

**EFFECT OF CHRONIC ETHANOL CONSUMPTION ON THE RESPONSE OF
PARATHYROID HORMONE TO HYPOCALCEMIA IN THE PREGNANT RAT**

by

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ABSTRACT

Drinking alcohol during pregnancy is known to have adverse effects on the offspring. Little is known, however, about the effects of drinking on the health of the pregnant female. Chronic ethanol consumption in the pregnant rat has been shown to alter the level of maternal blood calcium (Ca) and the major Ca-regulating hormone, parathyroid hormone (PTH). Specifically, ethanol decreases maternal blood ionized Ca (iCa) levels and, in spite of this decrease, PTH levels are unchanged or even decreased in ethanol-exposed dams. It is unknown if ethanol impairs the ability of PTH to respond to hypocalcemia, or if the ethanol-exposed dam can still regulate her serum Ca levels, but ethanol lowers the level of iCa that is maintained. The objective of my thesis was to determine the effect of chronic ethanol consumption on the response of maternal serum PTH levels to an acute decrease in blood iCa level.

Rats were fed a liquid diet with ethanol (36% ethanol derived calories, Ethanol, E rats) or without ethanol (Pair-Fed, PF or Control, C rats), 3 weeks prior to and throughout 21 days gestation. On day 21 gestation, the E and PF dams received an intraperitoneal (ip) injection of the Ca-chelating agent ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) at a dose of 300 or 500 $\mu\text{mol/kg}$ body weight (300-EGTA or 500-EGTA group, respectively), an equal volume of saline (Saline group), or no injection (Baseline group). The C rats received all treatments except the 300-EGTA dose. Maternal blood was collected from the Baseline group (0 min), and at 30 and 60 min post-injection in the Saline and EGTA groups. Blood was analyzed for iCa, pH, blood ethanol concentration (BEC), PTH, and corticosterone levels.

Saline injection had no effect on blood iCa and serum PTH levels. Injection of EGTA resulted in a significant decrease (vs. Saline) in blood iCa levels, which varied with both dose of EGTA and time post-injection, but did not vary with dietary treatment. Importantly, ethanol consumption did not prevent PTH from increasing in response to the EGTA-induced hypocalcemia, and PTH levels were significantly increased in all diet groups at 30 min post-injection. Ethanol did, however, appear to decrease the maximum PTH levels achievable in blood (vs. PF dams). This difference in maximum PTH levels between E and PF rats could not be explained by an increased response of the PF rats to stress, as measured by serum corticosterone levels.

These data suggest that chronic ethanol consumption does not impair the ability of the pregnant rat to raise serum PTH levels in response to acute hypocalcemia, but may decrease the maximum level of PTH achieved. Ethanol's effect on the maximal PTH level could potentially impair the ability of the pregnant female to sustain high PTH levels in response to a more chronic hypocalcemia, as might occur during consumption of a low Ca diet. Effects of ethanol on the ability to maintain Ca homeostasis during pregnancy could have adverse effects on both maternal and fetal health.

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LIST OF ABBREVIATIONS

1,25(OH) ₂ D	1,25-dihydroxy vitamin D
25-OH-D	25-hydroxy vitamin D
ANOVA	analysis of variance
BEC(s)	blood ethanol concentration(s)
C	control
Ca	calcium
CaSR	calcium-sensitive receptor
d	day
dl	decilitre
E	ethanol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
g	gram(s)
HPA	hypothalamic-pituitary-adrenal
hr	hour(s)
iCa	ionized calcium
ip	intraperitoneal
kg	kilogram
Mg	magnesium
mg	milligram(s)
min	minute(s)
mM	millimolar

mRNA	messenger ribonucleic acid
n	sample size
P	phosphorus
p	probability
PF	pair-fed
pg	picogram(s)
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
r^2	coefficient of determination
r	Pearson correlation coefficient
RIA	radioimmunoassay
SE	standard error
VIF	variance inflation factor
Zn	zinc
μg	microgram(s)

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CO-AUTHORSHIP STATEMENTS

For the study conducted in this thesis, I was responsible for designing and conducting the experiments, analyzing the data and writing the manuscript. Chapter II, Effect of Chronic Ethanol Consumption on the response of Parathyroid Hormone to Hypocalcemia in the Pregnant Rat, included co-authors who made the following contributions:

- My supervisor, Dr. Kathy Keiver, was involved in designing and performing the experiments, advising on data analysis and editing the manuscript. Mary Elizabeth Simpson helped conduct the experiments and reviewed the manuscript.

CHAPTER I

1.1 INTRODUCTION

Despite warnings to the contrary, the prevalence of women that have one or more drink of alcohol (ethanol) during pregnancy is high (~20% in Canada and the U.S., U.S department of Health and Human Services, 1991; Roberts and Nanson, 2000), and approximately 2% of pregnant women consume ethanol at high levels (Ebrahim, et al., 1998; Flynn et al., 2003). It is well known that maternal ethanol consumption during pregnancy can have many adverse effects on the offspring. Such effects include increased neonatal morbidity and mortality (Abel, 1982), neurological deficits (Jones and Smith, 1973), and malformations of body systems (Chaudhuri, 2000). Further, prenatal ethanol exposure results in growth retardation, including decreased body weight and bone length and delayed skeletal development (Jones et al., 1973; Habbick et al., 1998). Many of ethanol's effects are permanent (Streissguth et al., 1991), and occur even at low levels of drinking. For example, subtle growth deficits have been demonstrated with ethanol intakes of <1 drink/day (Day et al., 2002).

In contrast to the effects on the offspring, the effects of drinking during pregnancy on the health of the mother have not been well studied. Using rodent models of prenatal ethanol exposure, we (Keiver et al., 1996; Keiver and Weinberg, 2003) and others (Baran et al., 1982) have shown that chronic ethanol intake during pregnancy in the rat results in maternal hypocalcemia and decreased mineral content (Keiver et al., 1996; Keiver and Weinberg, 2003) and density (Ronis et al., 2003) of maternal bone. The inability to maintain bone mass during pregnancy could affect the achievement or maintenance of peak bone mass and increase the risk for osteoporosis in later life (Heaney et al., 2000). Although the

mechanisms by which ethanol affects maternal bone are unknown, disruptions in calcium (Ca) homeostasis are often associated with negative effects on bone, and thus ethanol's effects on Ca regulation could contribute to its effects on maternal bone. Further, maternal hypocalcemia is associated with decreased birth weight, increased neonatal morbidity and mortality, and decreased mineralization of the fetal skeleton (Ibrahim et al., 1984; Chalon and Garel, 1985; Loughhead et al., 1990), and thus may underlie some of the effects of ethanol on offspring health.

The mechanisms by which ethanol decreases blood Ca levels are also not known. There is increasing evidence, however, that ethanol impairs the relationship between PTH and Ca, resulting in levels of PTH that are low relative to Ca levels. Parathyroid hormone levels fail to increase, or are even decreased, during ethanol-induced hypocalcemia in the pregnant (Keiver and Weinberg, 2003) and non-pregnant (Diez et al., 1997; Thomas et al., 1990; Keiver et al., 2000) rat and human (Laitinen et al., 1992, 1994; Perry et al., 1998). The response of PTH to changes in ionized Ca (iCa, the form of Ca that is regulated) is the first defense against the development of severe hypocalcemia, and is therefore vital to an organism's survival. This is especially true during pregnancy, when maternal requirements for absorbed Ca are increased due to fetal demands for Ca (Kovacs and Kronenberg, 1997). It is unknown if ethanol impairs the ability of PTH to respond to ethanol-induced hypocalcemia, or just lowers the level of iCa that is maintained. This is an important question because an inability to raise serum PTH levels could cause harm to both the pregnant female and her fetus. Alternatively, although a decrease in the level of iCa about which homeostasis is maintained may have negative long-term consequences for bone health and development, it is unlikely to be life-threatening.

This thesis examined the effect of ethanol on the ability of the rat to maintain Ca homeostasis during pregnancy. Our specific objective was to determine the effect of chronic ethanol consumption on the response of maternal serum PTH levels to an acute hypocalcemic challenge, which is a test of parathyroid gland function.

1.2 LITERATURE REVIEW

The following literature review will provide an overview of the function, metabolism and regulation of Ca in the body. Pregnancy is a physiological state that is associated with an increased Ca requirement to support fetal skeletal mineralization, and thus provides an effective and sensitive model to study ethanol-induced disruptions in Ca metabolism. This review will therefore include Ca regulation during pregnancy, as well as the effects of ethanol on Ca regulation. Moreover, as disruptions in Ca homeostasis often result in negative effects on bone, the effects of ethanol on bone will also be discussed. The review will end with an overview of rodent models of prenatal ethanol exposure, including the model used in this thesis, and a statement of the thesis objective.

1.2.1 Calcium

Calcium is estimated to make up 1 to 2 percent of adult human body weight (Institute of Medicine, 1997). The majority of Ca (over 99%) is found in the hard tissues of the body, particularly bones and teeth. The remainder is present in blood and soft tissues (Institute of Medicine, 1997). Intracellular free Ca concentrations are generally low (in the nm to μ m range), but can fluctuate greatly due to release from cellular stores or influx from extracellular fluid (Brown, 2000). In contrast, extracellular Ca levels are relatively high (in the mM range), but maintained within a narrow limit. This rigid control of extracellular Ca

ensures a constant supply of Ca for vital intracellular functions. In addition, extracellular Ca also has several other roles in the body, such as in blood clotting and regulation of plasma membrane potential.

Extracellular Ca exists in the blood in several forms. Of the total blood Ca, approximately 48% exists as free or ionized Ca (iCa), 46% is protein bound (of which about 70% is bound to albumin) and the remainder is complexed with anions such as citrate, sulfate and inorganic phosphates (Brighurst, 1995). Blood iCa is the biologically active and hormonally regulated form of extracellular Ca. Although total Ca is often measured as an indicator for changes in iCa, changes in total Ca do not always reflect changes in iCa. Disorders that decrease blood albumin levels also lower total blood Ca, without affecting iCa concentration. Moreover, binding of Ca to albumin is also affected by blood pH. A decrease in pH will decrease the amount of Ca bound to protein and increase iCa levels, without affecting total blood Ca levels (Broadus, 1996). Although algorithms exist to calculate iCa from measured total Ca, albumin and pH levels, they do not predict iCa accurately in abnormal states (e.g. disease) and are poor substitutes for direct measurements of blood iCa levels (Brighurst, 1995). It is, therefore, preferable to measure iCa directly. Further, the measurement of iCa is always accompanied by measurement of pH, and iCa measures are often corrected to pH 7.4. This eliminates effects of spurious changes in pH (due to sample handling and possible stress associated with sampling) on iCa levels and thus facilitates the interpretation of iCa measures.

Blood iCa and pH are measured by an ionized Ca analyzer, a computerized system that simultaneously measures the iCa concentration and pH of blood by means of Ca and pH selective electrodes, respectively (Radiometer, 1986). An electrolyte solution is present

between the ion selective electrode and an inner electrode. When a blood sample contacts the electrode, the difference in iCa concentration in the electrolyte and sample creates a potential across the ion sensitive membrane that is directly related to the Ca activity (which is automatically converted to concentration of iCa of the sample, Radiometer, 1986).

A number of substances can affect the measurement of iCa by ion selective electrodes, such as use of organic preservatives and oils during blood collection (Radiometer, 1986). Recently it has been shown that ethanol interferes with the measurement of iCa by the most commonly used analyzer, the Radiometer analyzer (Keiver, 2004). This methodological artifact results in an inverse relationship between measured blood iCa and ethanol concentration, with iCa decreasing by approximately 5% (0.06 mM) at a blood ethanol concentration (BEC) of 500 mg/dl. Although this interference does not fully account for the observed effects of ethanol consumption on blood iCa levels in vivo, it complicates the interpretation of such studies. The newer I-Stat Clinical analyzer (Abbott Laboratories Inc., Mississauga, Ontario), which uses disposable electrodes, is not affected by ethanol (Keiver, 2004). Thus, the I-Stat Clinical analyzer is most suitable for studies on ethanol's effects on Ca homeostasis.

1.2.2 Calcium Metabolism

Calcium in the body is obtained from the diet. Intestinal absorption of dietary Ca results from both passive diffusion and active transport across the intestinal mucosa. Passive diffusion of Ca is concentration dependent (i.e. high when Ca intakes are high) and involves the movement of Ca between mucosal cells. The active transport of Ca is mediated by carriers that transfer Ca into intestinal cells, and is regulated by 1,25-dihydroxyvitamin D (1,25(OH)₂D), the active form of vitamin D. The contribution of active transport is high at

low Ca intakes (i.e. $1,25(\text{OH})_2\text{D}$ is stimulated when Ca intake is low). The efficiency by which Ca is absorbed is influenced by many factors, such as the amount of Ca in the diet, the person's physiological state, and age (Institute of Medicine, 1997). The efficiency of calcium absorption varies inversely with dietary Ca intake (Malm, 1958; Spencer et al., 1969) and generally ranges from 70 percent at very low Ca intakes to 10 percent or less at high Ca intakes (Nordin and Marshall, 1988). During times of active growth and bone development (infancy and adolescence) and pregnancy, Ca demands are high and efficiency of absorption is increased by 30-60% (Institute of Medicine, 1997). In older age, there is a decrease in the efficiency of Ca absorption, and therefore an increased dietary requirement for Ca (Institute of Medicine, 1997). Unabsorbed Ca is lost in feces.

Once Ca is absorbed, it is transported in blood and equilibrates with the interstitial fluid surrounding the tissues of the body. From the interstitial fluid, the cells take up the Ca they need for normal function and growth. Most of the Ca absorbed by the body is used in the calcification of bones (Institute of Medicine, 1997). Although a small amount of Ca is secreted into the intestine, the major route of Ca excretion is through the urine. Thus, the regulation of extracellular Ca concentrations involves regulation of active transport of Ca across the gut, excretion of Ca in urine, and mobilization of Ca from bone.

