+TC 3.

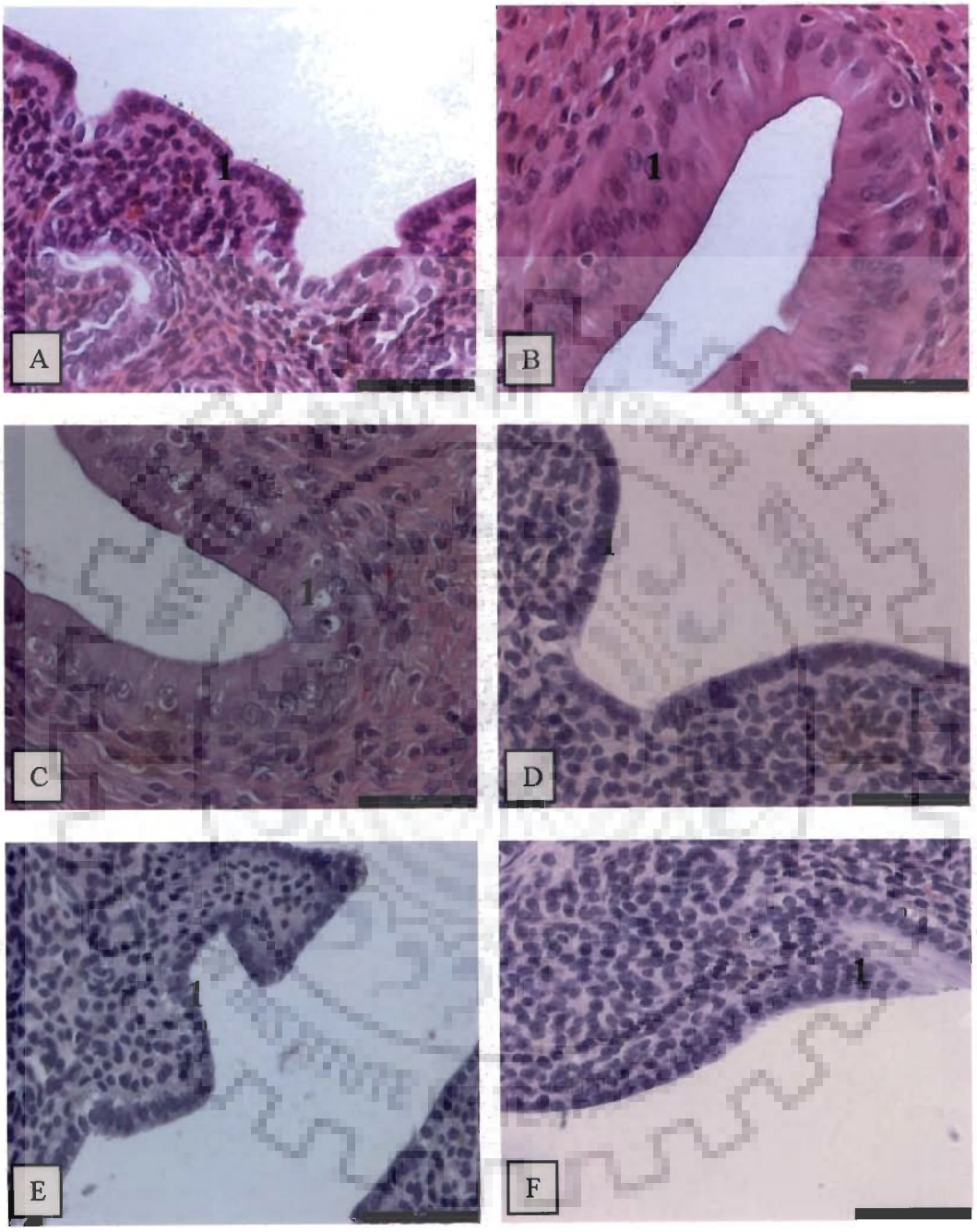


Figure 4.13: Photomicrographs of the transverse sections of uterine epithelium (1) layer. All slides stained with H & E and viewed at 40X.

A: Ovx control group, B: Ovx + E2, C: Sham ovx, D: ovx+TC 1, E: ovx+TC 2 and F: ovx+TC 3.

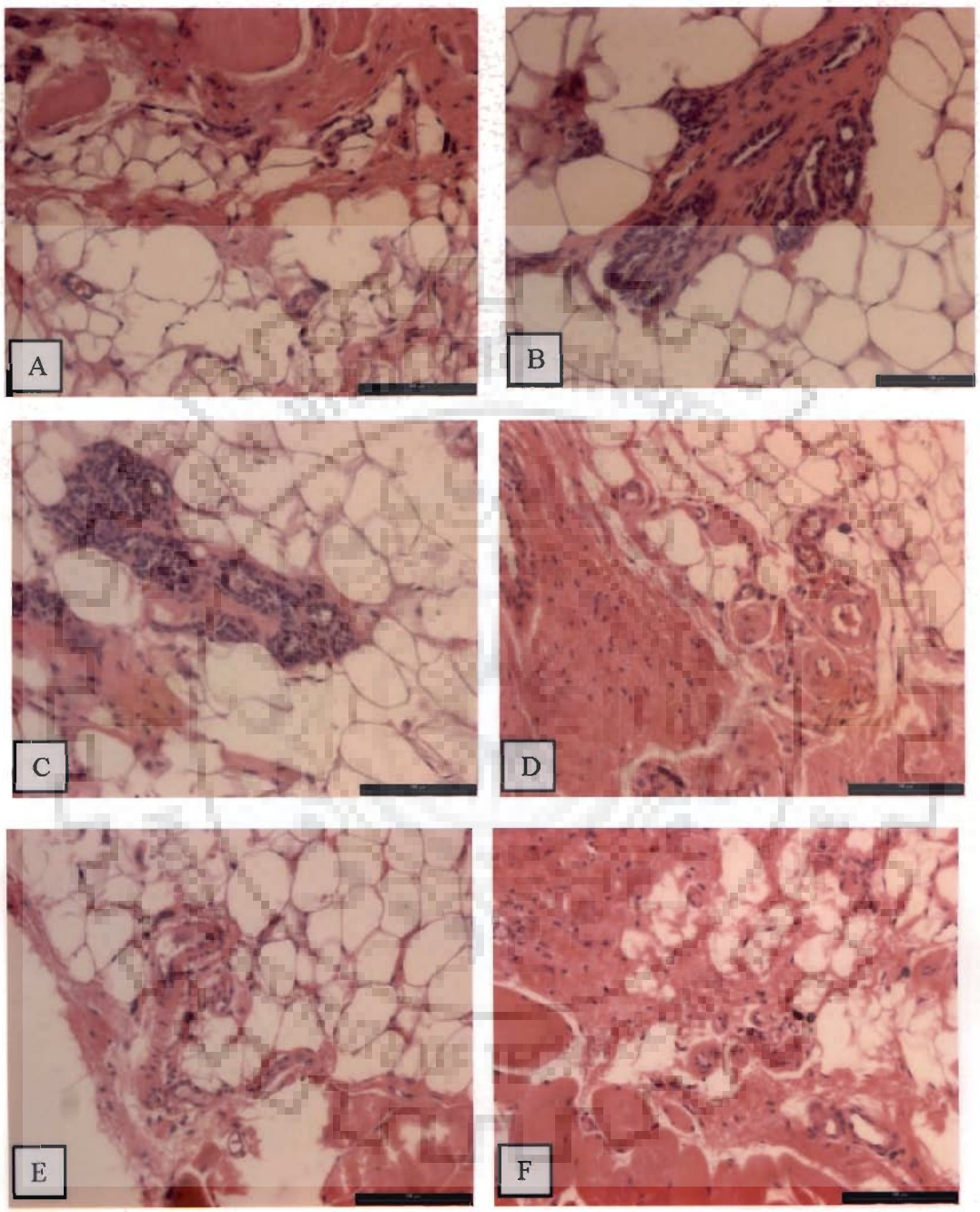


Figure 4.14: Photomicrographs of the transverse sections of mammary gland tissue. All slides stained with H & E and viewed at 20X.

A: Ovx control group, B: Ovx + E2, C: Sham ovx, D: ovx+TC 1, E: ovx+TC 2 and F: ovx+TC 3.

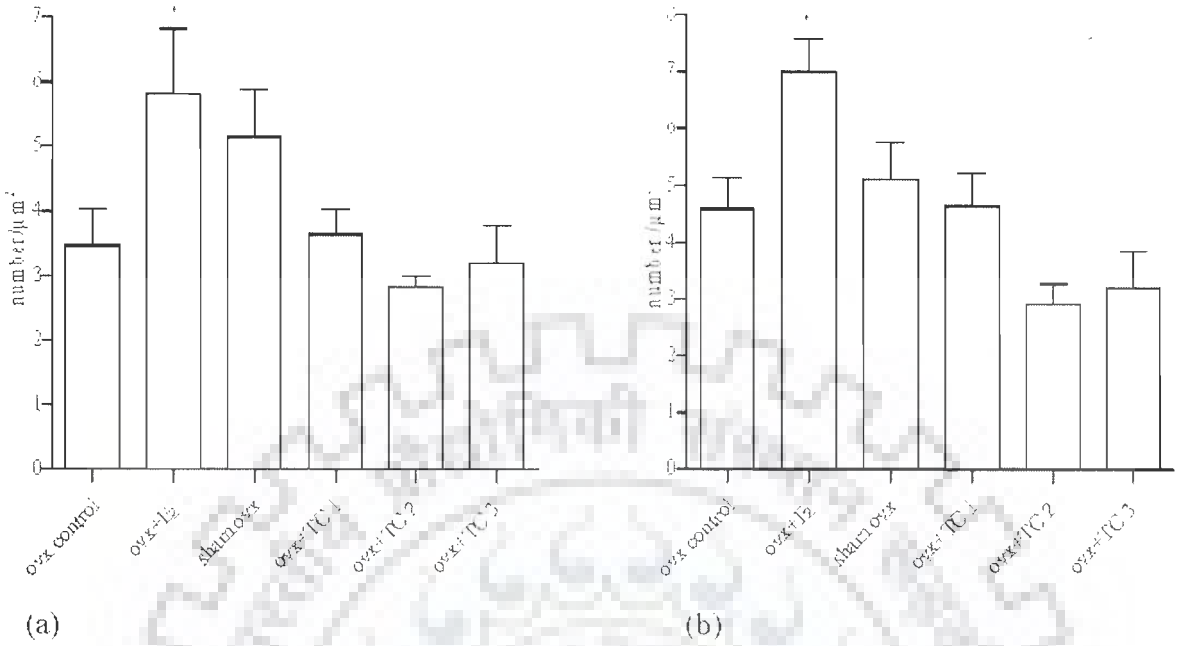


Figure 4.15: Comparison of the number of ducts (a) and lobuli (b) per µm^2 of the mammary gland area. Each value denotes mean ± S.E.M. (n=7/group). * p<0.05 vs. ovx control

4.3.2 Chronic treatment

From the acute treatment results, an effective dose of TC was found. In the chronic experimental design only this dose group was included along with the positive and negative control.

4.3.2.1 Body and uterine weight measurements

The effect of different treatments on increase in whole body weight upon 3 month treatment is shown in Table 4.4. Ovx and ovx + TC treated animals showed significant gain in weight in comparison to sham ovx and ovx+ E2 groups.

The changes in the absolute uterine weight are also shown in the table 4.4. No change was observed in ovx + TC treated animals in comparison to ovx even after the long duration of dosing. Higher uterine weights were observed in ovx + E2 group. Since the dosing was done on alternate days, E2 showed little response.