1.2.3 Regulation of Extracellular (Blood) Calcium

Blood Ca levels are regulated within a fairly narrow limit (2.2-2.7 mM total Ca; 1.1-1.3 mM iCa). Failure to maintain Ca homeostasis results in hypocalcemia (blood Ca levels below normal range) or hypercalcemia (blood Ca levels above normal range). Hypocalcemia may vary from being mild and asymptomatic to a severe life-threatening disorder, depending

on the duration, severity, and rapidity of development (Tohme and Bilezikian, 1993). Mild acute hypocalcemia is asymptomatic, while mild chronic hypocalcemia can result in decreased mineralization of bone and growth retardation. During fetal development or childhood, hypocalcemia can result in decreased bone mineral content and rickets (softening and weakening of the bones) (Root, 1999). In adulthood, this softening and weakening of the bones is referred to as osteomalacia. Adequate Ca supply during periods of maximal bone mass accrual are therefore important to maximize the peak bone mass attained and minimize the bone loss that inevitably occurs over the latter half of the lifespan (Weaver 1997; Weaver et al., 1999). This decreases the risk of osteoporosis, a condition characterized by a decrease in total bone mass, leading to bone fragility and an increased susceptibility to fractures (WHO, 1994). Acute or chronic severe hypocalcemia leads to abnormal cell function and can eventually result in tetany (uncontrolled muscle contraction) and death (Bushinsky and Monk, 1998).

Symptoms of hypercalcemia are also related to the severity and rate of change of the blood Ca (Bilezikian, 1993). Chronic or mild hypercalcemia can result in renal disorders such as diuresis, dehydration, or kidney stones and other soft tissue calcifications. An acute or chronic severe increase in Ca can lead to calcium rigor (inappropriate muscle fiber contraction) and central nervous system impairments which can result in decreased alertness, confusion, and coma (Bilezikian, 1993).

Severe effects of both hypocalcemia and hypercalcemia, such as tetany and calcium rigor, do not usually result from variations in dietary intake of Ca (excessively low or high, respectively) alone, because of the presence of a highly effective Ca regulating system

(Gertner, 1990). Such severe problems are usually associated with malfunction/problems with this Ca-regulating system.

The maintenance of normal blood Ca levels is controlled by the concerted action of three major hormones that control the movement of Ca in and out of blood: parathyroid hormone (PTH), $1,25(\text{OH})_2\text{D}$, and, to a lesser extent, calcitonin (Figure I-1).

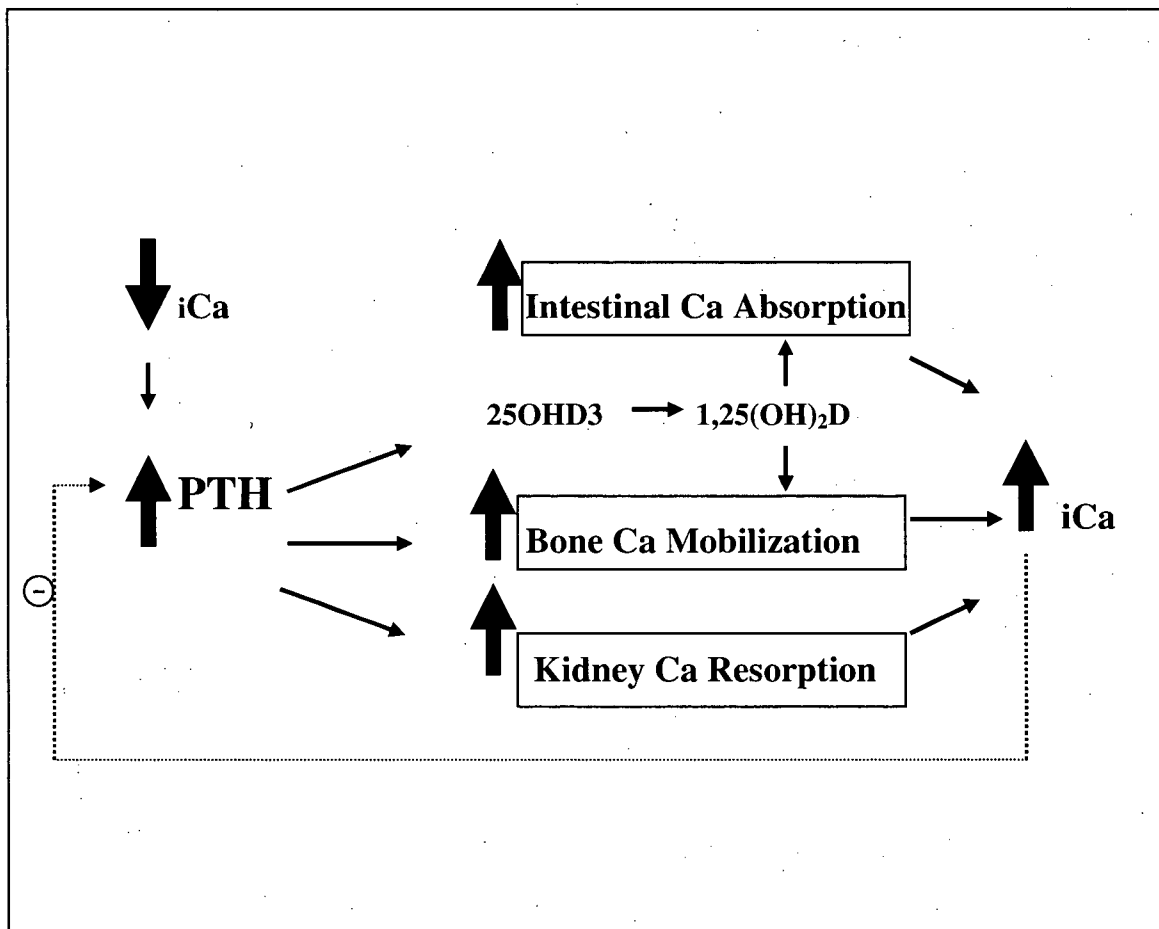


Figure I-1. Endocrine regulation of blood iCa by parathyroid hormone (PTH) and vitamin D (1,25(OH)₂D). The symbol \ominus represents the inhibition of PTH secretion and closure of the negative feedback loop.

In response to a decrease in blood iCa levels, there is an increase in PTH secretion from the parathyroid gland. Within minutes of its release, PTH stimulates the reabsorption of Ca in the kidney and thus suppresses Ca loss in urine (Yamamoto et al., 1989). Also in the kidney, PTH stimulates the enzyme 1- α -hydroxylase, which catalyzes the conversion of vitamin D to its active form, 1,25(OH)₂D and thus increases levels of 1,25(OH)₂D in the blood (Bushinsky and Monk, 1998; Brown, 2000). Active absorption of Ca in the small intestine is stimulated by 1,25(OH)₂D (Adams et al., 1979; Brown, 2000). Both PTH and 1,25(OH)₂D act synergistically to stimulate osteoclasts, or bone resorbing cells, to reabsorb bone mineral, liberating Ca into the blood (Adams et al., 1979). The receptors for PTH and 1,25(OH)₂D are expressed on osteoblasts (bone-forming cells), not osteoclasts (Sutton and Dirks, 1996). Thus, the osteoblasts, in response to these hormones, send a paracrine signal to osteoclasts to differentiate (vitamin D influence) and reabsorb bone (PTH influence). Ultimately, the increase in blood Ca from intestine and bone, together with renal re-absorption of Ca, return blood Ca to normal levels. This in turn inhibits PTH secretion and closes the negative feedback loop (Figure I-1). This increase in 1,25(OH)₂D (stimulated by hypocalcemia) also exerts direct negative feedback action on PTH release (Bushinsky and Monk, 1998). Further, the increase in 1,25(OH)₂D levels eventually inhibits 1- α -hydroxylase activity thereby limiting its own production (Brown, 2000).

When blood Ca levels increase above normal, PTH secretion and 1,25(OH)₂D synthesis are inhibited. Secretion of calcitonin from the C cells of the thyroid gland is stimulated and acts to lower blood Ca levels. Calcitonin inhibits bone resorption and thus the release of Ca into the blood by inhibiting the activity of osteoclasts (Kovacs and Kronenberg, 1997). Calcitonin also inhibits reabsorption of Ca in the kidney, leading to increased renal

excretion of Ca (Kovacs and Kronenberg, 1997). The secretion of calcitonin is inhibited by low levels of Ca (Foster et al., 1972).

There is a sequential time frame of response to hypocalcemia by the Ca regulating hormones and the tissues and organs involved (Silver et al., 1986; Yamamoto et al., 1989; Brown, 2000). Parathyroid hormone is the first to respond (within seconds) to changes in blood Ca levels. This PTH is from preformed stores of the hormone in the parathyroid gland. The most rapid changes in Ca handling in response to PTH stimulation occur in the kidney and bone. In vitro, the increase in Ca reabsorption in the kidneys takes place within a few minutes, whereas the release of Ca from bone takes about 2-3 hours (Robertson et al., 1972). If these changes are not sufficient to normalize Ca levels, PTH stimulates increased synthesis of $1,25(\text{OH})_2\text{D}$ within several hours to days, which further increases the activity of osteoclasts and increases intestinal Ca absorption (Adams et al., 1979). It is rare that this full response by the Ca regulating hormones, organs and tissues is unable to restore normal Ca levels.

Hypercalcemia results in reduced PTH levels which in turn induces renal hypercalciuria, reduces the release of Ca from the skeleton and eventually suppresses the intestinal absorption of Ca by inhibiting the synthesis of $1,25(\text{OH})_2\text{D}$ (Brown, 2000). Calcitonin's Ca-lowering effect begins within hours, with a nadir in blood Ca within 12–24 hr, but the effect on Ca levels is modest and transient (Root, 1999).

1.2.3 a) PTH

PTH is a straight chain peptide of 84 amino acids that is secreted from chief cells of the parathyroid glands (Brown, 1982). The parathyroid glands are located on the dorsal

surface of the thyroid gland. Removal of the parathyroid gland (without any medical intervention) results in hypocalcemia, leading to tetany, convulsion, and death. As a result of the metabolism of PTH, either peripherally or within the parathyroid glands, there are several fragments of the hormone in the circulation (Potts et al., 1995). Intact PTH is cleaved during degradation, mainly in the liver, between the amino acid residues 34-37 into fragments which include the N-terminal or the C-terminal amino acid sequence of the hormones (Riond et al, 1993; Rucinski et al, 1995). The structural requirements for target cell receptor binding and signaling exist within the N-terminal fragment (1-34) of the PTH molecule; thus, only those PTH peptides that contain this sequence are biologically active (Potts et al., 1995). Intact PTH and N-terminal fragments have a short half-life in blood and disappear quickly from the circulation. In contrast, the biologically inactive mid-region and C-terminal fragments disappear less rapidly from the circulation and thus are present in higher concentrations (Riond et al., 1993; Rucinski et al., 1995).

A calcium-sensitive receptor (CaSR) on the chief cells of the parathyroid gland detects small changes in blood iCa levels and couples this information to intracellular signaling pathways that modify PTH secretion (Brown and Hebert, 1997). A decrease in blood iCa leads to a decrease in intracellular iCa levels and an increase in PTH secretion. The CaSR is a member of the 7-membrane spanning G-protein-coupled receptor family. It has a large extracellular N-terminal domain that contains multiple low-affinity calcium-binding sites, seven membrane-spanning regions, and an intracellular portion that couples to G proteins and other signal transduction pathways. Several disorders of Ca homeostasis have been linked to mutations in the CaSR (Chen and Goodman, 2004).

An increase in secretion of preformed PTH from the parathyroid glands occurs within seconds of a decrease in blood iCa levels. Within 20 to 30 min of continued hypocalcemia, there is an increase in net synthesis of PTH without a change in PTH messenger RNA (mRNA), possibly due to reduced intracellular degradation of PTH (Habener and Potts, 1975). Thus, a larger proportion of PTH secreted is the intact and biologically active form. If hypocalcemia persists over 1-3 days, there is an increase in levels of PTH mRNA (Yamamoto et al., 1989) and thus PTH synthesis. Chronic hypocalcemia extending over days and weeks is associated with an increase in parathyroid cell proliferation and gland hypertrophy (Lee and Roth, 1975).

The relationship between blood PTH and iCa levels follows an inverse sigmoid curve (Figure I-2), which is best described by the four-parameter model of Brown (1983):

$$PTH = \frac{A - D}{1 + (Ca/C)^B} + D$$

where PTH= blood PTH concentration (pg/ml), Ca = blood iCa concentration (mM), A = maximal value of PTH (pg/ml), D = minimal value of PTH (pg/ml), C = the set-point, or the calcium level at which one-half of the maximal stimulation of PTH occurs, and B = slope of the PTH-iCa relationship at the set-point.

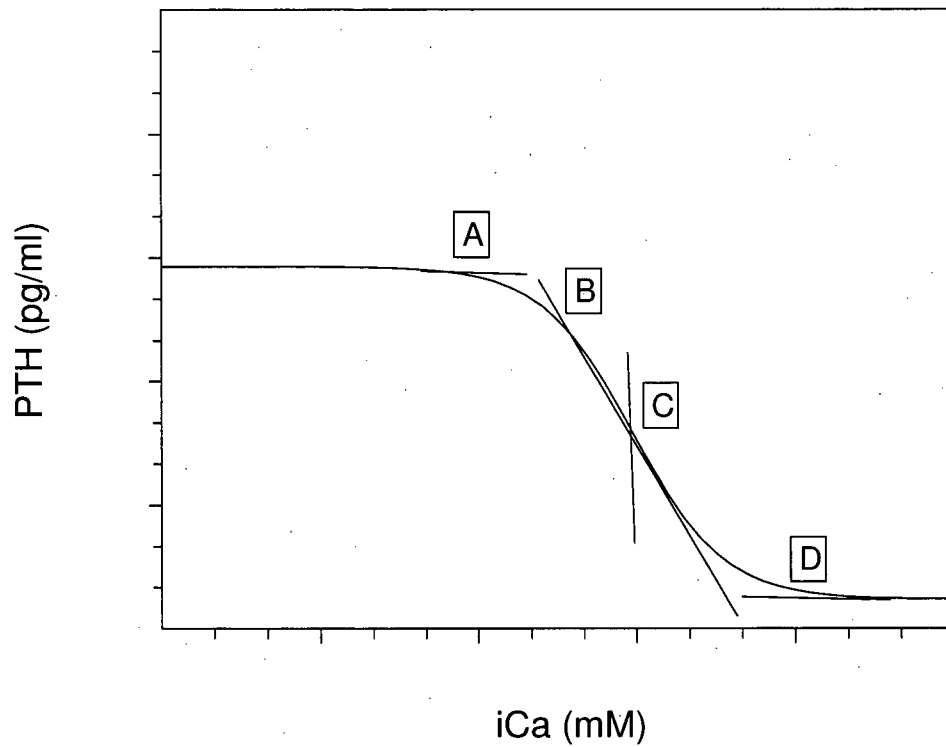


Figure I-2. Four-parameter model of the inverse sigmoid relationship between blood PTH and iCa levels. The curve is based on the equation $PTH = \frac{(A-D)}{(1 + (Ca/C)^B)} + D$. Ca, blood iCa levels; A, maximal PTH; D, minimal PTH; C, set-point; B, slope of PTH-iCa relationship at set-point. (Modified from Brown, 1983.)

This four-parameter model has been shown to accurately describe the PTH-iCa relationship for parathyroid cells in vitro, and in vivo for a variety of animal species and physiological states (Habener, 1978; Brown, 1983,1991; Fox, 1991; Ramirez et al., 1993; Imanishi et al., 2002). The four parameters represent four biologically relevant characteristics of PTH secretion. The two extremes of the curve represent the maximum (A) and minimum (D) levels of PTH achievable in blood. The maximum PTH level (parameter A) is indicative of the acute secretory reserve of the parathyroid gland (Brown, 1983, 2000). The minimum PTH level (parameter D) represents the non-suppressible PTH level. The secretion of PTH is never completely inhibited, even at very high levels of iCa (Brown, 1983). Parameters B and C describe the part of the curve in which PTH secretion is highly sensitive to small changes in iCa level. For example, a decrease in blood iCa of only approximately 0.2-0.3 mM below basal levels is sufficient to increase serum PTH to maximal levels (Fox, 1991; Imanishi et al., 2002). Parameter B is the slope of the line and its steepness describes the sensitivity of PTH secretion to a change in blood iCa level. As basal blood iCa levels are normally maintained towards the bottom of this line (between C and D) (Grant et al., 1990; Uden et al., 1992), the steepness of the slope of the line is responsible for maintaining blood iCa levels within a very narrow range, with low variation (percent coefficient of variation of basal iCa is $\leq 2\%$, Brown, 2000). Parameter C represents the set-point, or the iCa level at which PTH secretion is half maximally stimulated. A change in the set-point is an indication of a change in the level of iCa that is regulated or defended. Measurement of these four parameters is used to assess changes in parathyroid gland function due to gland abnormalities or changes in physiological state (Brown et al., 1983; Grant et al., 1990). When assessing parathyroid cell function in vitro, the PTH level reflects PTH secretion. In vivo, however, although

measurements of serum PTH levels are typically used to evaluate secretion, they actually reflect the net effect of the processes of degradation and clearance from blood, as well as secretion (Aguilera-Tejaro et al., 1996; Imanishi et al., 2002).

The relationship between PTH and iCa may be affected by many factors, such as age (Fox, 1991; Uden et al., 1992; Ledger et al., 1994; Portale et al., 1997; Haden et al., 2000), physiological state (Schultz, 1997) or abnormalities of the parathyroid gland, such as tumors (Brown et al., 1978; Brown, 1983). The increase in PTH levels that occurs with age in both humans and rats appears to be the result of an increase in the set-point (Uden et al., 1992; Portale et al., 1997), although an increase in maximum and/or minimum levels of PTH have also been observed (Ledger et al., 1994; Portale et al., 1997; Haden et al., 2000). In the rat, the relationship between iCa and PTH levels has also been shown to be altered during lactation and pregnancy (Schultz et al., 1997). During lactation, there is a decrease in set-point and an increase in minimal PTH level which is due to a higher rate of PTH secretion from parathyroid cells at high Ca levels, but a lower rate of secretion at low Ca levels, compared with non-lactating rats. During pregnancy, although there is no change in the parameters of the PTH-iCa relationship, there is an increase in PTH release from parathyroid cells which helps to maintain both PTH and iCa at normal levels despite the hemodilution or expanded plasma volume that occurs during pregnancy. Parathyroid adenomas have shown increased set-point values (Brown et al., 1978; Brown, 1983), which contribute to high circulating PTH and iCa levels.

To test parathyroid gland function and examine the relationship between PTH and iCa, varying degrees of hypocalcemia and hypercalcemia are induced, including a level sufficient to stimulate maximal and minimal PTH secretion (Brown, 1983). Hypocalcemia is

readily induced by injection of Ca chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Although both bind to iCa, EDTA also binds (with higher affinity) to other divalent cations, such as magnesium and zinc (Mg^{+2} and Zn^{+2} , respectively), lowering their levels in blood (Segura-Egea et al., 2003). EGTA preferentially binds iCa with a significantly greater affinity than the other divalent cations, and thus induces hypocalcemia without significant decrease in blood Mg^{+2} and Zn^{+2} levels (Segura-Egea et al., 2003). The minimum or non-suppressible PTH level is assessed by inducing hypercalcemia by injection of Ca, usually in the form of Ca gluconate or Ca citrate. When assessing the relationship between PTH and iCa, it should be noted that PTH secretion has been shown to be affected by the direction of change of blood iCa levels (Aguilera-Tejaro et al., 1996). For a given iCa level, PTH is higher during induction of hypocalcemia than it is during recovery from hypocalcemia. This is not due to depletion of PTH stores or the rate of change in blood Ca, but appears to result from an ability of the parathyroid gland to sense when blood iCa levels are decelerating or stabilizing and thus decrease PTH secretion accordingly to prevent an over-correction in iCa levels (Aguilera-Tejaro et al., 1996).

Although the major determinant of PTH secretion is the blood iCa level, PTH secretion can be affected by other factors such as $1,25(OH)_2D$, amino acids and corticosterone. $1,25(OH)_2D$ is a physiological regulator of PTH secretion [discussed in section 1.2.3 b)], while the functional significance of the effects of amino acids and corticosterone on PTH levels is unclear. Amino acids have been shown to suppress PTH secretion in normal human parathyroid cells in vitro (Conigrave et al., 2004). Corticosterone has been shown to increase serum PTH levels (Williams et al., 1974; Fucik et al., 1975; Au,

1976), although some studies have found no effect (Paz-Pacheco et al., 1995; Suliman et al., 2004).

1.2.3 b) Vitamin D ($1,25(\text{OH})_2\text{D}$)

Vitamin D is considered to be a steroid hormone as well as a vitamin (Falkenstein et al., 2000). In the skin, 7-dehydrocholesterol is converted by ultra violet light to pre-vitamin D_3 (Goff et al., 1990). Vitamin D_3 is converted in the liver to 25-hydroxyvitamin D (25-OH-D), which can be further hydroxylated by $1\text{-}\alpha$ -hydroxylase in the kidney to $1,25(\text{OH})_2\text{D}$. $1,25(\text{OH})_2\text{D}$ is the biologically active metabolite of vitamin D (Falkenstein et al., 2000). The activity of $1\text{-}\alpha$ -hydroxylase is primarily regulated by PTH, but is also regulated by iCa, phosphorus, and $1,25(\text{OH})_2\text{D}$ itself (Bushinsky and Monk, 1998). Hypocalcemia stimulates $1,25(\text{OH})_2\text{D}$ synthesis, which in turn exerts direct negative feedback action on PTH release (Bushinsky and Monk, 1998; Figure I-1). Increasing levels of $1,25(\text{OH})_2\text{D}$ eventually inhibits $1\text{-}\alpha$ -hydroxylase activity thereby limiting its own production (Brown, 2000).

$1,25(\text{OH})_2\text{D}$ acts by binding to intracellular receptor proteins in the cell nucleus of various tissues, including the intestine, bone, and kidney (Goff et al., 1990), and regulating mRNA synthesis. Because of this genomic mode of action, the effects of $1,25(\text{OH})_2\text{D}$ on blood Ca levels occur several hours to days after PTH stimulation, but are longer lived. Recently, non-genomic effects of $1,25(\text{OH})_2\text{D}$ have been found that rapidly stimulate a variety of signal transduction systems including Ca transport across the duodenum, release of Ca from intracellular stores, modulation of protein kinase activities, and changes in the phosphorylation of cellular proteins (Norman et al., 1993; de Boland and Boland, 1994;

Falkenstein et al., 2000). The physiological relevance of these rapid effects, however, is presently unclear.

1.2.3 c) Calcitonin

Calcitonin is a 32 amino acid peptide cleaved from a larger pro-hormone (Kalu and Foster, 1976). In mammals, the major source of calcitonin is from the parafollicular or C cells in the thyroid gland, but it is also synthesized in a wide variety of other tissues including the lung and intestinal tract (Kovacs and Kronenberg, 1997). Calcitonin secretion is stimulated by hypercalcemia, and the hormone acts acutely to lower blood Ca levels.

The importance of calcitonin in regulating blood Ca on a chronic basis is unclear. Although it has been shown that calcitonin opposes the action of PTH on osteoclasts, thus decreasing PTH-stimulated bone resorption (Taylor et al., 1975), humans with chronically increased (medullary thyroid cancer) or decreased (surgical removal of the thyroid gland) levels of calcitonin in blood have normal blood Ca concentrations (Melvin et al., 1972; Kalu and Foster, 1976). However, calcitonin levels are increased during pregnancy in all species studied (Kovacs and Kronenberg, 1997). It is suggested that an important function of calcitonin is the protection of the healthy maternal skeleton from excessive resorption (Taylor et al., 1975; Stevenson et al., 1979), partly by antagonizing the effects of fetal PTH related peptide (PTHrP) on placental Ca transfer (Barlet, 1985).

1.2.4 Calcium Regulation during Pregnancy

During pregnancy, the female must maintain her own blood Ca levels while also meeting the demands of the fetus for Ca. Failure to do so may result in deleterious effects on both maternal and fetal/neonatal health, and chronic maternal hypocalcemia is associated

with decreased maternal and fetal/neonatal skeletal mineralization, decreased birth weight, and increased incidence of neonatal morbidity and mortality (Ibrahim et al., 1984; Chalon and Garel, 1985; Loughhead et al., 1990). The major physiological adaptation of the mother (human and rat) to meet this increased Ca requirement is increased efficiency in intestinal Ca absorption (Heaney and Skillman, 1971; Quan-Sheng and Miller, 1989). This increase in absorption is partly mediated by an increase in $1,25(\text{OH})_2\text{D}$ (Pitkin et al., 1979; Wilson et al., 1990; Seki et al., 1991), but may also be stimulated by other hormones such as prolactin and placental lactogen (Kovacs and Kronenberg, 1997; Kovacs et al., 2001). Increased levels of PTH and/or PTHrP are believed to stimulate the increase of $1,25(\text{OH})_2\text{D}$ (Kovacs and Kronenberg, 1997; Kovacs et al., 2001).

The increase in intestinal Ca absorption begins early in gestation, prior to the period of fetal skeletal mineralization (Heaney and Skillman, 1971; Cross et al., 1995). This early increase allows Ca to be stored in the maternal bone until the fetal skeleton begins to mineralize, at which time it is mobilized back into the blood for transfer to the fetus (Flynn et al., 2003). In humans and other animals, as long as dietary Ca is adequate, there is no net change in maternal bone mineral density over the course of the pregnancy (Ellinger et al., 1952; Miller et al., 1982; Ghannam et al., 1999). However, if sufficient Ca cannot be obtained from the diet (e.g. low Ca intake) or if absorption is impaired (e.g. vitamin D deficiency), a decrease in bone mineral content of the maternal skeleton occurs (Miller et al., 1983; Kovacs and Kronenberg, 1997; Ghannam et al., 1999). Calcitonin is elevated during pregnancy and may serve to protect the maternal skeleton from excessive resorption of Ca (Samaan et al., 1975; Garel and Jullienne, 1977; Kovacs and Kronenberg, 1997).

As in the non-pregnant state, PTH is crucial for the maintenance of Ca homeostasis during pregnancy. Tetany and death can occur if PTH levels are prevented from rising during late gestation in the rat (Garel et al., 1981; Kovacs and Kronenberg, 1997), and PTH levels rise earlier in gestation if dietary Ca levels are low (Garner et al., 1988). In humans, the symptoms of hypoparathyroidism are identical to that of hypocalcemia, and can range from a mild tingling sensation to severe muscle cramps, preterm labor, abortion and/or convulsion (Callies et al., 1998). Moreover, Bezerra et al. (2002) found that comparison between pregnant adult and adolescent women with low Ca intake showed that pregnant adolescents had elevated PTH and lower blood Ca levels, likely due to increased Ca requirements due to bone growth.