Table 4.4 Comparison of body and uterine weight in different treatment groups over a period of three months

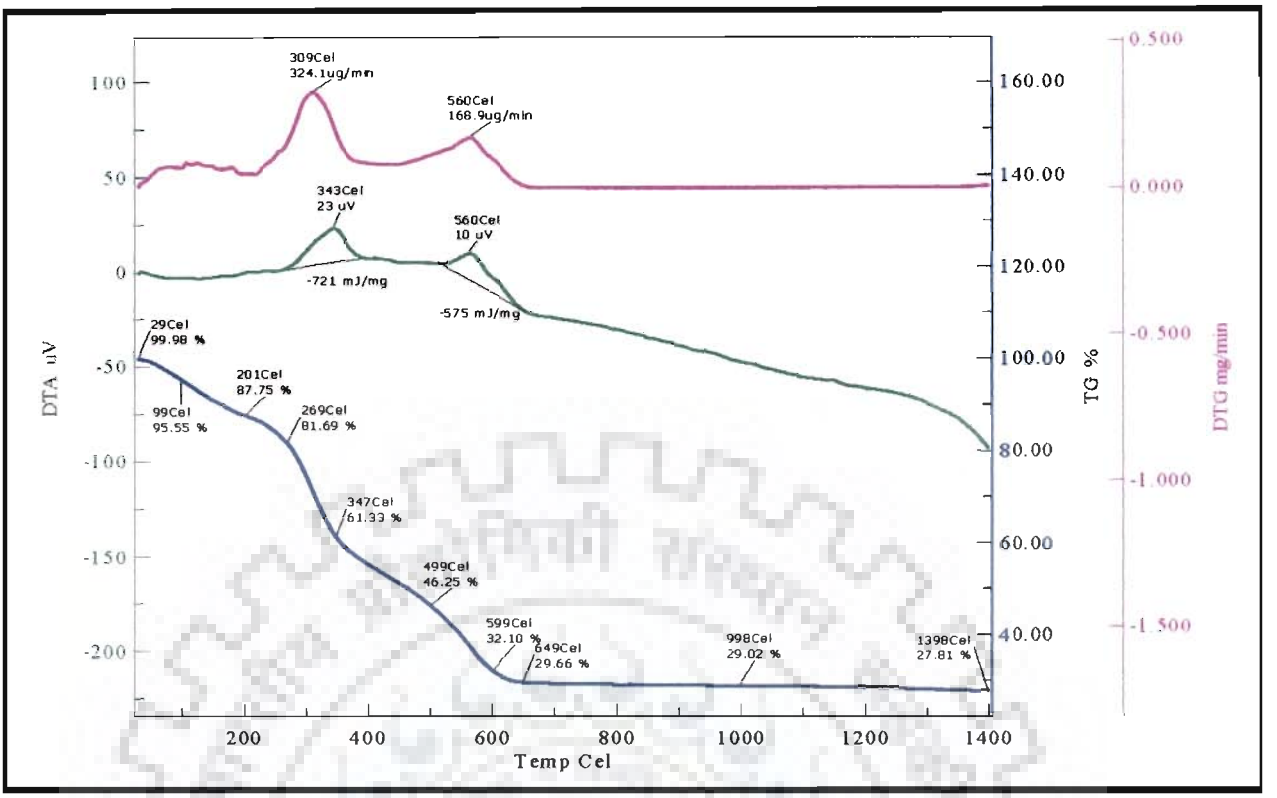
	Sham	Ovx	Ovx+E2	Ovx+TC
Initial BW (g)	246.7 ± 4.7	243.3 ± 6.5	250.8 ± 4.36	240.8 ± 5.9
Final BW (g)	279 ± 6.2	321.7 ± 7.6	290 ± 5.47	308 ± 8.5
Increase in wt (%)	13	32	16	28
Uterus wt, (g)	0.5 ± 0.04*	0.08 ± 0.01	0.17 ± 0.01	0.07 ± 0.01

Each value denotes mean ± S.E.M. (n=7/group), *p<0.05 vs. ovx.

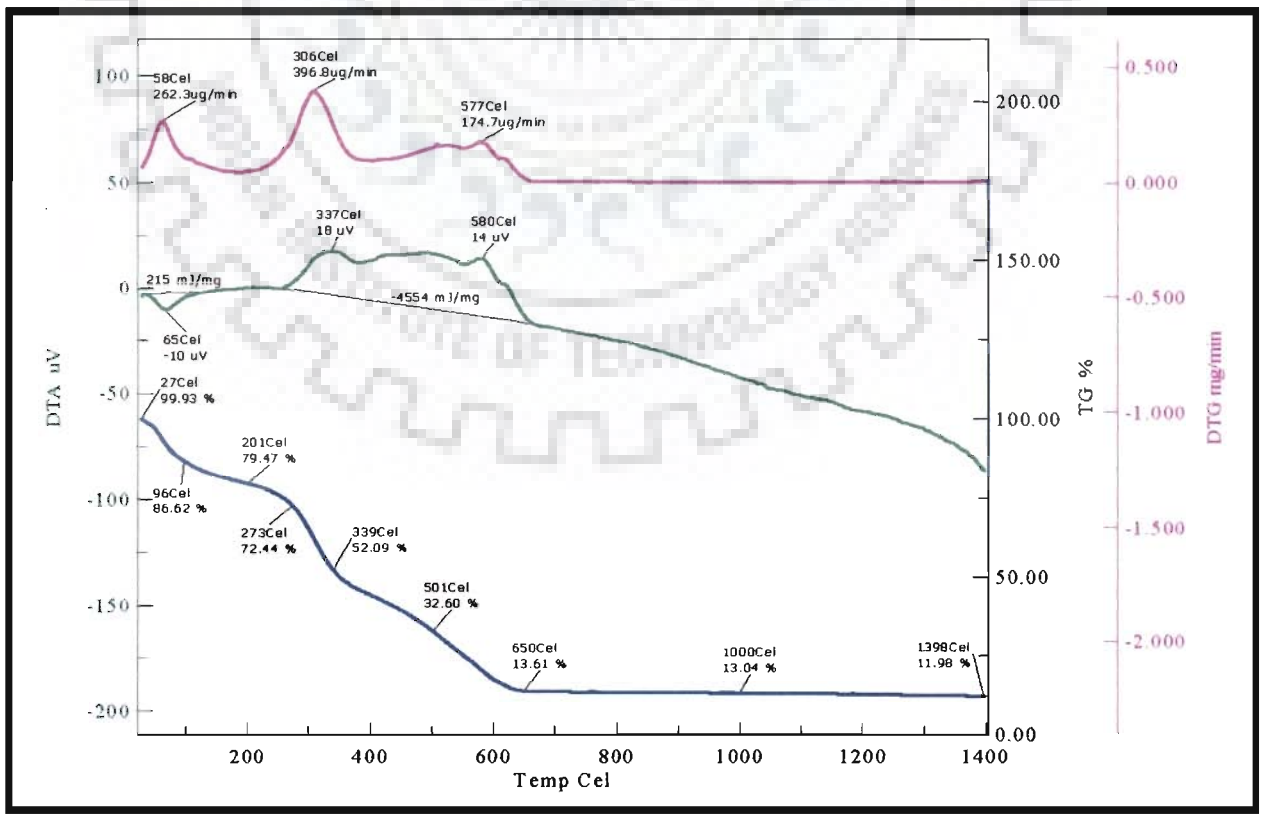
4.3.2.2 Thermal analysis

Thermogravimetry (TG), Differential thermo gravimetry (DTG) and Differential thermal analysis (DTA) has been used for the bone tissues. Thermo gravimetry curves record the weight changes in a material as a function of temperature, while differential thermal analysis shows the endothermic and exothermic heat effects which accompany the various chemical and physical processes.

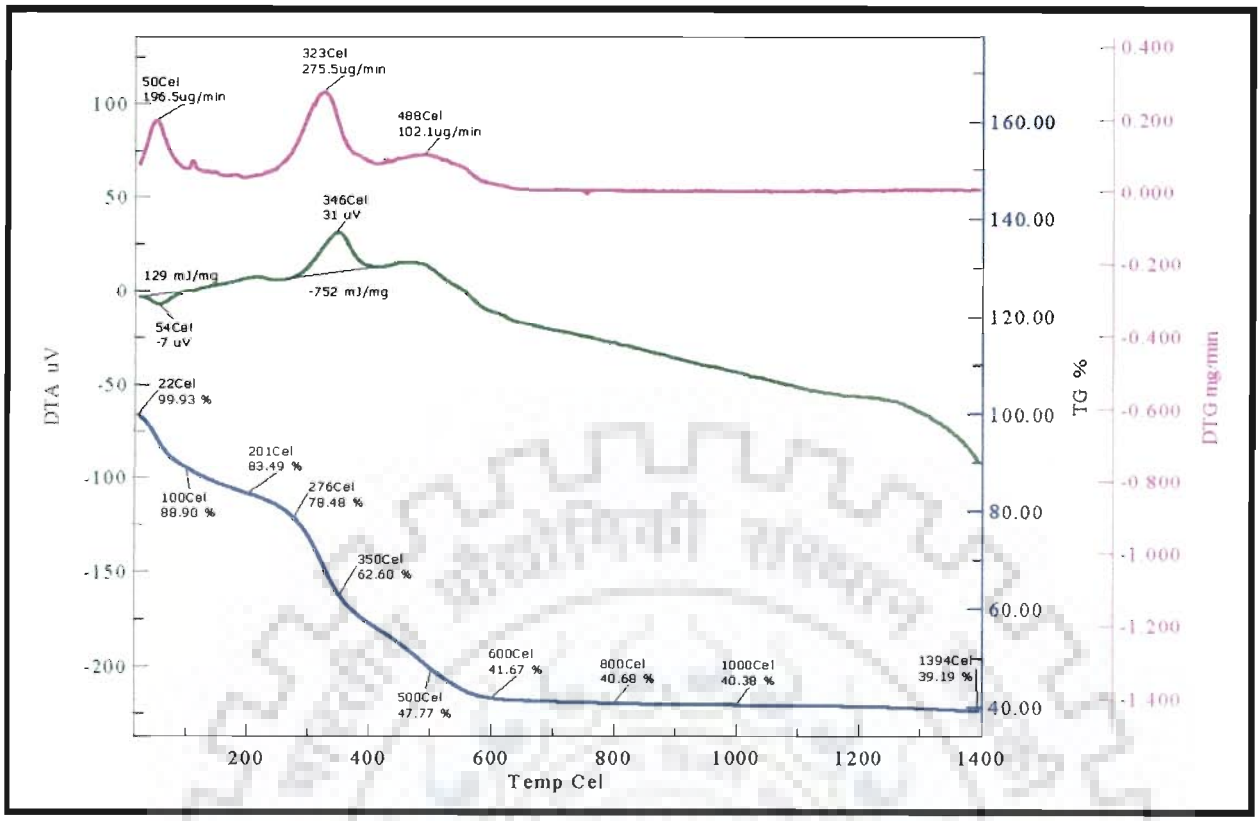
Representative data for TG-DTA-DTG for various treatment groups are shown in Fig. 4.16. TG curves present gradual mass loss in all the groups with changing slope as the temperature rose from 50 to 1500°C. The DTG curve shows three peaks due to three different effects of mass loss (step 1-3). The DTA curves also show three peaks. An endothermic peak was found at temperature ranging from 50-70°C. This represents the dehydration process; mostly the water molecules removed from surfaces of the samples (step 1). Large exothermic peaks were observed between temperatures 338-600°C. These peaks represent the decomposition of organic substances in the bone (step 2). Mass loss of step 3 in the oxidizing atmosphere can be attributed to decomposition of inorganic phase, notably the removal of carbon dioxide from the carbonated apatite