1.2.5 Ethanol & Calcium Regulation

Many studies have shown that ethanol consumption disrupts Ca regulation (e.g. Petroianu et al, 1991; Keiver et al, 1996, 1997; Perry et al, 1998; Sampson et al, 1999; Keiver and Weinberg, 2003; Keiver, 2004). The specific effects of ethanol on Ca and the Ca regulating hormones, however, have not been entirely consistent, particularly among studies involving human subjects. This is thought to be due, in large part, to lack of recognition or control of important confounding factors (Keiver and Weinberg, 2004). Although no studies have examined the effect of ethanol on Ca homeostasis during pregnancy in humans, some consistent trends have been observed in both non-pregnant human and rat studies. Acute ethanol administration decreases total blood Ca levels (Peng et al., 1972; Peng and Gitelman, 1974; Shah et al, 1978; Thomas et al., 1990; Laitinen et al, 1991; 1992; Diez et al., 1997) and, although the effects are somewhat more variable, chronic ethanol consumption also generally decreases total Ca levels (Bjorneboe et al., 1988; Perry et al., 1998; Sampson et al.,

1999). Moreover, the decrease in total Ca during both acute and chronic ethanol administration has been shown to result from a decrease in iCa levels, rather than changes in levels of albumin or pH (Peng and Gitelman, 1974; Thomas et al., 1990; Laitinen et al., 1992, 1994; Keiver et al., 1996; Keiver and Weinberg, 2003).

The mechanism by which ethanol decreases blood iCa levels is unknown. Although ethanol increases urinary Ca loss (Kalbfleish et al., 1963; Laitinen et al., 1991, 1992; Perry et al., 1998), nephrectomy does not diminish the effects of ethanol on blood Ca levels (Peng and Gitelman, 1974), indicating that the hypocalcemia does not result from increased urinary excretion. Ethanol can also affect the circulating levels of the Ca-regulating hormones. However, these changes do not appear to be responsible for the initial decrease in blood iCa. For example, PTH levels are not always decreased during ethanol-induced hypocalcemia (Neilson et al., 1990; Pepersack et al., 1992; Laitinen et al., 1994; Keiver et al., 1997), and a decrease in blood Ca level occurs with acute ethanol injection even after thyroparathyroidectomy (Peng et al., 1972; Peng and Gitelman, 1974). However, the possibility that ethanol may contribute to hypocalcemia through effects on PTH function (rather than secretion) cannot be ruled out, as Peng et al. (1972) have shown that ethanol blunts the ability of administered PTH to raise blood Ca levels.

Increased levels of calcitonin also do not appear to initiate the hypocalcemia. Although chronic ethanol intake increases maternal calcitonin levels in the pregnant rat (Keiver et al., 1997; Keiver and Weinberg, 2003), calcitonin levels are not usually increased during chronic or acute ethanol consumption in non-pregnant rats and humans compared with controls (Bjorneboe et al., 1988; Laitinen et al., 1991, 1994; Diez et al., 1997; Keiver et al., 2000). Further, Peng et al. (1972) demonstrated that the hypocalcemic response to acute

ethanol injection is still present with removal of the thyroid gland, which would prevent an increase in calcitonin levels. Ethanol has, however, been shown to increase the rate of disappearance of ^{45}Ca from blood (Peng et al., 1972). This has led to the hypothesis that the hypocalcemia results from an ethanol-induced shift of iCa from the extracellular compartment to the intracellular compartments within soft tissues and bone (Peng et al., 1972; Laitinen et al., 1994).

Although ethanol-induced alterations in the Ca regulating hormones do not appear to be responsible for the initial decrease in blood Ca levels, studies suggest that ethanol impairs the ability of PTH to respond to, and thus correct, the hypocalcemia. Acute ethanol administration has been reported to alter the relationship between PTH and blood Ca levels in both humans and rats (Thomas et al, 1990; Laitinen et al, 1991, 1992; Diez et al, 1997). Diez et al. (1997) demonstrated that both total Ca and PTH levels were decreased 1 hr after acute ethanol administration, suggesting that ethanol blunts the PTH response to this hypocalcemia. However, blood iCa levels were not measured and so the actual stimulus for PTH secretion is unknown. Importantly, Thomas et al., (1990) observed that PTH levels failed to increase in response to a large decrease in blood iCa levels (~ 0.2 mM) induced by acute ethanol administration. Since maximal secretion of PTH is stimulated by a decrease in iCa of $\sim 0.2 - 0.3$ mM (Fox, 1991; Imanishi et al., 2002), this suggests that ethanol prevents PTH levels from increasing in response to hypocalcemia. In addition, levels of PTH that are low relative to blood iCa or total Ca levels have also been reported to occur with chronic ethanol consumption in the non-pregnant human and rat (Turner et al, 1987; Laitinen et al., 1994; Perry et al., 1998; Keiver et al., 2000), as well as in the pregnant rat (Keiver and Weinberg, 2003).

It is not known if ethanol prevents PTH levels from increasing in response to hypocalcemia, or if the ability to regulate blood Ca levels is intact (i.e. the parathyroid gland is capable of responding to a decrease in blood Ca) but ethanol lowers the level of Ca maintained. The ability to increase PTH levels is pivotal to the maintenance of normal blood Ca levels and is the first line of defense against severe or chronic hypocalcemia (Brown, 2000). Without this increase, the ability to maintain Ca homeostasis at low dietary intake is compromised and negative Ca balance and Ca loss from bone can result. During pregnancy, this could cause harm to both the pregnant female and her fetus. In contrast, although a decrease in the level about which Ca is regulated may have negative consequences on cell function and bone health, it is unlikely to be life threatening. Distinguishing between these two alternatives is the focus of this thesis.

1.2.6 Ethanol & Bone

Exposure to ethanol can have deleterious effects on both adult and developing bone. In the adult human and rat, these deleterious effects tend to be associated with chronic exposure to high levels of ethanol (Turner, 2000), and include an inhibition of osteoblast differentiation and function (Gonzalez-Calvin, 1993; Sampson et al., 1997; Turner et al., 2001), decrease in mineral density (Bikle, 1993; Turner, 2000; Turner et al., 2001) and bone strength (Hogan et al., 2001), increase in fracture incidence (Brown et al., 2002), and a delay in fracture healing (Elmali et al., 2002). Chronic ethanol abuse during adulthood is a known risk factor for osteoporosis (Olszynski et al., 2004). Further, ethanol consumption during pregnancy in the rat results in decreased mineral content (Keiver et al., 1996; Keiver and Weinberg, 2003; Ronis et al., 2003) and density (Ronis et al., 2003) of maternal bone, and thus may have detrimental effects on maternal bone health.

The effects of moderate ethanol consumption on adult bone are less certain (Rapuri et al., 2000; Turner, 2000). In humans, epidemiological studies have shown both negative and positive correlations between bone mineral density and ethanol at low to moderate levels (Hernandez-Avila et al., 1991; Feskanich et al., 1999; Rapuri et al., 2000; Turner, 2000). These conflicting results may be at least partly due to the populations studied (Turner, 2000). In men and premenopausal women, studies suggest a negative correlation between moderate ethanol intake and bone mineral density and risk of fractures (Hernandez-Avila et al., 1991; Turner, 2000). In postmenopausal women, however, some studies indicate a positive correlation between bone mineral density and moderate ethanol consumption (Feskanich et al., 1999; Rapuri et al., 2000; Turner, 2000). This protective effect of ethanol has been suggested to be a result of decreased bone remodeling (Rapuri et al., 2000; Turner, 2000), possibly due to ethanol-induced decrease in PTH levels or increase in estrogen (Rapuri et al., 2000). Importantly, the effect of moderate ethanol intake on bone remodeling does not likely represent an increase in bone mass in postmenopausal women, but suggests that this population is losing bone more slowly compared to age-matched women who are losing bone. Studies on the effects of moderate ethanol consumption in rats, including ovariectomized rats (a model of postmenopausal bone loss) have failed to find positive effects on bone, and instead have shown no effect or a negative effect (Sampson and Shipley, 1997; Kidder and Turner, 1998; Turner, 2000).

Ethanol also affects bone growth and development, and there is no level of exposure known to be without effect. In humans and animal models, prenatal exposure to levels of ethanol ranging from low to high can result in growth retardation, including decreased pre- and postnatal body weight and length (Jones et al., 1973; Detering et al., 1979; Day et al.,

2002; Keiver and Weinberg, 2004), and delayed skeletal development (Leichter and Lee, 1979; Lee, 1987; Keiver et al., 1997; Habbick et al., 1998; Keiver and Weinberg, 2004). Recent studies suggest that prenatal exposure to moderate to high levels of ethanol disrupts multiple stages of bone formation, including those associated with the onset of mineralization and ossification in the rat (Simpson et al., 2005). Moreover, the effects of prenatal ethanol exposure on bone do not appear to resolve after birth. In ethanol-exposed rats, growth plate abnormalities are apparent at 2 - 4 weeks of age (Miralles-Flores and Delgado-Baeza, 1992), and mean bone age is delayed in children with Fetal Alcohol Syndrome at least until puberty (Habbick et al., 1998). In addition, ethanol administered during the postnatal period also affects bone development. Ethanol consumption by adolescent weanling rats results in a significant reduction in bone growth, volume, density, and strength (Hogan et al., 1997; Sampson et al., 1997).

Although the mechanisms by which ethanol affects bone at the various life stages are unknown, disruptions in Ca homeostasis occur with ethanol exposure at every life stage (Baran et al., 1982; Turner et al., 1988; Sampson, 1997; Keiver et al., 2000; Keiver and Weinberg, 2003, 2004). As alterations in Ca homeostasis are usually associated with negative effects on bone, ethanol's effects on Ca regulation could contribute to its effects on bone. Further, maternal hypocalcemia is not only associated with decreased fetal skeletal mineralization, but also with decreased birth weight and increased neonatal morbidity and mortality (Ibrahim et al., 1984; Chalon and Garel, 1985; Loughhead et al., 1990), and thus may underlie some of the other effects of prenatal ethanol exposure on offspring health.

1.2.7 Appropriateness of Animal Models for the Present Study

Animal models of ethanol consumption have been developed to overcome the limitations inherent with performing studies on human subjects, including the inability to perform controlled studies involving ethanol administration during some life stages (e.g. pregnancy and fetal life). Human studies are often difficult to interpret because of a small number of patients who can be studied and wide variation of the patient population in age, duration and pattern of ethanol abuse, nutritional status and multi-drug use (Driscoll et al., 1990). It is also difficult to get an accurate assessment of recent or lifelong ethanol consumption and inaccurate reporting can compromise interpretation of results. This is particularly true for pregnant women consuming ethanol because of the social stigma associated with drinking during pregnancy, and fear of intervention by social services. In contrast, the dose and duration of ethanol exposure, nutritional effects, confounding environmental factors, and sample size can be controlled in animal models. Although a variety of animal models are used in ethanol research, rats are widely used because they are easy to handle, economical to maintain, have a low rate of spontaneous malformations, short gestation period, produce large litters, and extensive scientific data on them is available (Palmer, 1971). Importantly, many ethanol-induced effects that are seen in human alcoholics and their ethanol-exposed offspring have been replicated in the rat model (Riley and Meyer, 1984).

Since rats metabolize ethanol at a higher rate than humans, a higher dose of ethanol must be given to rats to achieve comparable tissue exposure levels to human subjects. Thus, cross species comparisons are made on the basis of BECs achieved rather than the amount of ethanol consumed (Zajac and Abel, 1992). Because rats (and most other animals) will not

voluntarily consume large amount of ethanol, a variety of methods to administer ethanol at the levels required to produce relevant BECs have been developed.

The simplest method of administering ethanol is placing it in the drinking water while providing rat chow *ad libitum* (*ad lib*). However this method of administration often neither provides substantial elevations in BECs nor proper nourishment for the animal, as the animal rarely consumes high levels of ethanol when it is placed alone in drinking water (Weiner, 1980). Furthermore, the animal often reduces its water intake which can result in dehydration and decreased food intake. Another method of ethanol administration is via a feeding tube directly into the stomach (intragastrically). This procedure produces high BECs and the dose of ethanol and timing of administration can be controlled. However, it involves excessive handling of the animal, which may cause unnecessary stress, especially during pregnancy. Ethanol can also be administered via inhalation in a vapor chamber. This method results in high BECs and involves minimal handling of the animal, but is not a route by which ethanol is normally administered in humans.

Adding ethanol to a liquid diet is one of the most effective and least stressful methods of ethanol administration, particularly during pregnancy (Weinberg, 1984; Pierce and West, 1986). This method results in greater and more consistent ethanol intakes overall, which leads to more reliably elevated BECs. Blood ethanol concentration varies with food intake with this method, and peak BECs (taken approximately 2-4 hr after lights out, which corresponds with a period of high food intake, Weiner, 1980) generally range from 100-200 mg/dl (Keiver and Weinberg, 2003). This is comparable to the lower end of BECs seen in human alcoholics, which can exceed 500 mg/dl (Urso et al, 1981).