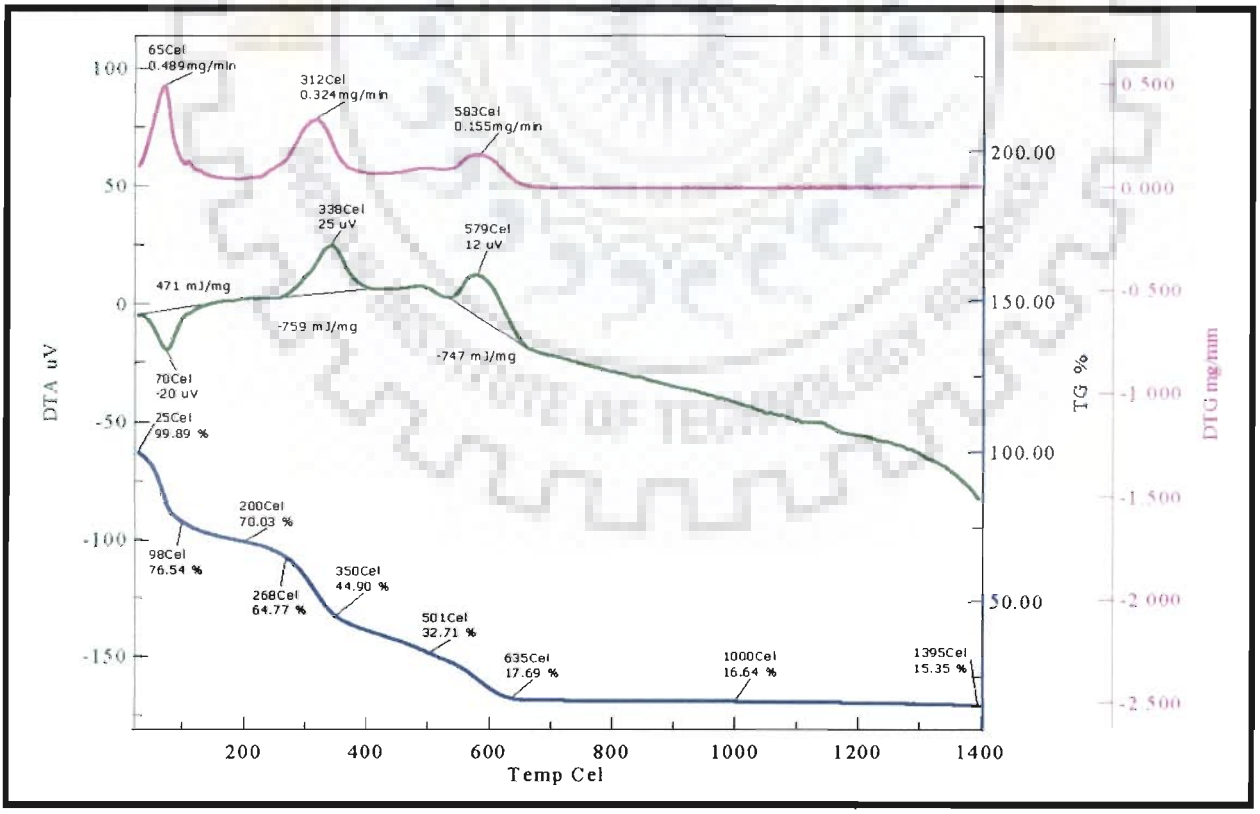
A--- Sham ovx



B--- ovx control



C--- ovx+E2



D—ovx + TC1

Figure 4.16: Representative curves of Thermogravimetry (TG), Differential Thermo Gravimetry (DTG) and Differential Thermal Analysis (DTA).

Table 4.5 and 4.6 summarizes the results of the thermal analysis. Table 4.5 reports the DTA peak temperatures and the recorded values for the observed endothermic and exothermic peaks. Among various groups, significant difference in water loss percentage was observed (8.7-23%). Percentage loss of organic phase was highest in ovx control group (Table 4.6). Absence of distinctive exothermic peaks with very high energy values (-4554 mJ/mg) in ovx control shows the seriousness of pathology. A slight difference in the temperature of exothermic processes i.e. 338-346°C and 560-580°C was observed among the bone samples. In ovx+ E2 group temperature was distinctly different since it has only one peak at 346°C (Table 4.6). TC administration generated two distinct peaks at 338°C and 579°C with values 759 and 747 mJ/mg respectively.

Table 4.5 Comparison of the peak temperature of DTA curves among various groups

Group	Water loss (°C); Endo peak (mJ/mg)	Organic loss (°C), Exo peak I (-mJ/mg)	Organic loss (°C), Exo peak II (-mJ/mg)
Sham ovx	66 ; 128	343 ; 721	560; 575
Ovx control	65 ; 215	337---580 ; 4554	
Ovx + E2	54 ; 129	346 ; 752	
Ovx + TC 1	70 ; 471	338 ; 759	579 ; 747

Table 4.6 Mass loss (%) in various treatment groups

Group	Ambient-100°C (%)	Organic phase loss (%)	≥ 1400°C (%)	Total (%)
Sham ovx	8.75	38.94	3.63	51.32
Ovx control	13.26	69.4	5.24	87.9
Ovx + E2	9.97	47.82	2.49	60.28
Ovx + TC 1	22.72	56.13	5.57	84.42

4.3.2.3 Histology of decalcified tibiae

Figure 4.17 shows photomicrographs of the sections of decalcified bone samples from sham ovx, ovx, E2 and TC treated rats. Results revealed that there was a significant reduction in the amount of metaphyseal cancellous bone in the ovx group compared to sham, E2 and TC treated animals. Photomicrographs of the ovx group also indicated greater inter-trabecular separation, decreased trabecular number and trabecular area. Normal and compact trabecular architecture was observed in sham and E2 treated animals. When compared with ovx moderately thick trabeculae with narrowed inter trabecular spaces were visualized in the animals receiving TC treatment.

4.3.2.4 Scanning electron microscopy (SEM)

The representative scanning electron micrographs of the tibiae taken at 3 months after ovariectomy are depicted in Fig. 4.18. Deleterious effects of ovariectomy on bone tissue are clearly observed. Compared with that of sham ovx rats, ovx animals exhibited greater loss in the trabecular bone region of metaphysis. Resorption sites are more in number in ovx control than in sham ovx animals. Administration of estradiol restored the connectivity in the metaphysis. The administration of TC at dose level 10 mg/kg b.wt. improved the integrity and intactness of the bone microarchitecture as compared to ovx control animals.

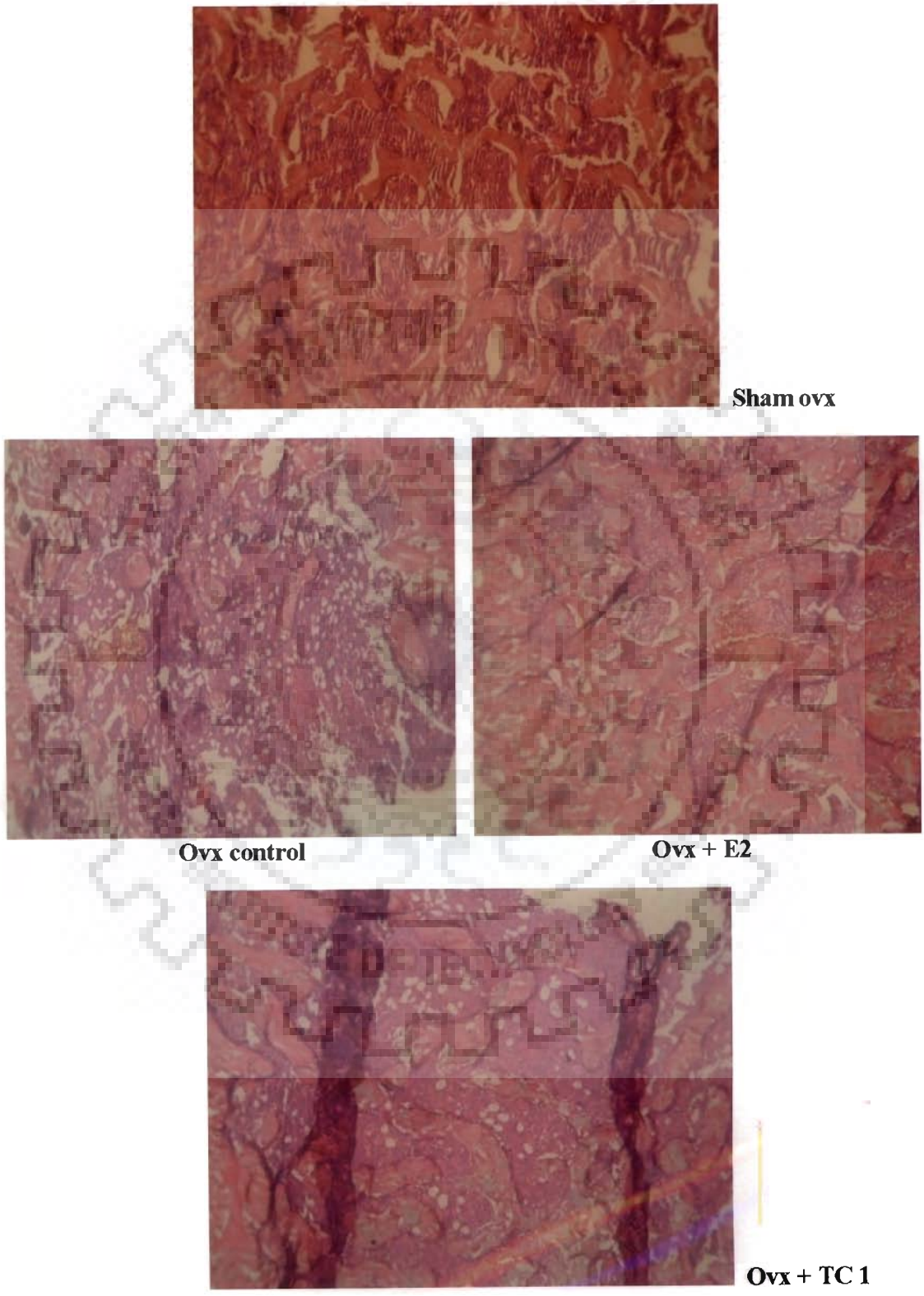


Figure 4.17: Photomicrographs of the sections of the decalcified tibiae. All slides stained with H & E and viewed at 10X.

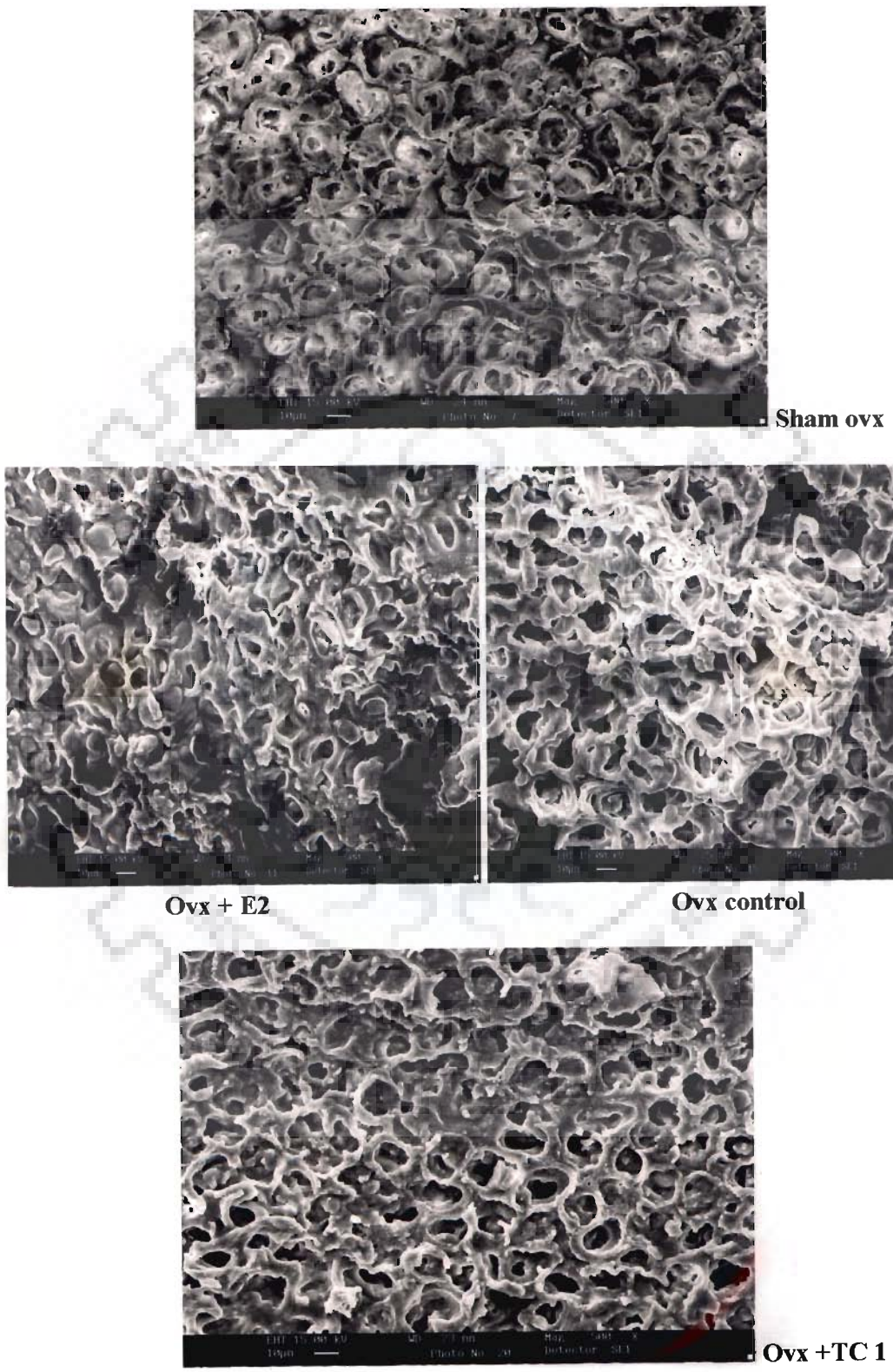


Figure 4.18: Scanning electron photomicrographs (500 X) of the frontal view of the metaphyseal region of decalcified tibiae.