Regardless of the method of ethanol administration, rats receiving ethanol typically reduce their food intake, especially when ethanol is first introduced. To control for the reduced nutrient intake associated with ethanol consumption, a Pair-Fed (PF) control group is included and fed an isocaloric liquid diet (with the ethanol calories usually replaced with maltose-dextrin) in amounts equivalent to that consumed by the ethanol animals (DeCarli and Lieber, 1967; Weiner, 1980). Thus, differences between the Ethanol and PF groups can be attributed to the effects of ethanol. The ethanol-derived calories are not replaced with protein since differences in dietary protein content have been shown to have effects on fetal development (Institute of Medicine, 1999). Likewise, fat is not used to replace ethanol-derived calories as high fat intake in rodents can result in fatty liver syndrome (Li et al., 2003; Lai et al., 2005), and thus potentially confound results. Since the food intake of the PF rats is restricted to that of their ethanol-exposed partner, the PF rats often consume less food than they would desire overall (Weinberg, 1984), and this results in the consumption of a larger proportion of their food shortly after presentation, compared with rats fed ad lib (Weiner, 1980). This involuntary reduction in food intake and change in food intake pattern is thought to affect the hypothalamo-pituitary-adrenal (HPA) axis. Although pair-feeding does not alter basal corticosterone levels, it has been shown to prolong the elevation in corticosterone levels following stress (Weinberg and Gallo, 1982). Thus, pair-feeding, in addition to serving as a nutritional control group, is itself a treatment condition. Some researchers (particularly those investigating the HPA axis) also include an ad lib fed Control (C) group in animal models of ethanol exposure. By comparing the PF group to the C group, the effects of altered nutritional status, such as reduced intake of calories and the associated stress, can be assessed.

1.3 SUMMARY

Using rodent models of prenatal ethanol exposure, we (Keiver et al., 1996; Keiver and Weinberg, 2003) and others (Baran et al., 1982) have shown that chronic ethanol intake during pregnancy results in maternal hypocalcemia. Moreover, chronic ethanol intake decreases mineral content (Keiver et al., 1996; Keiver and Weinberg, 2003) and mineral density (Ronis et al., 2003) of maternal bone. Disruptions in Ca homeostasis are often associated with negative effects on bone and thus ethanol's effects on Ca regulation could contribute to its negative effects on maternal bone.

Although the mechanism by which ethanol decreases blood iCa levels is not known, ethanol appears to impair the normal compensatory response to hypocalcemia. Importantly, ethanol impairs the ability of PTH to respond to the hypocalcemia, resulting in PTH levels that are low relative to blood iCa or total Ca levels (Petriouanu et al., 1991; Laitinen et al., 1994; Perry et al., 1998; Keiver and Weinberg, 2003). However, it is unknown if ethanol prevents PTH levels from increasing in response to hypocalcemia, or if the ability to regulate blood Ca levels is intact but ethanol lowers the level of Ca maintained. This is an important question and the focus of this thesis. An increase in PTH is the first defense against the development of severe hypocalcemia, and is therefore vital to an organism's survival. This is especially true during pregnancy, when maternal Ca requirements are increased due to fetal demands for Ca (Kovacs and Kronenberg, 1997). An inability to raise serum PTH levels in response to a Ca challenge could cause harm to both the pregnant female and her fetus. In contrast, although a decrease in the level about which Ca is regulated may have negative consequences on cell function and bone health, it is unlikely to be life-threatening.

1.4 THESIS OBJECTIVE

The objective of this study was to determine if chronic ethanol consumption impairs the ability of the pregnant rat to increase her serum levels of PTH in response to an acute hypocalcemic challenge.

1.4.1 Hypotheses

- H_0 : Ethanol does not prevent the pregnant dam from increasing her serum PTH levels in response to an acute EGTA-induced hypocalcemic challenge.
- H_1 : Ethanol prevents the pregnant dam from increasing her serum PTH levels in response to an acute EGTA-induced hypocalcemic challenge.

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CHAPTER II

EFFECT OF CHRONIC ETHANOL CONSUMPTION ON THE RESPONSE OF PARATHYROID HORMONE TO HYPOCALCEMIA IN THE PREGNANT RAT¹

2.1 INTRODUCTION

Maternal alcohol (ethanol) intake during pregnancy is well known to have adverse effects on the offspring, including increased neonatal morbidity and mortality (Abel, 1982), and retarded growth and skeletal development (Day et al., 2002; Jacobson et al., 1994; Jones et al., 1973; Keiver et al., 1996, 1997; Habbick et al., 1998; Keiver and Weinberg, 2004; Lee and Leichter, 1983; Simpson et al., 2005; Weinberg et al., 1990). The effects of drinking during pregnancy on the health of the mother, however, have not been well studied. We (Keiver et al., 1996; Keiver and Weinberg, 2003), and others (Baran et al., 1982) have shown that chronic ethanol consumption during pregnancy in the rat results in maternal hypocalcemia and decreased mineral content (Keiver et al., 1996; Keiver and Weinberg, 2003) and density (Ronis et al., 2003) of maternal bone. As disruptions in calcium (Ca) homeostasis are often associated with adverse effects on bone, ethanol's effects on Ca regulation could contribute to its effects on maternal bone. Moreover, maternal hypocalcemia is associated with decreased birth weight, increased neonatal morbidity and mortality, and decreased mineralization of the fetal skeleton (Chalon and Garel, 1985; Ibrahim et al., 1984; Loughhead et al., 1990), and thus may underlie some of the effects of ethanol on fetal/neonatal health and skeletal development.

¹ A version of this chapter will be submitted for publication. S. Duggal, ME Simpson, K Keiver. Effect of chronic ethanol consumption on the response of parathyroid hormone to hypocalcemia in the pregnant rat.

The mechanism(s) by which ethanol decreases blood Ca levels is unknown. It has been confirmed, however, that the hypocalcemia represents a decrease in the level of ionized Ca (iCa), the form of Ca that is regulated, and that the decrease in iCa does not result from altered pH or albumin levels (Keiver et al., 1996; Keiver et al., in press; Keiver and Weinberg, 2003; Laitinen et al., 1994; Thomas et al., 1990). Moreover, ethanol's effect on blood iCa levels is not unique to the pregnant state or to chronic exposure; it also occurs in the non-pregnant human and rat (Keiver et al., 2000; Laitinen et al., 1994), and with acute ethanol exposure (Keiver et al., in press; Thomas et al., 1990). Although alterations in the levels of all the major Ca-regulating hormones (parathyroid hormone, [PTH], calcitonin and 1,25-dihydroxyvitamin D [$1,25(\text{OH})_2\text{D}$]) can occur with ethanol consumption (Sampson, 1997; Turner, 2000), it is becoming increasingly evident that ethanol alters the relationship between PTH and blood Ca, resulting in levels of PTH that are low relative to Ca levels. Parathyroid hormone levels fail to increase, or are actually decreased, during ethanol-induced hypocalcemia in the pregnant (Keiver and Weinberg, 2003) and non-pregnant (Diez et al., 1997; Keiver et al., 2000; Thomas et al., 1990) rat and human (Laitinen et al., 1992, 1994; Perry et al., 1998). Whether or not alterations in PTH level or function contribute to the initiation of the ethanol-induced hypocalcemia, or just perpetuate it, has not been clearly established.

The ability of PTH to respond to changes in blood iCa is the first defense against the development of severe or chronic hypocalcemia, and is therefore vital to an organism's survival and well-being. This is particularly true during pregnancy, when maternal physiological Ca requirements are increased due to the demands of the fetus for Ca. It is unknown, however, if the lack of response of serum PTH to the ethanol-induced

hypocalcemia represents an inability to raise PTH to regulate blood iCa, or if the ability to regulate iCa is intact, but ethanol lowers the level of iCa that is maintained. This is an important question because an inability to raise serum PTH levels could cause harm to both the pregnant female and her fetus. In contrast, although a decrease in the level of iCa about which homeostasis is maintained may alter cell function and long-term bone health, it is unlikely to be life-threatening. The purpose of the present study was to determine if chronic ethanol consumption impairs the ability of the pregnant rat to increase her serum levels of PTH in response to an acute hypocalcemic challenge, which is a test of parathyroid gland function. The effect of ethanol on the ability of the fetus to maintain Ca homeostasis during the maternal hypocalcemia was also examined and will be reported elsewhere.

2.2 MATERIALS AND METHODS

Virgin female Sprague Dawley rats (3 months old, Charles River, St. Constant, Quebec) were weighed (mean body weight = 278 g, range = 249 - 313 g), and assigned to one of three weight-matched groups: Ethanol (E, n = 46), Pair-Fed (PF, n = 48), and Control (C, n=25). There were originally 48 rats in the E group, but 2 of these failed to breed. The E rats received a liquid diet ad libitum containing 36% ethanol-derived calories. Consumption of ethanol typically reduces food (and therefore nutrient) intake, particularly during the initial 1 - 2 weeks on the ethanol-containing diet (Keiver and Weinberg, 2003). To control for the reduced food intake, a PF group, which received an isocaloric liquid diet (with the ethanol calories replaced with maltose-dextrin) in amounts equivalent to that consumed by the E rats, was included. However, the restriction of food intake by pair-feeding is a treatment in itself (Weinberg, 1984), therefore an ad libitum control (C) group was also included. The C group

received the same liquid diet as the PF group, but ad libitum. Diets were supplied by Dyets Inc. (Bethlehem, PA), and had Ca and phosphorus (P) contents of 0.5% and 0.3%, respectively. Nutrient contents of the diets conformed to the American Institute of Nutrition (1993) standards for pregnant rats.

After 3 weeks on their respective diets, the rats were bred with Sprague Dawley males and then continued to receive the experimental diets throughout 21 days of gestation. While with the males in the breeding racks, the female rats in the E group were offered their experimental diet, but also had access to rat lab chow. Mean time for the rats to breed was 3 days (range = 1-7 days) and day 1 of gestation was marked by the appearance of a vaginal plug. Rats were maintained in a temperature-controlled room with lights on between 0600 and 1800 hr. Diet was given daily at 1700 hr and water given ad libitum. The rats were weighed weekly and kept in compliance with the Canadian Council for Animal Care guidelines. Experimental procedures were approved by the University of British Columbia Animal Care Committee.

The experimental design is shown in Figure II-1. On day 21 gestation, the E, PF and C rats were divided into weight-matched groups: Baseline, Saline and EGTA. The E and PF rats received the following treatments: no injection (Baseline group), or an intraperitoneal (ip) injection of one of two doses of ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 300 or 500 $\mu\text{mol/kg}$ body weight, 300-EGTA and 500-EGTA groups, respectively), or an equal volume of saline (Saline group). To conserve animals, the C rats received all treatments except the 300 $\mu\text{mol/kg}$ body weight dose of EGTA. The EGTA doses were chosen for their ability to produce decreases in blood iCa concentrations that would result in maximal PTH secretion (decrease in iCa of $\sim 0.2 - 0.3$ mM, Fox, 1991;

Imanishi et al., 2002) or submaximal PTH levels. The doses of EGTA required were determined in preliminary studies (Appendix I) that were based on the studies of Imanishi et al. (2002) and Thomas et al. (1990). All solutions were adjusted to pH 7.4 prior to injection and rats were injected between 0800 and 1200 hr. Blood was collected by cardiac puncture under light ether anesthesia from the Baseline group, and at 30 or 60 min post-injection (Saline and EGTA groups). Preliminary studies (Appendix I) were conducted to determine the time course of the EGTA-induced decrease in blood iCa levels. Blood iCa levels in E rats appeared to reach their lowest point at 30 min post-injection (compared with 15 and 45 min). Blood was also collected by tail nick from seven E rats 2-4 hr after lights out (following the period of maximal food intake, Weiner et al., 1981) on day 18 of gestation for determination of peak blood ethanol concentration (BEC).

At termination, blood was collected using syringes containing Ca-balanced heparin (Radiometer, Copenhagen) and analyzed immediately for iCa and pH using the I-Stat Clinical Analyzer (Abbott Laboratories Inc., Mississauga, Ontario). Measurement of iCa by the I-Stat Clinical Analyzer is not affected by the presence of ethanol in blood, whereas ethanol has been shown to interfere with the measurement of iCa in some other commonly used analyzers (Keiver, 2004). Blood was also collected without anticoagulant, allowed to clot at room temperature for 10 min, centrifuged at 4°C and serum stored at -70°C for analysis of PTH, corticosterone and BEC. Radioimmunoassays were performed on serum for PTH (intact molecule and N-terminal assay; Nichols Institute, San Juan Capistrano, CA) and corticosterone (by a modification of Kaneko et al, 1981). BEC was measured using kit #A7504-150 from Pointe Scientific (Lincoln Park, MI). Serum was analyzed for calcitonin by radioimmunoassay (DiaSorin, Stillwater, MN; Appendix II).

Group differences were determined by ANOVAs, followed by Newman-Keul's post hoc tests. Differences among diet groups for baseline values were determined using 1-way ANOVAs, while the effects of saline (sham) injection were analyzed by 2-way ANOVAs (diet [E, PF, and C] by time [0, 30, and 60 min]). As C rats only received the 500-EGTA dose, the effects of EGTA were analyzed by two separate 3-way ANOVAs : 1) diet (E, PF, C) by time (30 and 60 min) by treatment (saline, 500-EGTA) ANOVAs were run to analyze the effects of the high dose of EGTA; and 2) the two EGTA doses were compared in a diet (E, PF) by time (30 and 60 min) by treatment (saline, 300-EGTA, 500-EGTA) ANOVA. The relationships between iCa and PTH were analyzed by non-linear regression analyses using Sigma-Stat. Coefficients of determination are presented as r^2 values. The number of parameters to include in the regression was determined by examination of the variance inflation factor (VIF), which assesses multicollinearity. A VIF value greater than 4 was considered to indicate that insufficient data were available to specify a parameter. Level of significance was $p < 0.05$. Post hoc comparisons between 0.05 and 0.10 were considered marginally significant. Values presented are the mean \pm SE.

2.3 RESULTS

iCa:

Blood iCa levels are shown in Figure II-2. Consistent with previous studies (Keiver et al., 1996; Keiver and Weinberg, 2003), ethanol consumption resulted in significantly lower levels of blood iCa at baseline compared with that in PF and C rats (ANOVA, diet, $p=0.037$, Newman-Keul's $E < PF = C$, p 's < 0.05). This difference in blood iCa with diet was still apparent overall after saline injection (ANOVA, diet, $p=0.018$), with E rats having

significantly lower blood iCa levels than C rats ($p=0.010$), but only marginally lower levels than PF rats ($p=0.081$). There was, however, no significant effect of the saline injection itself on blood iCa levels (ANOVA, time, $p=0.071$).