4.4 DISCUSSION

Osteoporosis is a disease, governed by shift in the balance between bone formation and bone resorption. In women, cessation of ovarian function (estrogen decline) as a result of menopause causes negative shift leading to osteoporosis. The ovariectomized rat has been well established as a suitable model for studying postmenopausal bone loss. Both the postmenopausal women and ovariectomized rat due to estrogen decline develop similar characteristics like increased bone turnover, greater loss of cancellous than cortical regions of long bones, obesity and restorative response towards oestrogen therapy (Kalu, 1991). Treatments available like HRT (hormone replacement therapy), bisphosphonates, raloxifene etc protect against bone loss but associated risks like nausea, esophagitis and especially hyperplasia has driven patients toward seeking alternatives like herbs. Many studies have evaluated the potential of plants as osteoprotective agents. Present study is the first attempt to evaluate the potential of *Tinospora cordifolia* as antiosteoporotic agent in ovariectomized rat model for bone loss.

Antiosteoporotic effects of plant extracts are thought to be due to compounds called phytoestrogens which act as estrogen receptor agonists. In the present study, yet unidentified compounds of the TC extract, though osteoprotective *in vivo*, showed only weak displacement properties *in vitro* in the estrogen receptor ligand binding assay. It is known that in plants phytoestrogens are synthesized and stored as glycosides. Due to the sugar moiety, such glycosilated phytoestrogens only weakly bind to estrogen receptors, however, after hydrolysis by the intestinal bacteria, the aglycons have strong binding affinity. Since glycosides are very common in plants, we assume that TC extract also contains such estrogenic compounds which act *via* the estrogen receptor after *in vivo* hydrolysis. Based on studies with ER knockout mice and with subtype specific agonists it appears that the osteoprotective effects of estrogens are mediated *via* the ERα (Hillisch et al., 2004; Mueller and Korach, 2001). Since cytosol preparations from uteri contain both types of estrogen receptors, we suggest that the

osteoprotective effects of TC reported in the present study are due to estrogenic compounds which bind to ERα or both receptor subtypes.

Ovariectomy is associated with an increase in bone turnover, negative bone balance and bone mineral density loss in trabecular region of long bones (Omi and Ezawa, 1995). Particularly the metaphysis of the tibia of mice and rats was shown to react excessively sensitive to withdrawal or replacement of estrogens and by a variety of methods it was shown that demineralization of the metaphysis of the tibia amounts to 50% or more within 3 months following ovx (Seidlova-Wuttke, 2003a; Seidlova-Wuttke et al., 2008). Similar results were observed in the present study as 3 months old rats after weeks of ovariectomy showed a decline in BMD at metaphysis of tibia. E2 treatment showed osteoprotective effect by sustaining BMD in ovx animals. This is also in confirmation with earlier studies which showed that trabecular structures of long bone such as metaphysis of tibia are protected against ovariectomy induced osteoporosis by E2 (Rickard et al., 1999; Seidlova-Wuttke, 2008; Seidlova-Wuttke et al., 2005). Quantitative computer tomography (qCT) proved to be a mean to measure a variety of bone parameters in living small animals and was therefore used by others and ourselves to assess effects of a variety of estrogenic compounds on several bone parameters including BMD.

Biochemical markers of bone turnover respond sensitively towards change in bone remodelling and therefore widely used to study putative antiosteoporotic effects of test substances (Swaminathan, 2001). There are surrogate parameters of bone metabolism available: following ovx the osteoblast products osteocalcin increases in the serum and markers of osteoclast activity; cross-laps are also high in ovx rats (French et al., 2008; Seidlova-Wuttke, 2003a). This indicates that both osteoblast and osteoclast activities are increased in the absence of E2 and in ovx animals the activity of osteoclasts are more stimulated than of osteoblasts which results in more bone resorption than formation (Seidlova-Wuttke, 2003b; Wronski TJ, 1991). Estradiol maintains bone integrity and this is accomplished by stabilizing the serum

levels of the markers stated above (Wronski et al., 1988). In the present study also similar results were observed. Levels of the studied markers were highest in ovx control whereas in ovx+ E2 these were significantly lowered.

The primary proof for an effective antiosteoporotic agent is believed to be a prominent inhibitory effect against bone loss (Hidaka et al., 2006). Ovx animals treated with TC showed a paradox effect. While the bone mineral density was significantly restored (15%) only by TC 1 (10mg/kg b.wt), all the three dose levels i.e. TC 1 (10mg/kg b.wt), TC 2 (50mg/kg b.wt) and TC 3 (100mg/kg b.wt) were beneficial in normalizing the elevated bone marker levels. The differential response of each of these parameters to TC extract may be explained in the following manner. We would like to emphasize that our results with TC extract cannot directly be compared with those of E2 treated group since ethanolic extract of TC have a number of active constituents each of which are present in different proportions. The individual effects of each of these ingredients may not be the same as their combined effects. This in turn may bring about a dissimilar influence on the response of individual parameters to various concentrations of TC extract. It also implies that factors other than phytoestrogens could also be involved. Further detailed studies perhaps with purified individual active elements of TC extract are required to confirm the exact mechanism involved behind this seemingly paradox effect.

TC extract increased alkaline phosphatase activity while E2 caused a significant reduction. At present this opposed effect cannot be explained however, this finding may indicate that TC compounds exert a selective estrogen receptor modulator activity in the bone. Regardless of the molecular mechanisms of TC action in bone, the data indicate that osteoblast activity is modulated by TC which is a prerequisite for an action in bone.

Weight gain in ovariectomized rats has been well documented. This is considered as a response of the body to protect long bones such as femur and tibia against osteopenia (Roudebush et al., 1993). In our studies estrogen was found to maintain body weight close to sham operated levels. It is likely that estrogens act by exerting anti lipogenic effects on adipose

tissue as proposed by earlier workers (Anwar et al., 2001). Substantial gain in body weight was observed in all three groups of TC treated ovx animals and this increase in weight was significantly higher than the E2 treated group. This implies that the weight gain may have been achieved by factors in TC extract other than estrogen. Besides, the estrogen loss may have led to accumulation of energy stores leading to accumulation of fat (reflected in the rise in cholesterol, HDL and LDL) and hence weight gain.

Ovariectomy enhanced the levels of both total and high density lipoproteins (Table 4.2). A study by Lundeen *et al* (1997) reported that increased levels of lipoproteins were normalized by the administration of estrogens in ovx rats (Lundeen et al., 1997). In our study, the subcutaneous injections of estradiol in ovx rats normalized these lipid parameters to the level of sham rats. Administration of TC to ovx rats depresses the lipid metabolism (Table 4.2) which strongly suggests that TC would be beneficial for cardiovascular disease for women after menopause.

Uterus is the most sensitive organ towards estrogen. Hormone decline after ovariectomy rapidly regresses the organ and hence weight loss (Hidaka et al., 1997). Estrogen therapy can restore the tissue. Stimulation of IGF-1 and inhibition of ERβ gene expression are the reported mechanisms by which E2 mediate this effect (Adesanya et al., 1999; Klotz et al., 2002). IGF 1 is a growth factor which causes proliferation in uterus by increasing the thickness of endometrium and myometrium layers. Similar results were noticed in the present study. E2 treated ovx animals showed significantly thick layers and therefore heavier uteri compared to ovx control and TC treated ovx animals.

Mammary glands are also target for estrogens and it has been shown that E2 in ovx animals stimulate proliferation by acting through ERα (Tekmal et al., 2005). Just as in the uterus, the mammary gland showed a similar response in E2 treated ovx animals compared to ovx control. Proliferation is such that secretion is also demonstrable in ducts. Number of ducts and lobuli are also increased confirming the stimulation. On the other hand, no changes were

seen in TC treatment groups compared to ovx control. This indicates that the non proliferative action of TC extract in reproductive tissues is governed by the factors that are not sensitive enough like estrogens for binding to the receptors responsible for proliferation.

Bone is a complex composite material consisting of approximately 10% water, 30% organic phase (mainly collagen fibrils) and 60% inorganic material (predominately carbonated hydroxyapatite) (Onishi et al., 2007). The strength and hardness of bone is a function of the interaction between the hydroxyapatite crystals and collagen fibres. Largely the assessment of osteoporotic status is based on bone densitometry techniques such as quantitative computed tomography, bone histomorphometry and biochemical markers study. Although, trabecular bone density is a widely used method for assessing fracture risk and therapeutic efficacy, it does not always predict the risk of individual fractures, explain the pathophysiology of osteoporotic changes or assess the impact of a particular intervention completely (Hopper et al., 2004). The quantitative analysis of trabecular bone structure and the elucidation of relationship between structural properties and bone strength are therefore important. Many authors have studied and compared the structure and physico-chemical properties of normal and osteoporotic bones. Findings revealed that osteoporotic bone mineral is monotonically different in its properties expressed as crystallinity/maturity (Paschalis et al., 1997), showed less strength and stiffness (Dickenson et al., 1981), showed decrease in reducible collagen cross links without an alteration in collagen concentration which tend to increase bone fragility (Burr, 2002) and possess collagen with higher level of cross linking (Mansell and Bailey, 2003) than the normal bone. With an aim to further assess the beneficial effects of TC administration in ovx animals the structural and physico-chemical studies were performed. Methods like thermal analysis, histological and scanning electron microscopic studies were used to elucidate the micro architectural and physico-chemical properties change in bones samples after the administration of different compounds.

Thermogravimetry (TG) and Differential thermal analysis (DTA) in combination with DTG has been used for calcified tissues providing parameters related to water, protein and carbonate content (Okamoto, 1998). Pathologic bones present a completely different behaviour during thermal analysis compared to healthy bones. Therefore, this method may be a useful qualitative tool to measure the seriousness of pathology similar to the measurement of bone mineral density for assessing osteoporosis and the effects of drugs upon it.

Larger peak values generated from thermal analysis are often associated with the porous or eroded appearance of the trabecular bones (Hidaka, 1997). Similar results were observed in the present study. Ovx group not only showed lack of normal microarchitecture but also exhibited high energy values for exothermic peaks. The smaller energy value of peaks for the sham rats may be ascribed to a fine particle structure of the surface of trabecular bone. TC improved the integrity of structure, energy pattern and generated distinct peaks. Only the temperature of the exothermic process of ovx+E2 was distinctly different indicating that the effect of E2 was different from that due to TC.