Injection of EGTA resulted in a decrease in blood iCa levels which did not vary with diet, but varied with both dose of EGTA and time post-injection. For the 500-EGTA dose, significant main effects were found for treatment (ANOVA $p<0.001$) and time post-injection (ANOVA $p=0.005$), and there was a significant time by treatment interaction ($p=0.001$). Treatment with EGTA (500-EGTA) significantly decreased blood iCa levels, compared with saline treatment, in all diet groups at 30 min post-injection ($p's<0.001$), and mean iCa levels decreased by 0.32, 0.34 and 0.42 mM in E, PF and C rats, respectively (Figure II-2A). Blood iCa levels were clearly recovering by 60 min (Figure II-2B, 30 min vs. 60 min, $p<0.001$), but were not yet back to saline-injected levels (500-EGTA vs. Saline, $p<0.001$).

Comparison of the two EGTA doses revealed significant main effects of treatment (ANOVA, $p<0.001$) and time post-injection (ANOVA, $p<0.001$), and a significant time by treatment interaction ($p=0.002$). Injection of the 300-EGTA dose resulted in a decrease in blood iCa levels at 30 min (Saline vs. 300-EGTA, $p<0.001$), but the decrease was not as great as that produced by the 500-EGTA treatment (300-EGTA vs. 500-EGTA, $p=0.007$). Moreover recovery was quicker after the 300-EGTA treatment, as iCa levels were no longer different from saline-treated rats by 60 min. There were no significant differences between E and PF animals.

Adjustment of blood iCa levels to a uniform pH (pH 7.4, data not shown) did not significantly change the results of the analyses examining the effects of diet, treatment and time on blood iCa levels. Blood pH levels are given in Table II-1. Although some small

changes in blood pH occurred with diet, treatment and time (Table II-1), they were not the cause of the observed trends in blood iCa levels. Blood pH did not differ with diet at baseline, and was not significantly affected by saline injection. However, a significant diet by time interaction ($p=0.038$) was found for the 500-EGTA condition. Post-hoc tests showed that regardless of treatment, the blood pH of C rats was lower than E ($p=0.042$) and PF ($p=0.012$) rats at 60 min. In addition, significant effects of time (ANOVA, $p=0.003$) and treatment (ANOVA, $p=0.023$) were found for the 300-EGTA treatment. Overall, blood pH at 30 min was lower than at 60 min ($p=0.005$), and marginally higher in the 500-EGTA compared with Saline ($p=0.075$) or 300-EGTA ($p=0.062$) groups.

PTH:

The response of serum PTH to the treatment-induced changes in blood iCa levels is shown in Figure II-3. Serum PTH did not differ with diet at baseline, and saline injection did not significantly affect PTH levels. Importantly, ethanol consumption did not prevent PTH levels from increasing in response to the EGTA-induced decrease in blood iCa levels. For the 500-EGTA treatment, significant effects of diet (ANOVA, $p=0.010$) and treatment (ANOVA, $p<0.001$), and a diet by treatment interaction ($p=0.005$) were found. Serum PTH levels were increased by EGTA injection in all diet groups ($p's < 0.001$), but overall, the increase in PTH levels was higher in PF, compared with E or C, rats ($p's \leq 0.002$).

For the 300-EGTA treatment, significant effects of diet, treatment and time (ANOVA, $p's \leq 0.002$) and a diet by treatment by time interaction ($p=0.037$) were found. The response of serum PTH to the 300-EGTA treatment did not differ between E and PF rats at either 30 or 60 min. Injection of the 300-EGTA dose resulted in an increase in serum PTH levels at 30 min in both E ($p=0.007$) and PF ($p<0.001$) rats compared to saline, but PTH had

recovered to saline levels by 60 min. Levels of PTH were not significantly affected by dose of EGTA injected at 30 min, but were lower in the 300-EGTA, compared with 500-EGTA, group at 60 min (p 's=0.010 and <0.001 for E and PF rats, respectively).

Relationship between PTH and iCa:

The effect of diet on the relationship between serum PTH and blood iCa levels was further examined by regression analyses on the values for individual rats (Figure II-4). Although the theoretical relationship between PTH and iCa levels is best described by a sigmoid curve following the four-parameter model of Brown et al. (1983), insufficient data (as assessed by the variance inflation factor [VIF]) were obtained for the minimum (maximally suppressed) PTH levels to fit the four-parameter model. Therefore sigmoid curves described by a three-parameter model were fitted (it is common to fit other models when data are insufficient for a four-parameter model, Aguilera-Tejero et al., 1996; Fox, 1991) to the data for E, PF and C rats (Figures II-4A, B and C, respectively), and the values for maximal serum PTH level, slope and the PTH-iCa set-point (the level of iCa at which half maximal stimulation of PTH secretion occurs) were determined. These values and their associated VIFs are given in Table II-2. In all rats, serum PTH varied inversely with iCa (Figure II-4). The relationships were significant for all diet groups (p 's < 0.001) and the changes in iCa explained 67% ($r^2=0.67$), 73% ($r^2=0.73$) and 60% ($r^2=0.60$) of the variation in PTH levels in E, PF, and C rats, respectively. As the C rats did not receive the 300-EGTA dose, their data were insufficient to adequately fit even a three-parameter model, and the VIFs were greater than four for the estimates of maximal PTH level and set-point (Table II-2).

Visual inspection of the PTH-iCa curves for the E and PF rats suggested that ethanol consumption may have lowered the maximal serum level of PTH and altered the sensitivity of PTH secretion to a decrease in blood iCa level (i.e. decreased the slope), but had no effect on set-point (Figures II-4A and B, Table II-2). Although the PTH levels attained with the 300 and 500-EGTA doses were compared among dietary groups, the degree of hypocalcemia obtained with each dose varied considerably and thus the statistical analyses of the effect of diet on the PTH response to EGTA by dose does not necessarily reflect the effect of diet on maximal PTH level or the sensitivity of PTH to a decrease in iCa level. Inspection of the curves in Figure II-4 suggest that in all diet groups, maximal PTH levels were elicited only when blood iCa levels fell below 1.00 mM, and that decreases in blood iCa levels sufficient to elicit maximal PTH levels occurred at both the 300 and 500-EGTA doses, but did not always occur with the 500-EGTA dose. Thus to determine the effect of ethanol consumption on maximal PTH levels, we examined the effect of diet on PTH levels by 1-way ANOVA only for rats whose iCa values were < 1.00 mM. By this criterion, mean maximal PTH levels were 199 ± 16 (n=14), 298 ± 30 (n=10) and 259 ± 20 (n=5) for the E, PF and C rats, respectively. This analysis indicated a significant effect of diet on maximal PTH level (ANOVA, $p=0.010$), and levels were significantly lower in E compared with PF rats ($p=0.028$). However, there were no significant differences between the C and either E ($p=0.110$) or PF ($p=0.286$) rats. Because we were unable to identify the minimum PTH level, we did not perform any further analyses on slope.

Corticosterone:

Although maximal PTH levels were decreased in E compared with PF rats, maximal PTH levels in C rats were between, and not significantly different from, those of the E and

PF rats. The data for the C rats is insufficient (due to a low n) to make firm conclusions regarding their maximal PTH levels; however it is possible to interpret the difference between E and PF rats as representing an increase in maximal PTH levels due to pair-feeding, rather than an ethanol-induced decrease in levels. One of the limitations of the model of ethanol administration used in this study is that there is no perfect control for ethanol's effects on nutrition. Although the PF group controls for ethanol-induced reductions in food intake, pair-feeding in itself can be considered a treatment condition (Weinberg, 1984). Pair-fed rats are subjected to involuntary food restriction and can have a prolonged response of corticosterone levels to stress (Weinberg, 1984). Comparison of the PF group with an ad lib fed C group allows some assessment of the effects of reduced food intake and altered stress response in this model. As stress, or sympathetic nervous system stimulation, may increase PTH levels (Brown et al., 1977; Kukreja et al., 1975), we analyzed serum corticosterone levels to determine if the higher maximal PTH levels in the PF rats resulted from an increased response to the stress of EGTA injection.

Serum levels of corticosterone are shown in Figure II-5. Overall, corticosterone levels were higher in E, rather than PF, rats suggesting that the higher PTH levels in the PF rats were not due to an increased response to stress (and supporting the interpretation that maximal PTH levels were decreased in E rats). Saline injection had no significant effect on corticosterone levels, but levels were higher in E, compared with PF and C, rats (ANOVA, diet, $p=0.011$; $E>PF=C$, $p's<0.005$) at baseline. Injection of EGTA increased corticosterone levels in all rats (ANOVAs, treatment, $p's<0.001$) and levels were not significantly affected by time post-injection. In response to the 500-EGTA treatment, E rats had significantly higher corticosterone levels than C rats ($p=0.004$), and marginally higher than PF rats

($p=0.057$). For the 300-EGTA treatment, corticosterone levels were higher in E, compared with PF rats ($p<0.001$).

Mean Terminal Body Weight, E intake, BEC, peak BEC:

Mean ethanol intake throughout gestation was 11.8 ± 0.1 g ethanol/kg body weight and mean peak BEC on day 18 gestation was 169 ± 7 mg/dl ($n=6$). The BECs at termination are given in Table II-3, and did not differ among E rats with treatment or time post-injection; thus changes in blood iCa levels with treatment were not confounded by changes in BECs. Mean food intakes during the experimental period are shown in Figure II-6. Food intake, and therefore Ca intake, varied significantly with diet during the gestation period (food and Ca intakes, ANOVAs, $p's<0.001$). Mean Ca intakes during gestation were 0.29 ± 0.004 , 0.27 ± 0.004 and 0.34 ± 0.006 mg/g body weight for E, PF and C rats, respectively. Mean food and Ca intakes were significantly higher in C, compared with E and PF, rats ($p's<0.001$), and slightly but significantly higher in E, compared with PF, rats ($p's=0.001$). Final body weights (as well as mean body weight and weight gain, Appendix III) showed similar trends ($C>E>PF$, $p's<0.001$) and were 451 ± 8 , 383 ± 3 , and 347 ± 5 g for the C, E and PF rats, respectively.

2.4. DISCUSSION

It is well established that ethanol decreases blood Ca levels, and that the magnitude of the decrease varies with BEC or dose of ethanol (Keiver and Weinberg, 2003; Krishnamra & Limlomwongse, 1983; Krishnamra et al., 1987; Laitinen et al., 1994; Petriouanu et al., 1991; Peng et al., 1972; Sampson et al., 1999). Although the decrease in blood Ca appears to represent a change in iCa (Keiver, 2004; Keiver et al., 1996; Keiver and Weinberg, 2003;

Laitinen et al., 1992, 1994; Thomas et al., 1990), we have recently demonstrated that ethanol interferes with the measurement of iCa by the analyzer (Radiometer, Copenhagen) most commonly used in these studies. This methodological artifact results in an inverse relationship between measured blood iCa and ethanol concentration, with iCa decreasing by approximately 5% (0.06 mM) at a BEC of 500 mg/dl (Keiver, 2004). We have previously confirmed (Keiver et al., in press) that acute ethanol administration decreases blood iCa levels when measured by the I-Stat Clinical Analyzer, whose performance is not affected by the presence of ethanol (Keiver, 2004). However, the present study is the first to show that chronic ethanol consumption also decreases blood iCa levels independent of this methodological artifact.

The mechanism underlying ethanol's effect on blood iCa is unknown, but studies suggest that the ability of PTH to respond to the hypocalcemia is impaired by ethanol (Diez et al., 1997; Laitinen et al., 1992, 1994; Thomas et al., 1990). The ability to increase PTH is the first defense against the development of severe or chronic hypocalcemia and thus ethanol's effects on PTH may contribute to its effects on blood iCa. It is not known, however, if ethanol actually prevents PTH levels from increasing in response to hypocalcemia, or if Ca regulation is intact, but ethanol alters the level of iCa regulated. The results of the present study clearly showed that ethanol did not prevent serum PTH levels from increasing in response to acute hypocalcemia induced by EGTA injection in the pregnant rat. Ethanol did, however, appear to decrease the maximal PTH level achievable in blood, and thus altered the relationship between PTH and iCa levels.

Although ethanol can affect the circulating levels of the Ca-regulating hormones (refer to Sampson, 1997; Turner, 2000 for reviews), such effects do not appear to initiate the

ethanol-induced decrease in iCa. As in the present study, PTH levels are not always decreased during ethanol-induced hypocalcemia (Keiver et al., 1997; Laitinen et al., 1994), and the decrease in blood Ca with acute ethanol injection occurs even after thyroparathyroidectomy (Peng et al., 1972; Peng and Gitelman, 1974). The possibility that ethanol may contribute to hypocalcemia through effects on PTH function cannot be ruled out, however, as ethanol has been shown to blunt the ability of administered PTH to raise blood Ca levels (Peng et al., 1972). Increased levels of calcitonin also do not appear to initiate the hypocalcemia. Although calcitonin levels can be increased during chronic ethanol consumption in pregnant rats (Keiver et al., 1997; Keiver and Weinberg, 2003), they are not usually increased during chronic or acute ethanol consumption in non-pregnant rats and humans (Diez et al., 1997; Keiver et al., 2000; Laitinen et al., 1991, 1994). Moreover, removal of the thyroid gland (and so prevention of an increase in calcitonin secretion) does not prevent hypocalcemia after acute ethanol injection (Peng et al., 1972). The decrease in Ca also does not depend on an increase in urinary Ca loss. Although ethanol increases urinary Ca excretion (Kalbfleisch et al., 1963; Laitinen et al., 1991, 1992; Perry et al., 1998), nephrectomy does not abolish the effect of ethanol on blood Ca (Peng and Gitelman, 1974). Ethanol has been shown, however, to increase the disappearance of ^{45}Ca from blood, and it has been hypothesized that ethanol decreases blood Ca by inducing a shift of Ca from the extracellular compartment into the intracellular compartment within soft tissue and bone (Peng and Gitelman, 1974). Although direct evidence to support this hypothesis is lacking, it is the only hypothesis suggested that is consistent with the evidence to date.