Ovariectomy results in increased bone turnover and net bone loss with a permanent deficit of bone mass at several skeletal sites rich in cancellous bone such as proximal femur, vertebral bodies and the metaphysic of long bones (Nagareddy, 2006). The microarchitectural alteration in cancellous bone network is very similar to that seen in postmenopausal osteoporosis, including thinning of trabecular elements (Wronski et al., 1989). This phenomenon is also reflected in our histological and microscopic findings. Observations of photomicrographs suggested a decreased bone mass with increase in the number of resorption sites. TC treatment reduced the thinning of trabeculae and also showed a minimum number of such trabeculae. It improved the normal integrity, structure and compactness of bone and therefore appears to be beneficial for the prevention of osteoporosis.

Since TC produced estrogen-like effects in bone and serum markers but not in reproductive tissues, it might be possible that the tissue specific response arises due to different

constituent present in TC extract. This is a classical example of how the harmful effects of active ingredients are neutralized by other factors present in plant extracts. It is for this reason that ayurvedic medicine advocates the use of plants in their native form.

CHAPTER 5

20-β HYDROXYECDYSONE- A POSSIBLE CANDIDATE FOR THE ANTIOSTEOPOROTIC ACTION OF *Tinospora cordifolia*

5.1 INTRODUCTION

Literature related to natural products, evaluated specifically for the prevention of postmenopausal bone loss unveils plethora of phytochemicals that have been responsible for their antiosteoporotic action. They belong to different classes such as flavonoids, lignans, carbohydrate, alkaloids, saponins, stilbene and tannin. In search for the active compounds in *Tinospora cordifolia* stem extract, responsible for the antiosteoporotic action of extract we identified an ecdysteroid namely 20-β hydroxy ecdysone (β-Ecdysone = Ecd) as a possible candidate.

Ecdysteroids are the steroid hormones of arthropods and probably of many other invertebrate phyla. In insects, they regulate moulting, metamorphosis, reproduction and diapause. Ecdysteroids are also present in 5-6% of plant species, generally at far higher concentrations than those typically found in arthropods. Their presumed function is to mostly act as protection agents (toxins or antifeedants) against herbivore predators (Dinan, 2001a). The archetypal ecdysteroid in both arthropods and plants is 20-β hydroxyecdysone (Fig. 5.1).

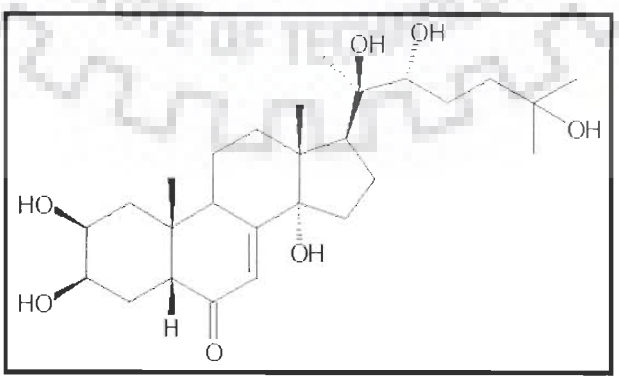


Figure 5.1: Structure of 20β-hydroxyecdysone

Ecdysteroids differ markedly from vertebrate steroid hormones:

1. Polarity: They possess good water solubility caused by the high number of hydroxyl groups

2. Bulk: Zooecdysteroids are mainly C27-C29 steroids in contrast to C18, C19 and C21 of hormones.

3. Shape: Bear a cis-fused A/B ring junction compared to trans-fused A/B.

Phytoecdysteroids are not endogenous products of mammalian metabolism and are non-toxic to mammals and may even have a number of beneficial pharmacological and medicinal applications (Dinan et al., 2001b). This is consistent with the use of several ecdysteroid containing plant species in traditional medicines. The ready availability of large amounts of ecdysteroids from certain plant sources has led to a boom in recent years in its inclusion in many commercial anabolic preparations for body builders and sportsmen (Dinan and Lafont, 2006). Wide array of pharmacological effects of ecdysteroids on mammals are shown in Table 5.1.

Table 5.1 Pharmacological effects of ecdysteroids in mammals

Ecdysteroids
Protein metabolism Increase muscle mass by enhancing protein synthesis and decreasing protein catabolism (Otaka et al., 1968)
Lipid metabolism Stimulate bile secretion, improve liver regeneration after chemically induced damage Reduced lipid peroxidation in membranes (Kuzmenko et al., 1997)
Carbohydrate metabolism Anti-diabetic effects are known for ecdysteroid-containing plants used in traditional medicine Increase glucose consumption in insulin independent fashion (Chen et al., 2006) Reduce hyperglycaemia induced by administration of glucagon or alloxan treatment (Yoshida et al., 1971)
Skin Promotes differentiation of human keratinocytes in vitro (Detmar et al., 1994) Improves skin quality by accelerating the healing of wounds and burns (Dinan, 2006)
Brain Induction of enzymes related to neurotransmitter synthesis or degradation Protection of neurons against drugs

5.2 MATERIALS AND METHODS

5.2.1 Identification of Ecd in extract

20β-hydroxyecdysone (Ecd) has already been established as one of the important biomarkers present in TC species. With few modifications method of Ahmed et al (Ahmed, 2006) i.e. HPLC-UV method employing gradient elution was used to identify and estimate Ecd amounts present in TC stem extract used for the present study.

5.2.1.1 Solvents and chemicals

All the solvents used in this study were of HPLC grade. Both phosphoric acid and acetonitrile were purchased from J. T. Baker (Germany). Water used was prepared with a Milli-Q water purification sytem. The standard powder form of marker was purchased from Sigma. The purity of the compound was >95%.

5.2.1.2 Instrument

- HPLC Pump K-501, Dynamic interface box and Chromgate (integrator and analysis program) --- Knauer
- 851-AS Intelligent auto sampler ----- Jasco
- LC-95 UV7 Visible Spectrophotometer Detector---- Perkin Elmer
- Column: NC-04 250 x 4,0mm Hypersil-OD1 5,0µm P/N 2504 F180HY050 ---- Bischoff
- Precolumn : 10µm C18 Shandon ODSiiRP --- Shandon

5.2.1.3 Characterization

UV detection of Ecd was performed at 254nm. The stock solution of standard ecdysone and plant extract were prepared at the concentration of 1mg/ml in ethanol and dimethyl sulphoxide respectively. Ten and hundred µl were injected respectively for each solution. The solutions were filtered through a Millipore filter (0.45µm) before injection onto the HPLC

system. The chromatographic profiles of the standard Ecd and the extract solution were obtained and analyzed. The gradient method used for analysis is shown in Table 5.2.

Table 5.2 Gradient method employed in HPLC-UV method

Time (min)	Flow (ml/min)	Solvent A (%)	Solvent B (%)
0	1	80	20
15	1	0	100
20	1	0	100
30	1	80	20
32	1	80	20

A: 0.085% phosphoric acid in water; **B**: Acetonitrile (100%)

5.2.1.4 Chromatograms

The representative HPLC-UV chromatograms of the standard Ecd and crude plant extract are shown in the Fig. 4.3 and Fig. 4.4. The chromatograms exhibited a smooth baseline with excellent resolution and the biomarker peak can be identified. The presence of biomarker peak in crude extract under similar experimental conditions as that of standard authenticates the plant as *T.cordifolia*.

5.2.2 Estrogen ligand binding assay

To test the estrogenicity of Ecd in a cytosolic estrogen receptor preparation the method of Jarry et al (Jarry, 2003) as explained in section 4.2.2 was employed. Samples were measured in triplicates.

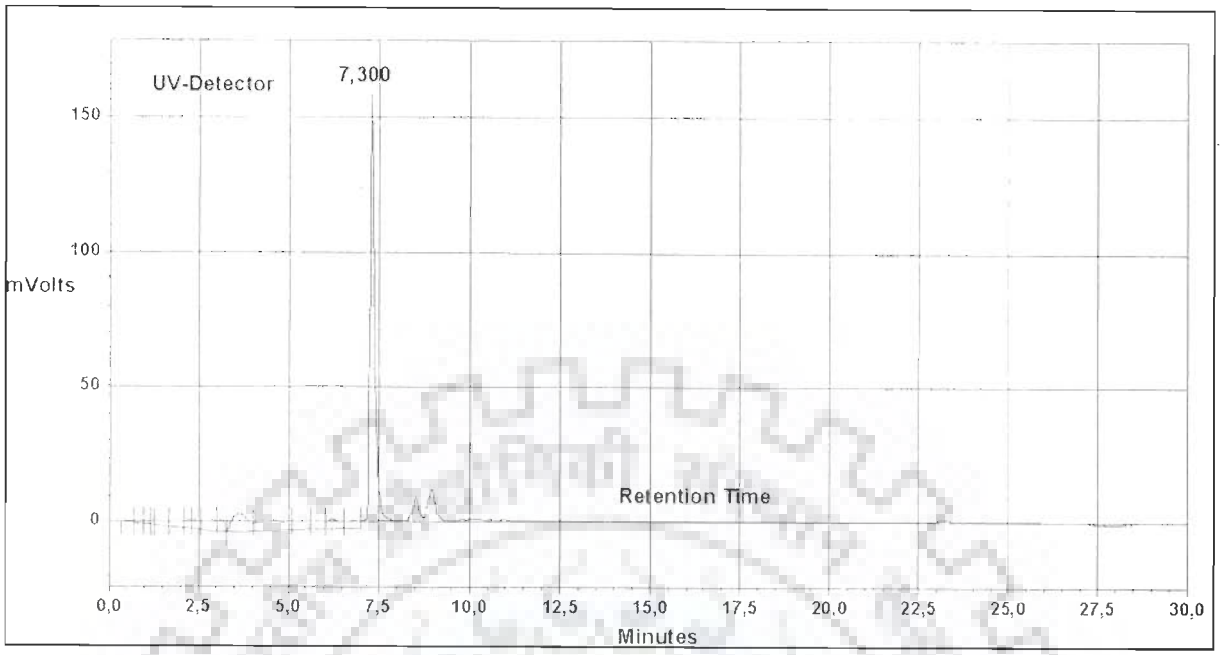


Figure 5.2: HPLC-UV chromatogram of 20β-hydroxyecdysone, retention time: 7.3 min

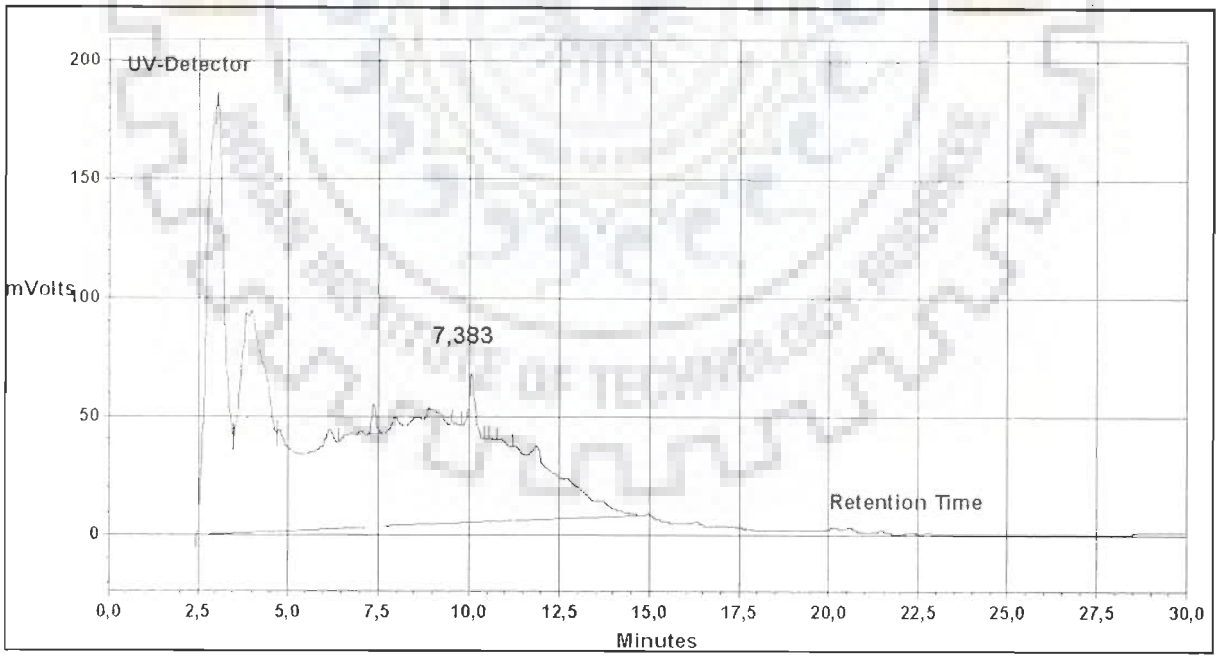


Figure 5.3: HPLC-UV chromatogram of crude plant extract, retention time: 7.38 min

5.2.3 Animals

5.2.3.1 Ethical approval

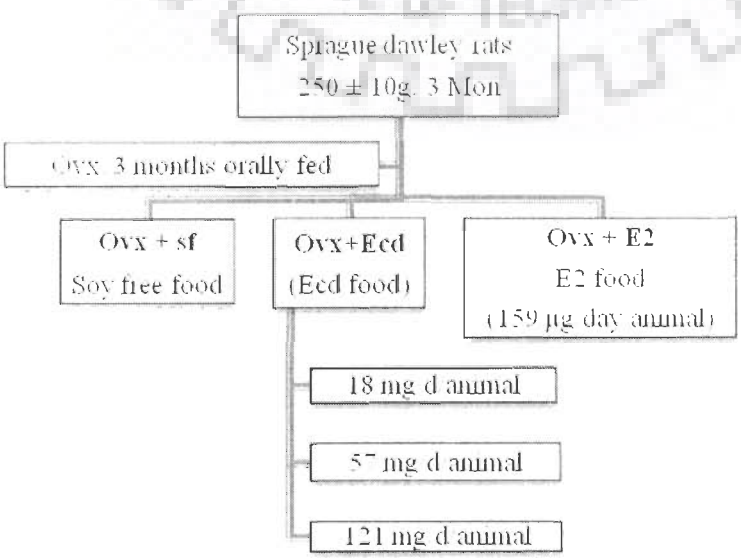
Allowance to perform the experiments was obtained from the Bezirksregierung Braunschweig, Germany (permission No. Az. 33.42502-082/06).

5.2.3.2 Strain and husbandry

Female Sprague Dawley rats were used for the present experiments. 3 months old rats (Winkelmann, Borken, Germany) weighing 250 ± 10 g were adjusted to our animal facilities (5 animals/cage; light on 06.00 a.m. to 06.00 p.m., relative humidity 55%). They were kept on soy-protein, isoflavone-poor pelleted food (ssniff, V 1355, R-Z, poor in phytoestrogens) in which isocaloric protein supplementation was secured by added potato proteins (details shown in Fig. 4.2).

5.2.3.3 Allocation and dosage

Three weeks after acclimatization, animals were anaesthetised with Isofluran (Forene®, Abbott), weighed and subjected to qCT for determination of BMD of the metaphysis of tibia (methodology explained in section 4.2.5.2.2). One week later rats were ovariectomized. The ovx rats (n=10) were separated into five groups as follows:



The test substance, 20-OH-Ecdysone (= ß-Ecdysone = Ecd), 97.2% pure was commercially available through Changzhou Dahua Import and Export (Group) Corp. Ltd. Changzhou, Jiangsu, China. The animals were maintained in the conditions for 3 months. At the end of the treatment the animals were subjected to qCT measurements of the metaphysis of tibia and then sacrificed under CO_2 anaesthesia. Blood samples were collected by decapitation. The uteri were removed, cleaned of adherent tissue and weighed.

5.2.4 Evaluation parameters

5.2.4.1 Body weight and food intake measurements

Body weights of animals and food intake were measured once per week. The estimation of E2 and Ecd daily intake per animal was calculated from the weekly measured food consumption per cage divided by 7 and the number of animals per cage.

5.2.4.2 Safety assessment

Sensitive parameters followed to assess the estrogenicity and safety of test compounds in *in vitro* and *in vivo* systems has already been explained in section 4.2.5.2.4.

5.2.4.3 Bone parameters

The following bone parameters were calculated utilizing the XCT 5.40 software package:

1. Density of cancellous bone, where mineral density was between 280 and 710 mg/cm^3.
2. Cortical density, the cortical area where mineral density was above 710 mg/cm^3.
3. On the basis of these data, a strain strength index (SSI) can be calculated which, after adjustment to the body weight of the animals gives an index of stability of the bone.

5.2.4.4 Serum analysis

The blood samples were centrifuged (3000 g, 20 min) and the serum stored at –20°C for further analysis. Serum E2 was assayed with a commercially available kit (DSL, Sinsheim, Germany). Serum osteocalcin was determined using a human system (Elecsys,

Roche, Mannheim, Germany) and the C-terminal telopeptides of type I Collagen were measured with an ELISA (RatLaps ELISA: Nordic Biosciences, Herlev, Denmark).

To recover Ecd from serum, enzymatic hydrolysis of potential metabolites was performed before serum extraction. A volume of 500 µl serum was extended with 500 µl NH acetate buffer (pH 5.0) containing 1 mg ß-glucuronidase (Helix Pomatia ß-Glucuronidase Type H1; Sigma, Taufkirchen) and incubated overnight at 37 °C. The Strata X solid-phase extraction method (8B-S100-UBJ, Phenomenex, Aschaffenburg) with a polymeric sorbent was used according to the instructions of the manufacturer. The eluted volume was evaporated to dryness and reconstituted with 100 µl ethanol. For HPLC a volume of 20 µl was chromatographed over a NC 2504.6 mm Hypersil-ODS 5.0 µm column (Bischoff, Leonberg, Germany). Ecd was detected at 254 nm.

5.2.5 Statistical analysis

Data were expressed as means ± standard errors of the means (SEM). Significant differences between control and treatment groups were analyzed by one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons (Prism™, Graph Pad, San Diego, USA). P values < 0.05 were considered statistically significant.

5.3 RESULTS

5.3.1 Estrogen ligand binding assay

Figure 5.4 depicts the interaction of Ecd with ER from cytosolic preparation of porcine uteri. While, E2 readily displaces the radio labelled ligand in the low nM range, Ecd was totally ineffective even at concentrations of 10^{-3} M.

5.3.2 Safety assessment

Figure 5.5 details a marked increase of uterine weights in the E2 treated controls. This effect was not shared by any of the three doses of Ecd.

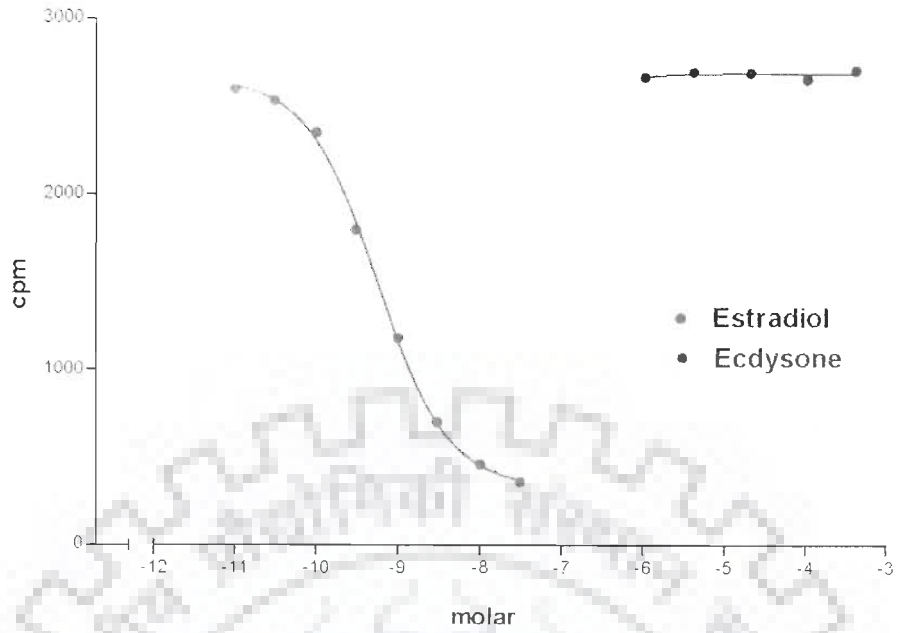


Figure 5.4: Displacement curve of E2 and Ecd extract in an ER-LBA with porcine uterine cytosol. (n=3 at each concentration).

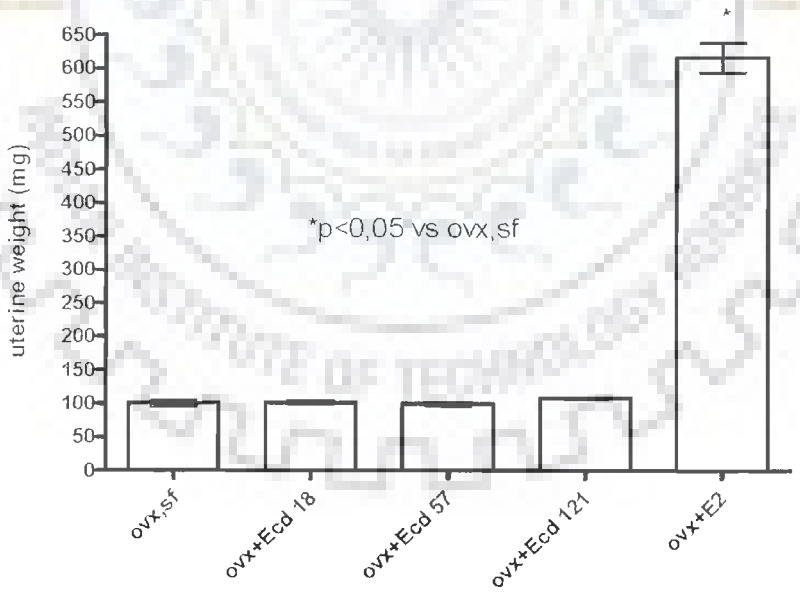


Figure 5.5: Comparison of uterine weight among various groups. Each value denotes mean ± S.E.M. (n=10/group). * $p<0.05$ vs. ovx, sf

5.3.3 Body weight and food intake measurements

Food intake in ovx control and Ecd treated animals did not vary significantly i.e. 18-20g/day/animal. In E2 fed animals, intake was significantly less i.e. 15.9 g/day/animal as compared to other two treatment groups. No significant difference in the body weights of ovx control and Ecd treated animals was observed.

Table 5.3: Average daily food and test substance intake and the final body weights of the animals.

Group	Avg. daily food intake (g/day/animal)	Avg. daily substance (mg/day/animal)	Final body weight (g)
ovx, sf	17.32 ± 1.3	-	324.7± 21.5
ovx+Ecd 18	18.0 ± 1.1	18.02	324.4 ± 30.5
Ovx+Ecd 57	18.9 ± 1.3	56.5	333.2 ± 21.1
Ovx+Ecd 121	19.3 ± 1.7	120.8	344.5 ± 10.9
Ovx+E2	15.9 ± 3.9	0.159	274.5 ± 23.7

Each value denotes mean ± S.E.M. (n=10/group)

5.3.4 BMD measurements

The effects of Ecd on cancellous and cortical densities in the metaphysis of the tibia are detailed in Fig. 5.6 (a and b)

Trabecular or cancellous densities (Fig. 5.6(a)) were reduced by more than 50% in ovx+sf group in comparison to the preovx values. The three doses of Ecd partially prevented the reduction of cancellous density. Interestingly, the intermediate dose of Ecd was less effective in this respect than the highest and lowest dose. Cortical BMD (Fig. 5.6 (b)) was significantly increased in all ovx animals in comparison to preovx values. The weight related strain strength index was lowest in the ovx, highest in the E2 and at intermediate value in the Ecd treated animals (Fig. 5.7).