Although ethanol-induced alterations in the levels of the Ca-regulating hormones do not appear to initiate the decrease in blood iCa levels, the failure of PTH to respond to the

hypocalcemia and thus initiate appropriate compensatory responses would contribute to its perpetuation. Chronic ethanol administration has been reported to result in PTH levels that are low relative to blood iCa or total Ca levels (Keiver et al., 2000; Keiver and Weinberg, 2003; Laitinen et al., 1994; Perry et al., 1998), but the hypocalcemia observed in these studies (and thus the stimulus for PTH secretion) is small. Few studies have documented the changes in both PTH and Ca after acute ethanol administration, which tends to be associated with a greater degree of hypocalcemia. Diez et al. (1997) observed that both total Ca and PTH were decreased at 1 hr after acute ethanol administration (ip) in the rat, suggesting that ethanol prevents PTH from increasing in response to hypocalcemia. However, blood iCa levels were not measured and so the actual degree of stimulus for PTH secretion is unknown. Importantly, Thomas et al. (1990) found that PTH levels did not increase despite a large decrease in blood iCa levels (~ 0.17 mM at 30 min) induced by acute ethanol administration (ip). This strongly suggests that ethanol prevents PTH levels from increasing in response to hypocalcemia, rather than just altering the sensitivity of PTH to iCa (or level of iCa regulated), as maximal PTH secretion is stimulated by decreases in iCa of approximately 0.2 – 0.3 mM (Fox, 1991; Imanishi et al., 2002). The interpretation of these results, however, are complicated because blood iCa was measured using a Radiometer ICA analyzer, whose performance is affected by the presence of ethanol (Keiver, 2004). Thus the true decrease in blood iCa levels in the study of Thomas et al. (1990) is not known. However, based on our results with the Radiometer ICA analyzer (Keiver, 2004) and estimates of BECs achieved with similar doses of ethanol (Keiver et al., in press; Roine et al., 1991), the magnitude of decrease in iCa due to the methodological artifact in Thomas et al. (1990) would have only

been approximately 0.03 – 0.04 mM, and thus would not have significantly affected the interpretation of their results.

In the present study with pregnant rats, chronic ethanol consumption resulted in a decrease in blood iCa, but PTH levels were not increased above control levels. However, ethanol did not prevent PTH levels from increasing in response to the additional decrease in blood iCa levels elicited by EGTA injection. The apparent difference in the effect of ethanol on the ability to raise serum PTH levels in response to hypocalcemia between the present study and Thomas et al. (1990) is not likely due to differences in the severity of hypocalcemic challenge, or in BEC. The decrease in blood iCa induced by ethanol in the study of Thomas et al. (1990) was at least 0.1 mM, regardless of any measurement error. A decrease in iCa of this magnitude is sufficient to raise PTH levels by approximately 2-fold when induced by EGTA injection in rats without ethanol (Aguilera-Tejero et al., 1996; Thomas et al., 1990), and in the rats in the present study, with or without ethanol. Moreover, mean BEC in the present study at the time of termination (30 – 60 min after EGTA injection) was approximately 90 mg/dl. Although BEC was not measured in the study of Thomas et al., the results of other studies in which ethanol was administered by ip injection (Keiver et al., in press; Roine et al., 1991) suggest BEC was probably between 100 – 150 mg/dl.

It is possible that the difference in the ability of PTH to increase in response to hypocalcemia between the present study and that of Thomas et al. (1990) reflects attenuation of ethanol's effect on PTH secretion with chronic exposure to ethanol. It is not uncommon for some adaptation to occur in the response of tissues to the effects of ethanol after repeated exposure, and thus tissue adaptation may cause the impairment in response of PTH to hypocalcemia to decrease over time.

Alternatively, it is possible that the difference in response of PTH may be due to differences in the mechanisms underlying the hypocalcemia. Laitinen et al. (1994) proposed a hypothesis for ethanol's interference with PTH secretion that we believe can explain the apparent difference in response of PTH to the hypocalcemia induced by ethanol and that by EGTA injection. This hypothesis states that if, as originally suggested by Peng et al. (1972), ethanol causes a shift of iCa from the extracellular to intracellular compartments, then PTH secretion may be impaired because the signaling cascade of the Ca-sensing receptor on the parathyroid gland involves changes in intracellular Ca concentrations. Specifically, intracellular Ca concentrations increase when extracellular iCa levels increase, and this inhibits PTH secretion (Chattopadhyay et al., 1996). Thus administration of ethanol would mimic the effect of increasing the extracellular iCa level and effectively decrease the sensitivity of PTH secretion to the ambient extracellular iCa level. The acute hypocalcemia induced by ethanol administration in the study of Thomas et al. (1990) would thus have been accompanied by an increase in intracellular iCa concentration in the parathyroid cells, explaining the observed lack of increase in serum PTH levels. In contrast, the hypocalcemia caused by EGTA injection in the present study would not have increased intracellular iCa levels (as EGTA causes hypocalcemia by binding iCa in the extracellular compartment), and would not interfere with PTH secretion. Thus ethanol may interfere with the PTH response to ethanol-induced hypocalcemia, but not to hypocalcemia induced by EGTA.

If the model described above is correct, then the effect of ethanol on PTH secretion might be predicted to vary with the degree of ethanol-induced hypocalcemia (and the degree of the shift of iCa from the extracellular compartment). Therefore in the present study, the small decrease in iCa that resulted from chronic ethanol consumption might have been

expected to result in a small decrease in the sensitivity of the PTH response to ambient iCa levels. Examination of the PTH-iCa curves (Figure II-4) suggests that ethanol may indeed have resulted in a small decrease in the response of the parathyroid cells to iCa.

Changes in the sensitivity of the PTH response to blood iCa are determined by examining the sigmoidal PTH-iCa curves generated across a range of iCa levels sufficient to elicit maximal and minimal (maximally suppressed) PTH secretion. Changes in PTH response to ambient iCa concentrations have been reported to occur in the rat during pregnancy and lactation (Schultz et al., 1997) and with aging (Fox, 1991; Uden et al., 1992), and in humans with tumors of the parathyroid gland (Brown, 1983), and can result from alterations in the set-point of PTH-iCa curves (due to right or left shifts of the curve), or the sensitivity of PTH secretion to changes in iCa (due to shifts of the curve up or down, or to changes in slope). Although changes in PTH-iCa curves are generally interpreted to reflect changes in PTH secretion, they may also reflect alterations in PTH clearance from blood (Imanishi et al., 2002). However, although studies looking at clearance rates are limited, they indicate that factors affecting the PTH-iCa curves act on PTH secretion rather than clearance (Fox, 1991; Fox and Mathew, 1991).

The present experiment was not designed to generate PTH-iCa response curves, but the variable response of blood iCa among individual rats to the two doses of EGTA resulted in near-complete curves for the E and PF rats. In all rats (E, PF and C), the EGTA injections resulted in a large enough decrease in blood iCa levels (0.32 – 0.42 mM) to elicit maximal serum PTH levels, although data were limited for the C rats for this parameter. However we did not administer Ca to the rats to increase their iCa levels sufficiently to examine the minimal PTH levels. Thus although an apparent set-point was estimated from the curves,

these values must be interpreted with caution, as they can only be accurately determined when both the maximal and minimal PTH levels are known. The actual set-point for the PTH-iCa relationships are probably slightly higher than estimated from the present study. Moreover, because the relationship between PTH and iCa differs during induction or recovery from hypocalcemia (Aguilera-Tejero et al., 1996), PTH-iCa curves should ideally be generated from data collected at only one time point.

Examination of the curves obtained for the pregnant rats suggested that the maximal PTH levels achieved in serum may have been decreased by chronic ethanol consumption (at least compared with PF rats), and this was confirmed by comparison of PTH levels by ANOVA when iCa levels were < 1.00 mM (and thus sufficient to elicit maximal PTH levels). Although corticosterone can increase secretion of PTH (Au, 1976; Brown, 1982), this difference in maximal PTH level could not be explained by differences in the response of E and PF rats to stress (as measured by corticosterone levels). Whether or not there are true differences in maximal PTH levels between the PF and C rats remains to be determined. However, as decreases in iCa of approximately $0.2 - 0.3$ mM are required to elicit maximal PTH secretion (Fox, 1991; Imanishi et al., 2002; the present study), a decrease in maximal PTH level alone is not sufficient to explain the altered relationship between PTH and iCa observed at baseline iCa levels in this study, and with more modest hypocalcemia in other studies (Keiver and Weinberg, 2003; Laitinen et al., 1991, 1992, 1994). Although it cannot be concluded from the present study, visual examination of the curves suggest that the minimal PTH level and the set-point were not affected by ethanol, but that the slope of the curve may have been decreased by ethanol. If substantiated, this would imply that ethanol

affects the relationship between PTH and iCa, and that this altered relationship between PTH and iCa would become more apparent as the iCa level decreased.

Our data are consistent with the hypothesis that ethanol decreases the sensitivity of PTH to changes in iCa by causing a shift of iCa from the extracellular to intracellular compartment, which interferes with intracellular signaling in the parathyroid gland. However, ethanol could alter PTH-iCa sensitivity by other mechanisms, including altering the number of Ca-sensing receptors on parathyroid cells, the interaction of iCa with the receptors, or the levels of proteins involved in the intracellular signaling pathway. The fact that acute ethanol administration also alters the PTH-iCa relationship suggests that ethanol's effects are not mediated by changes in parathyroid cell number, although this mechanism cannot be ruled out for chronic studies.

The mechanism by which ethanol may have decreased maximal PTH levels cannot be determined from this study. Decreased maximal PTH secretion with short-term stimulation is likely to reflect the amount of PTH stored in the parathyroid glands (Fox, 1991; Habener et al., 1975) and thus chronic ethanol consumption may decrease the PTH content of the parathyroid glands. This could result from fewer parathyroid cells, or from a decreased amount of PTH stored within each cell. With more prolonged stimulation, the amount of PTH secreted is also affected by the rate of PTH synthesis (Yamamoto et al., 1989), and it is possible that an ethanol-induced decrease in PTH synthesis may have contributed to the lower PTH levels in the E rats by 60 min.

The present study was designed to test the PTH response to an acute hypocalcemic stimulus. It is difficult to imagine a real-life situation in which an acute change in blood iCa levels would be large enough to elicit maximal PTH secretion. However, an effect of ethanol

on the amount of PTH stored in the parathyroid gland may have an impact on the ability to maintain Ca homeostasis during a chronic hypocalcemic challenge. Thus the effects of ethanol on the response to a more physiologically relevant challenge, such as chronic consumption of a low Ca diet, should be studied.

In summary, ethanol consumption did not prevent PTH levels from increasing in response to an acute EGTA-induced decrease in blood Ca levels in the pregnant rat. Ethanol did, however, appear to decrease the maximal PTH levels achievable in blood and thus altered the relationship between PTH and iCa levels. This altered relationship is unlikely to interfere with the ability of the pregnant dam to respond to acute perturbations in blood iCa levels, but could impair her ability to sustain high PTH levels in response to chronic hypocalcemia, as might occur with a low Ca diet. A significant number of people consume very low levels of Ca in North America (Institute of Medicine, 1997), and this could be exacerbated by the inadequate diets consumed by many alcoholics. As chronic maternal hypocalcemia has adverse effects on maternal/fetal health, the effect of ethanol on the ability to maintain Ca homeostasis during chronic consumption of a low Ca diet is an important area for further study.

Table II-1. The effect of treatment and time post-injection on blood pH levels in Ethanol (E), Pair-Fed (PF), and Control (C) dams. Values are mean \pm SE. Values for n are given in the brackets. See text for explanation of significant main effects and individual comparisons.

Treatment

Diet	Baseline	Saline	300-EGTA	500-EGTA
E – pre-injection	7.31 \pm 0.02 (n=5)			
E – 30 min		7.30 \pm 0.02 (n=5)	7.30 \pm 0.03 (n=8)	7.34 \pm 0.01 (n=8)
E – 60 min		7.33 \pm 0.03 (n=6)	7.32 \pm 0.02 (n=6)	7.35 \pm 0.01 (n=8)
PF – pre-injection	7.31 \pm 0.01 (n=5)			
PF – 30 min		7.28 \pm 0.02 (n=5)	7.29 \pm 0.02 (n=8)	7.31 \pm 0.02 (n=10)
PF – 60 min		7.34 \pm 0.01 (n=6)	7.32 \pm 0.01 (n=7)	7.37 \pm 0.03 (n=7)
C – pre-injection	7.31 \pm 0.02 (n=5)			
C – 30 min		7.30 \pm 0.02 (n=5)		7.31 \pm 0.02 (n=6)
C – 60 min		7.30 \pm 0.01 (n=4)		7.26 \pm 0.03 (n=5)

Table II-2. Values for maximal serum PTH level, slope, PTH-iCa set-point and variation inflation factor (VIF) for the relationship between serum PTH and blood iCa in Ethanol (E), Pair-Fed (PF), and Control (C) dams. The values for each parameter were determined from the sigmoid curves (described by a three-parameter model) shown in Figure II-4. Values are mean \pm SE.