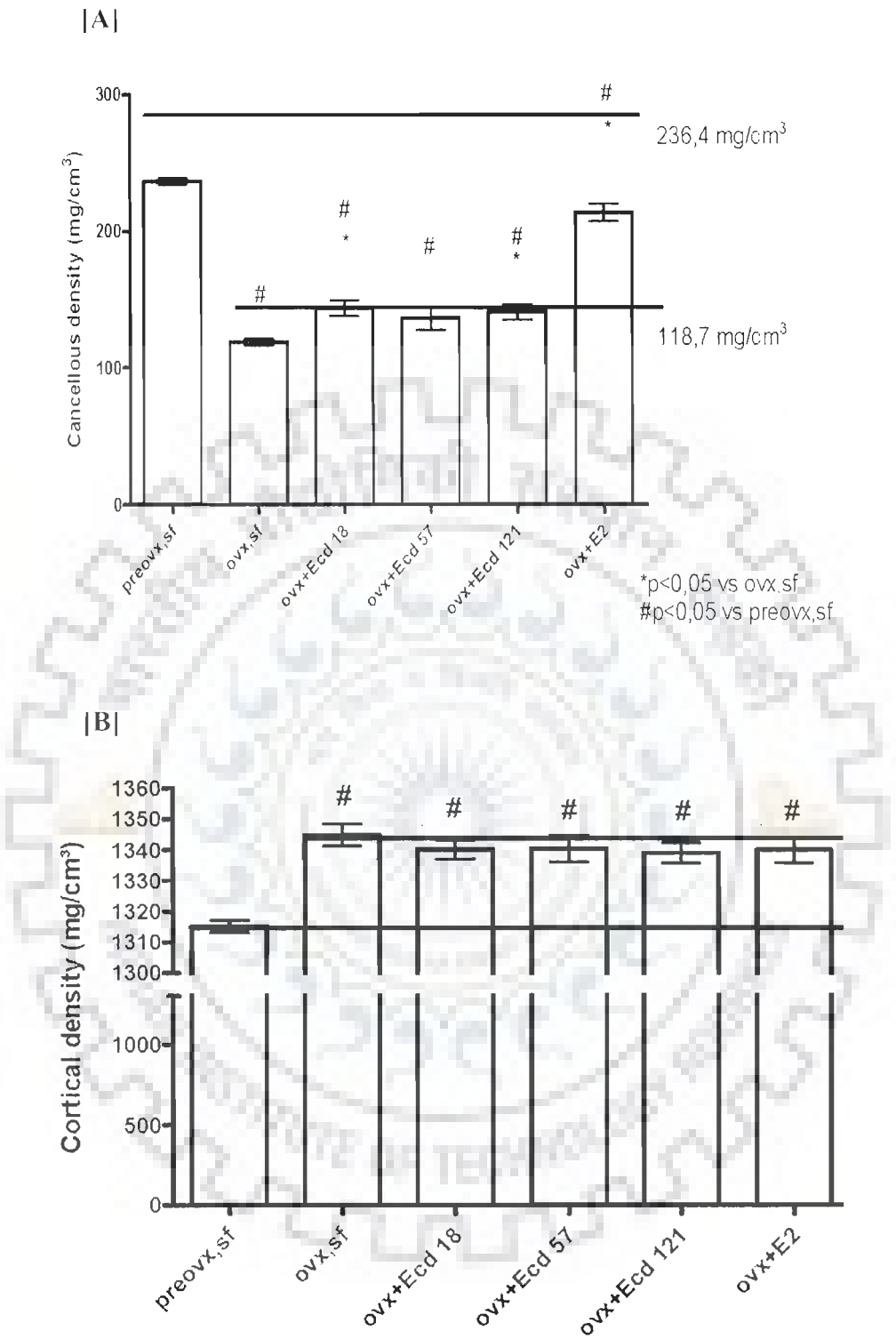


Figure 5.6: Trabecular |A| and Cortical |B| densities of the metaphysis of tibia for various groups plotted as compared to preovx. (n=10/group). * $p<0.05$ vs. ovx, sf and # $p<0.05$ vs. preovx, sf

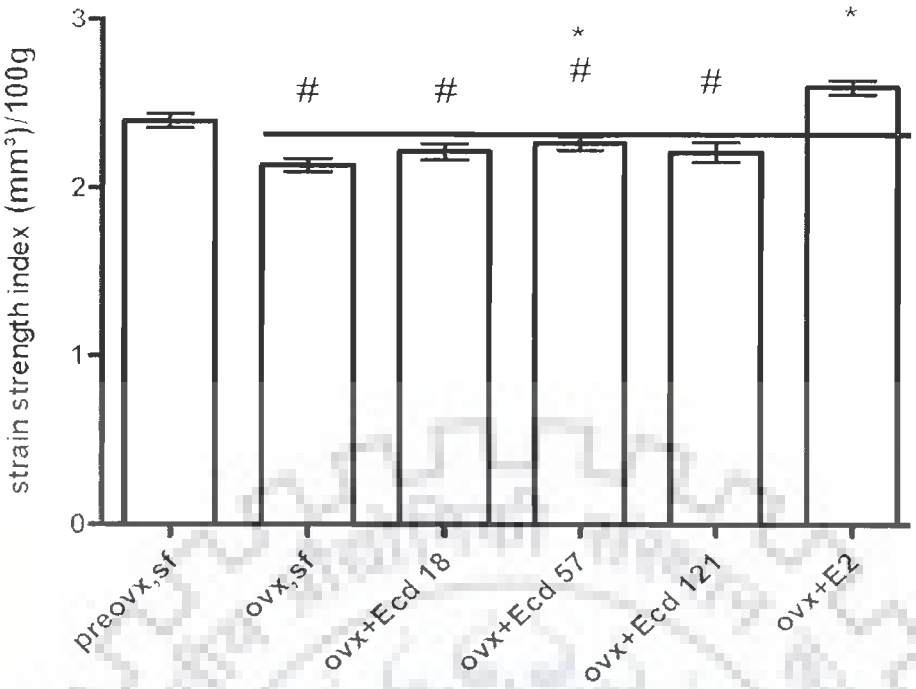


Figure 5.7: Strain strength index/100g (SSI) of tibia for various groups plotted as compared to preovx. (n=10/group). * $p<0.05$ vs. ovx, sf and # $p<0.05$ vs. preovx, sf

5.3.5 Serum analysis

Serum OC levels were high in ovx animals and remained at the high values in the Ecd treated animals while the E2 application resulted in a significant reduction of OC (Fig. 5.8). The metabolic breakdown products of bone specific collagen I, the cross-laps were also high in ovx animals and reduced by Ecd and E2 (Fig. 5.9).

Serum concentrations of Ecd in the animals receiving the food with the highest Ecd amounts were 1.2 x 10^{-6}M. In sera of animals receiving lower amounts of Ecd the assay sensitivity was at its limit. Serum E2 levels in the E2 fed animals were 73 ± 24.4 pg/ml. In the other animals E2 concentrations were below the detection limit of the assay, i.e. <5 pg/ml.

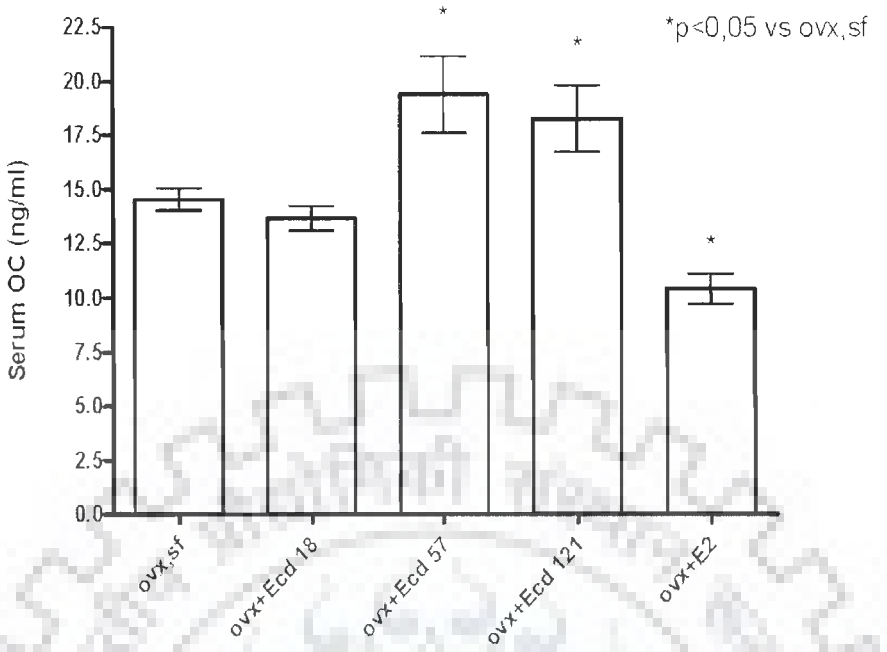


Figure 5.8: Effect of Ecd on serum osteocalcin level (ng/ml). (n=10/group). * p<0.05 vs. ovx, sf

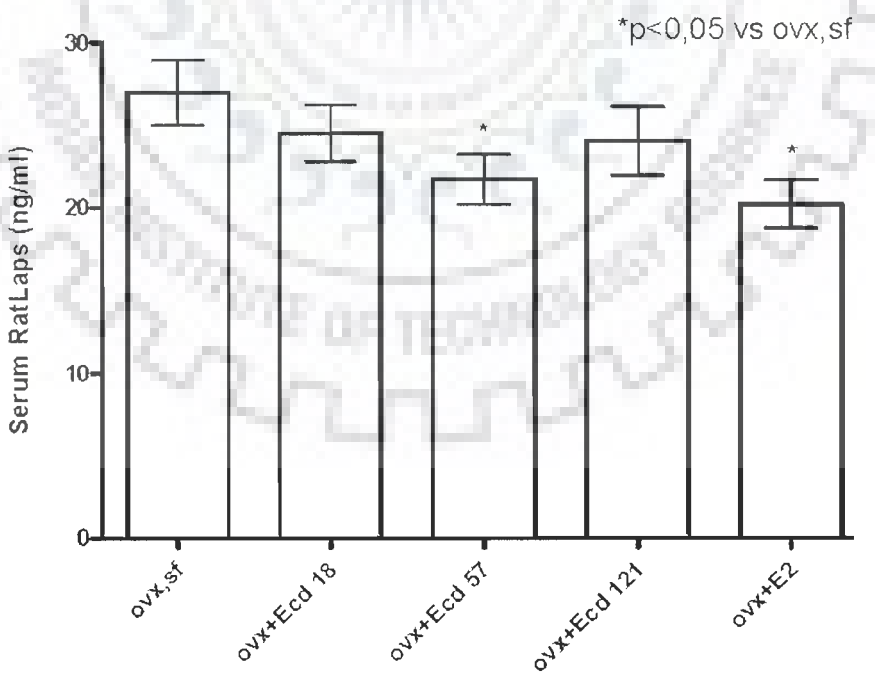


Figure 5.9: Effect of Ecd on serum cross-laps (C-terminal telopeptide) level (ng/ml).

*** p<0.05 vs. ovx, sf**

5.4 DISCUSSION

Ever since the report of increased breast cancer risk and cardiovascular incidences following hormone replacement therapy an intensive search for alternatives has begun. Phytoestrogens, such as isoflavones derived from soy, red clover or hops were promoted but they have similar though milder effects than estradiol-17ß benzoate (E2). In the present experiment we demonstrated a marked effect of Ecd to prevent osteoporosis in ovx animals.

Ecdysteroids differ markedly from vertebrate steroid hormones in their polarity, size and shape, thus one would hypothesize its little interaction with the steroid-hormone receptors or steroid-metabolizing enzymes in mammals. Lack of Ecd activity in E2-ligand binding assay supports the hypothesis. Lacking effect of any of the three Ecd doses to stimulate uterine weights further concludes that the observed effects in the bone are not due to an estrogenic effect of the ecdysteroids. Also androgenic effects of Ecd were not found previously (Gorelick-Feldman et al., 2008; Semeikin et al., 1991). What then are the mechanisms of action of Ecd?

Ecd is known to have protein anabolic effects in the mammalian organism (Gorelick-Feldman, 2008). In the human Ecd was shown to increase lean body mass. Particularly muscular training in combination with an Ecd treatment causes an increase in muscular mass (Bathori et al., 2008). Such increase in muscular mass may have also occurred in the ovx rats resulting in increased mechanical stimulation of the bone. Mechanical stimulation of the bone tissue is known to have bone protective effects (Ferretti et al., 2003; Flieger et al., 1998; Rubin et al., 2002).

Attempts to demonstrate Ecd receptors in mammalian tissues failed. There is some evidence however, that Ecd can stimulate transactivation via stimulation of the retinoid X receptor (RXR) (Thomas et al., 1993; Yao et al., 1993). Among others RXR may dimerize with the peroxisome proliferator activated receptors (PPARs), the retinoic receptor (RAR) (Germain et al., 2006) or the Vitamin D (VD) receptor (Bathori, 2008; Baudino et al., 1998; Farmer et al., 2000) Vitamin D promotes intestinal calcium resorption and stimulates the production of

osteocalcin (Nagpal et al., 2001) a protein necessary for mineralization of the protein bone matrix, hence, Ecd stimulated transactivation of a RXR/VD dimer may be one of the factors by which Ecd exerts its beneficial effects in the bone. In favor of this explanation is our observation that osteocalcin, an osteoblast product, was significantly increased in the Ecd treated animals, an effect also exerted by VD (Peleg et al., 2002; Shiraishi et al., 2000).

The PPARs are known to be intimately involved in fat metabolism. PPAR α agonists are clinically used as lipolytic drugs (Berger and Moller, 2002; Guerre-Millo et al., 2000; Michalik et al., 2006) while PPAR γ agonists predispose the development of lipocytes at the cost of osteoblasts. This action would not explain the antiosteoporotic effects of Ecd. Possibly, the ecdysteroid has selective PPAR modulator effects (SPPAR effects) effects and recruits in the bone more co-repressors than co-activators. Such organ selectivity is known for estrogen receptors where selective estrogen receptor modulators (SERM) have estrogen antagonistic effects in the mammary gland but act as estrogen agonist in the bone. Also retinoids are known to have bone protective effects (Weston et al., 2003) and the transactivational effects of the RXR/RAR dimer may result in increased osteoblast activity. Taken together Ecd appears to have fundamentally different mechanism of action from that of E2 which following activation of the ER-α decreases bone metabolism by decreasing osteoclast activity more pronounced than osteoblast activity, thereby preventing the development of osteoporosis (Seidlova-Wuttke, 2003a).

The findings derived from bone mineral density and biochemical parameters indicate that the antiosteoporotic effect may be attributed at least in part to the presence of Ecd in the extract.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Menopause, also called the change of life is defined as the permanent termination of a woman's menstruation and her fertility. In western women, it occurs on average at 51 years but there is a wide range of onset extending from 40s to 60s. The natural menopause occurs when the ovaries age and fail to produce female sex hormones; estrogen and progesterone. The fall in the levels of these regulating hormones cause a variety of unpleasant physiological and psychological symptoms. Prevalence rate of symptoms vary greatly among individuals. While many hardly notice the change, others find their lives severely affected.

In the past years, many population studies have been conducted, which enabled us to get insight into varied menopause experiences across India. Many cities, districts and states have contributed for the data but from state like Uttarakhand the questions have not yet been answered. Cross- sectional survey conducted by our group among the healthy women (35-60 yrs) of Haridwar district in Uttarakhand to reveal age at menopause, symptom prevalence and factors affecting its onset was the first attempt of its kind. Following is the summary and the major conclusions derived from the survey analysis,

I. Different methodologies used for analyzing even the same set of premenopause (54.26%), early postmenopause (25.58%) and late postmenopause (20.15%) women generated variations in the computed age of menopause. While recalled mean age was 45.02 ± 4.35 years, the age calculated by probit analysis rose by 2 years to 46.82 yrs. The recorded age is younger than that of other developed nations with onset at more than 50 years.

II. Menopause was rated as a positive change in life by majority (88.1%) of the participating women.

III. Socio-demographic factors like socio-economic status and lifestyles significantly affect the onset of menopause ($p= 0.04$ and 0.02). Lack of physical activity and malnutrition

depletes the energy sources, affect the neuroendocrine activity and therefore cause a decline in the reproductive span.

IV. Menopause transition significantly affects the frequency and severity of symptoms. Significant increase in the percentage occurrence of psychological, somatic, sexual, vasomotor and miscellaneous symptoms while transitioning from premenopause to early postmenopause and subsequent decline or stability towards late postmenopause was observed.

V. Most prevalent symptom among the whole women population under study were muscle and joint pains (55.81%), feeling tired or lack of energy (51.19%), eye problems (49.61%), headache (43.41%) and feeling unhappy or depressed (36.43%), hot flushes (33.33%), irritability (32.36), attacks of panic (31.78%), and feeling dizzy or faint (30.23%).

VI. The psychological cluster scored highest and vasomotor scored the least in all the categories representing different menopause status. Factors such as genetic variation and dietary habits are believed to protect Asian women against vasomotor symptoms like hot flushes.

VII. Specifically for postmenopausal women, muscle and joint pains ranked at the top with an occurrence of 22.03%, followed by attacks of panic (20.34%) and loss of interest in sex (18.64%).

Severe and frequent presence of muscle and joint pains among the postmenopause participants, points toward their debilitating bone health. Significant loss of bone tissue during postmenopause compared to premenopause has been attributed to factors like sedentary lifestyle, obesity, higher parity and estrogen deficiency. During the past decade, considerable evidence has accumulated which suggests that estrogen plays a pivotal role in maintaining bone health. Dramatic loss in its levels in the first 5-10 yrs after menopause leads to progressive

deterioration of the bone tissue. Unless the lost estrogen is restored, bone becomes thin and brittle quite rapidly. The available courses of therapy like estrogen replacement therapy, calcitonin and bisphosphonates to osteoporosis in postmenopausal women are limited by several side effects generated. A need therefore arises to explore alternatives that are safe and effective in curing this devastating ailment. Herbal drugs are one such alternative, which are maximally accepted among the women patients. They appear to improve the body imbalance, slow down the disease progression and are safe for long term use.

Several plants and plant derived compounds have been employed for preventing postmenopausal bone loss but there is still large variety of plants that are not studied yet. During the exploration for a new medicinal herb which can exert protective effect on skeleton, *Tinospora cordifolia* (TC) emerged as our lead plant.

In order to examine the protective effects of TC (stem extract, 95% ethanolic) on bone loss, we used ovariectomized (ovx) rat model system. Both acute (4 weeks) and chronic (12 weeks) treatment studies were performed. Summary and major conclusions drawn from the investigations are as follows.

I. Successful bilateral ovariectomy produces an estrogen deficient model appropriate for studying postmenopausal bone loss.

II. Ovariectomy results in an increased bone turnover, negative bone balance and net bone mineral density loss in the trabecular region of long bones (up to 50%).

III. TC administration (subcutaneously, 4 weeks) to ovx rats at the dose of 10 mg/kg b.wt. showed significant osteoprotective effects. Higher doses (50 mg/kg b.wt. and 100 mg/kg b.wt.) were not found effective.

IV. Ovariectomy induced elevation in the serum cross-laps and osteocalcin levels were normalized by all three dose levels of TC (10, 50 and 100 mg/kg b.wt.).

V. Ovariectomy enhanced the levels of both total and high density lipoproteins. Positive shift in the lipoprotein profile was achieved with TC administration, particularly at the

dose level 50 mg/kg b.wt. This strongly suggests that TC would be beneficial for cardiovascular disease for women after menopause.

VI. In chronic treatment studies, 3 months post ovariectomy; quantitative analysis of trabecular bone structure revealed anatomical changes in the bone microarchitecture. The alterations observed in cancellous bone network are very similar to that seen in postmenopausal osteoporosis, including thinning of trabecular elements. Both H&E (haematoxylin and eosin) staining and SEM confirmed that TC administration at dose level 10 mg/kg b.wt. attenuates estrogen deficiency induced decrease in the trabecular thickness and restored the increase in trabecular separation.

VII. Thermal analysis proved as a useful qualitative tool to measure the seriousness of pathology similar to the measurement of bone mineral density for assessing osteoporosis. Curves so obtained from TG, DTA and DTG record weight changes and associated endothermic and exothermic heat effects in bone as a function of temperature (50 to 1500°C). Information about the bone matrix composition in terms of water, protein and carbonate content can also be accumulated using these curves.

VIII. Ovariectomy induces severe pathology and hence completely different response during thermal analysis compared to healthy bones was observed. Bone mass loss; particularly in the organic phase of matrix was highest for ovx group (69.4%) followed by ovx+TC (56.13%), ovx+E2 (47.82%) and sham ovx (38.94%) rats. Additionally, absence of distinctive exothermic peaks and high energy values (4554 mJ/mg) in ovx rats confirmed the seriousness of pathology. Ovx+ E2 group was distinctly different since it has only one peak at 346°C with energy value 752 mJ/mg. TC administration improved the energy absorption pattern and generated distinct peaks. Two peaks were observed at 338°C and 579°C with values 759 and 747 mJ/mg respectively. Since, larger peak values are often associated with the porous or eroded appearance of the trabecular

bones, improvement observed by TC administration represents this as a potential anti-osteoporotic agent.

IX. Lack of TC binding to ER in ER-LBA and absence of proliferative effects in uterus and mammary gland make it unlikely that TC acts via estrogenic mechanism. Response is believed to be due to one or number of active constituents present in different proportions.

Literature related to natural products, evaluated specifically for the prevention of postmenopausal bone loss unveils plethora of phytochemicals that have been responsible for their antiosteoporotic action. They belong to different classes such as flavonoids (e.g. genistein, daidzein, tectorigenin), lignans (peltatin, podophyllotoxin), carbohydrate (xylitol), alkaloids (berberine, triptolide), saponin (escin), stilbene (resveratrol) and tannin. In search for the active phytochemical in TC extract that may be responsible for its osteoporotic potential, 20-β hydroxyecdysone (Ecd) was identified as the potential candidate. To investigate the bone sparing effect of Ecd, dose response studies were performed in ovx rats. Salient findings and conclusions inferred from the study are as follows.

I. HPLC-UV method was standardized for the identification and estimation of Ecd in the plant extract.

II. Bone protecting effects of Ecd was tested in ovx rats treated orally over 3 months with no Ecd (control) or 18, 57 or 121 mg Ecd/day/animal. Bone mineral density (BMD) at different doses of oral Ecd administration was determined and compared with the known antiosteoporotic effect of E2 after 3 months of treatment. BMD was reduced by more than 50% in the control but not in E2 animals. In the Ecd animals, BMD was dose dependently higher than in the control.

III. Serum osteocalcin levels were high in ovx and Ecd treated animals but with E2 application a significant reduction was observed. The metabolic breakdown products of

bone specific collagen I, the cross-laps were also high in ovx animals and reduced by Ecd and E2.

IV. Ecd lack binding to ER (even at concentration of 10^{-3}M) and also uterine weights of the Ecd group were not increased. Therefore, it is safe to conclude that the observed effects in the bone are not due to an estrogenic effect of the ecdysteroids.

V. The findings derived from bone mineral density and biochemical parameters indicate that the antiosteoporotic effect may be attributed at least in part to the presence of Ecd in the extract.

Taken together, findings derived from the present set of studies presented an overview of beneficial effects of TC and Ecd in ovariectomized rats. They prevent ovariectomy induced bone loss without influences on reproductive organs. If these findings can be approved in human they may provide a useful alternative to HRT for the treatment of postmenopausal osteoporosis.

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