Parameter						
Diet	Maximal PTH (pg/ml)	VIF	Slope	VIF	PTH-iCa Set-Point (mM)	VIF
E	217 \pm 20	2.31	18.19 \pm 5.89	1.89	1.15 \pm 0.02	2.40
PF	302 \pm 23	1.95	20.40 \pm 5.65	1.52	1.16 \pm 0.02	1.80
C	272 \pm 52	4.78	7.66 \pm 3.85	3.48	1.16 \pm 0.08	6.83

Table II-3. Blood ethanol concentration (BEC) in Ethanol (E) dams at termination. There were no significant differences in BECs among the different treatment groups or with time post-injection. Values are mean \pm SE. Values for *n* are given in the brackets.

Time	Baseline	Saline	300-EGTA	500-EGTA
Pre-injection	98 \pm 28 (n=5)			
30 min post-injection		125 \pm 31 (n=5)	78 \pm 23 (n=8)	80 \pm 21 (n=8)
60 min post-injection		126 \pm 17 (n=6)	91 \pm 19 (n=6)	62 \pm 16 (n=8)

Diet	Group	Treatment (per kg body weight)	Time Sampled (min post-injection)
Ethanol (E) Pair-Fed (PF)	Baseline	None	
	Saline (Sham)	Saline	→ 30 or → 60
	300-EGTA	300 μ mol EGTA	→ 30 or → 60
	500-EGTA	500 μ mol EGTA	→ 30 or → 60
Control (C)	Baseline	None	
	Saline (Sham)	Saline	→ 30 or → 60
	500-EGTA	500 μ mol EGTA	→ 30 or → 60

Figure II-1. The experimental design for the rat dams. Rats were fed diets containing ethanol (Ethanol, E) or control diets (Pair-Fed [PF] and Control [C] groups) for 3 weeks prior to breeding and throughout 21 days gestation. Treatments were administered on day 21 gestation.

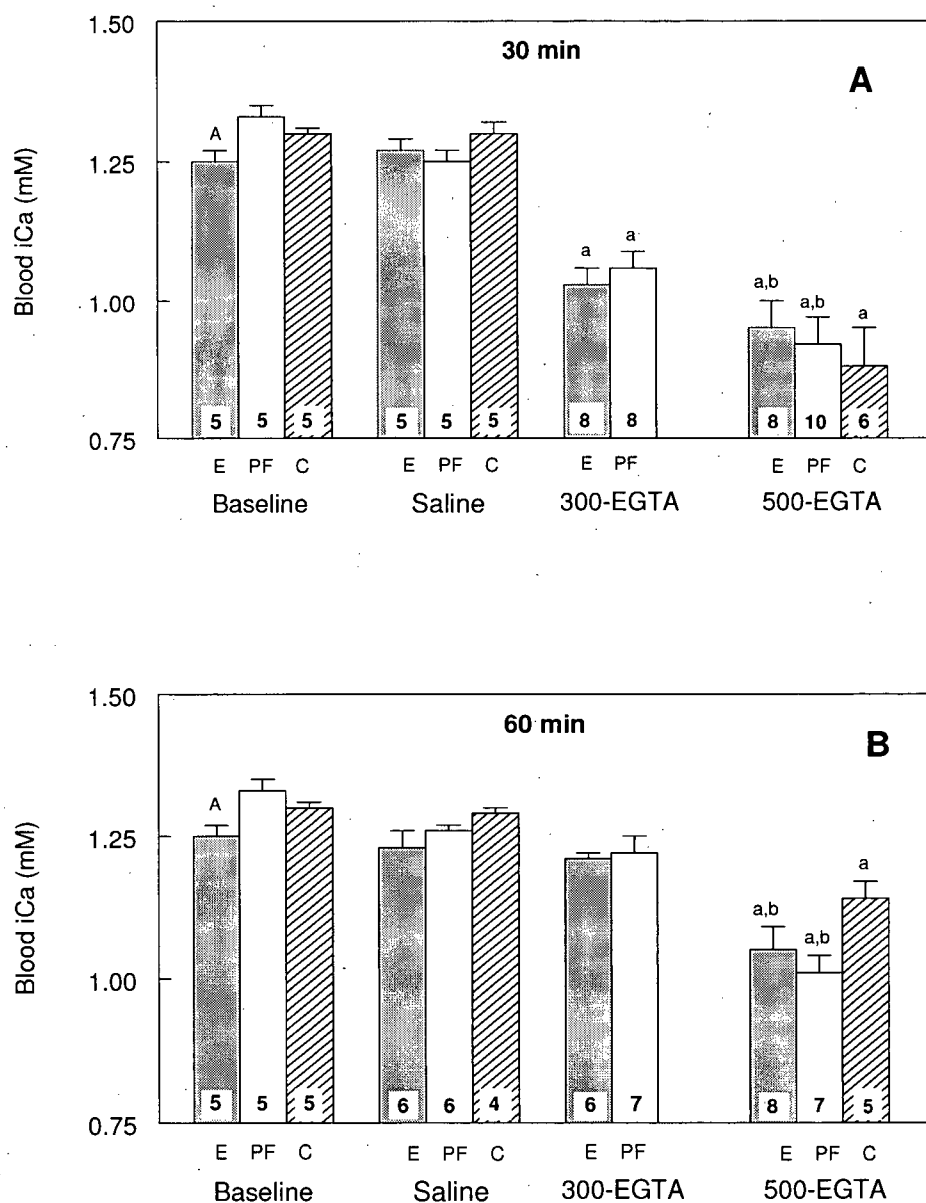


Figure II-2. Blood iCa levels in Ethanol (E), Pair-Fed (PF) and Control (C) dams at baseline, and at A) 30 min and B) 60 min after injection of saline or EGTA. Values are mean \pm SE. Values for n are given in the bars. The upper case letter represents significant differences ($p < 0.05$) between diets at baseline: A, significantly lower than PF and C dams. Comparison of Saline group to Baseline showed that saline injection had no significant effect on blood iCa levels, and blood iCa levels were significantly decreased in E, compared with C, rat overall (across both 30 and 60 min). The lower case letters represent significant differences ($p < 0.05$) within the same diet: a, significantly different from Saline; b, significantly different from 300-EGTA. Blood iCa levels were significantly increased at 60 vs. 30 min after both 300 and 500-EGTA treatments, regardless of diet

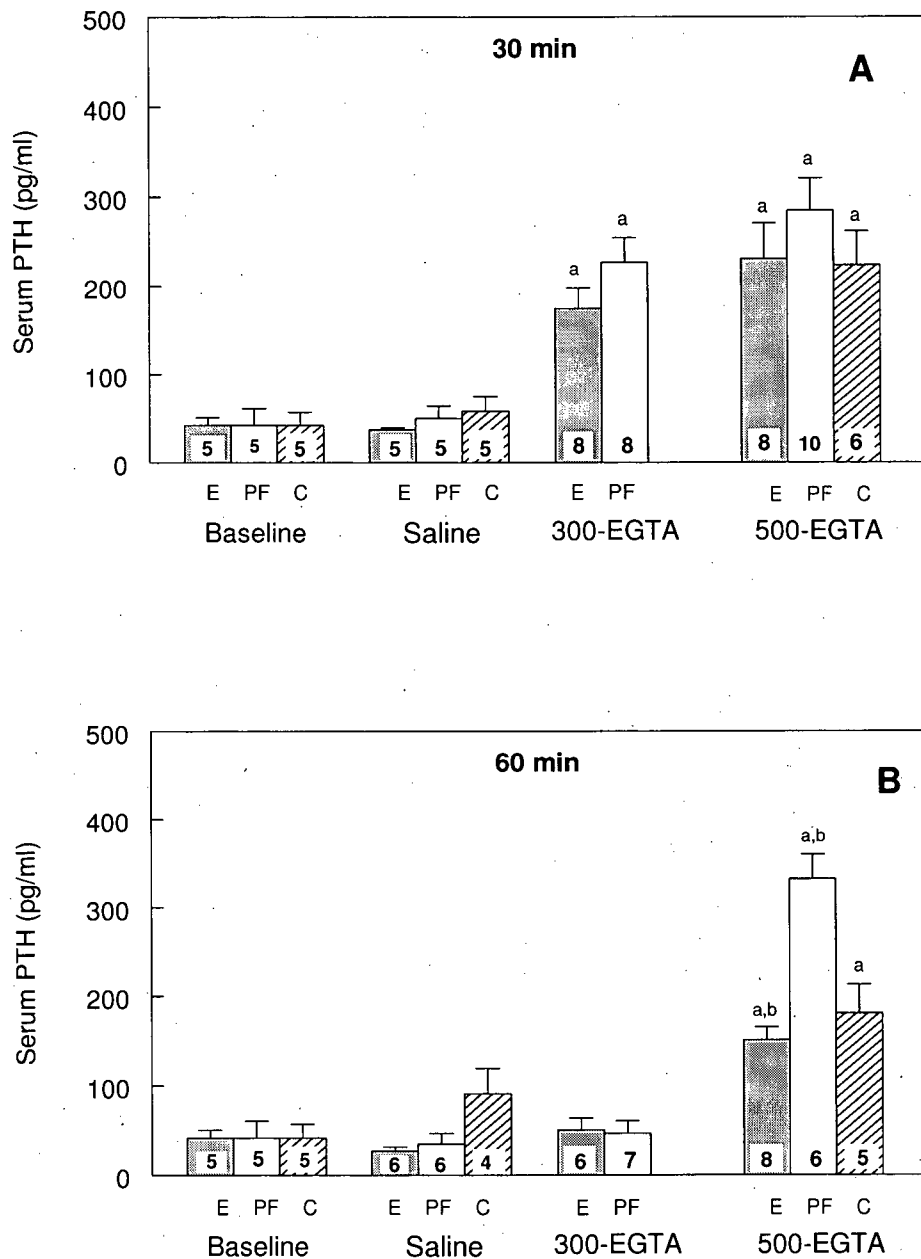


Figure II-3. Serum PTH levels in Ethanol (E), Pair-Fed (PF) and Control (C) dams at baseline, and at A) 30 min and B) 60 min after injection of saline or EGTA. Values are mean \pm SE. Values for *n* are given in the bars. The lower case letters represent significant differences ($p < 0.05$) within the same diet: a, significantly different from Saline; b, significantly different from 300-EGTA. At the 500-EGTA dose, time after injection was not a significant factor, and PTH levels were significantly ($p < 0.05$) higher overall in PF, compared with E or C, rats.

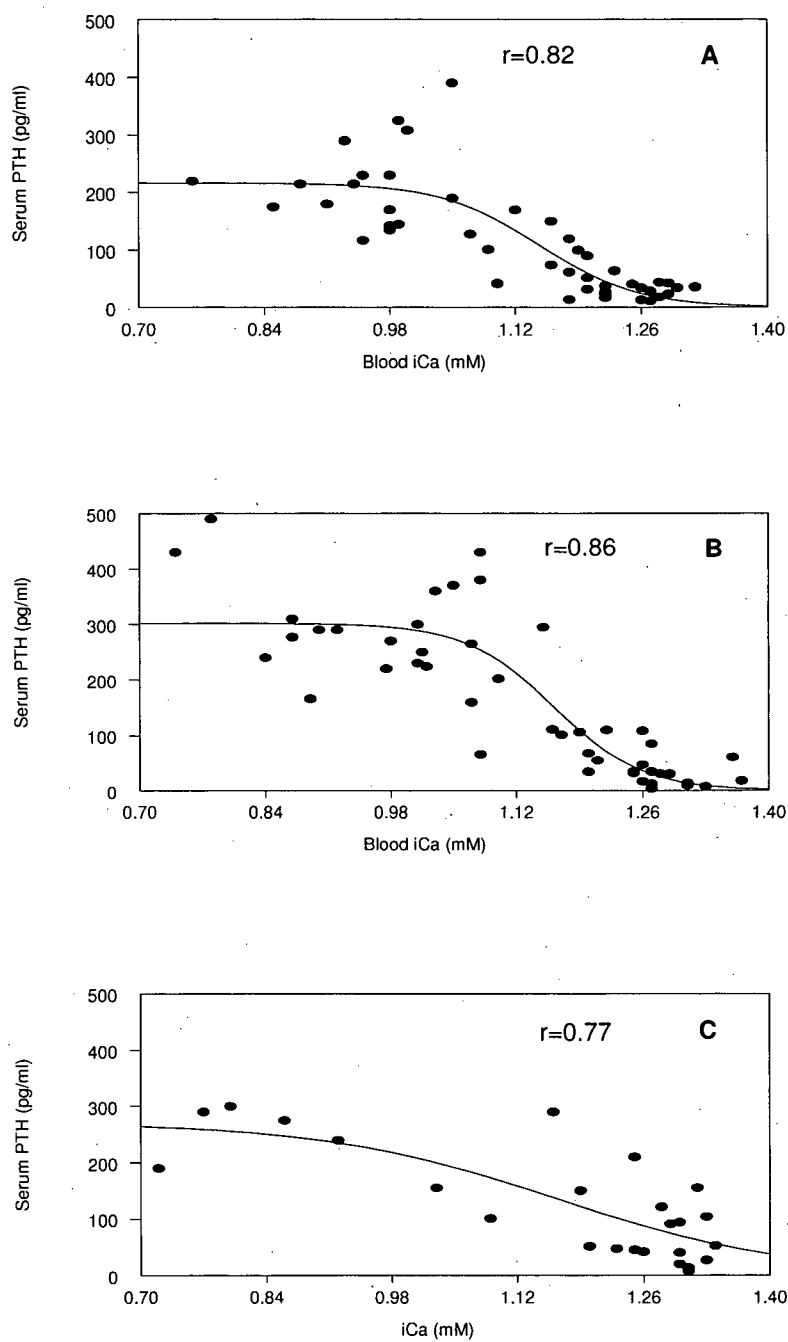


Figure II-4. The relationship between serum PTH and blood iCa levels in A) Ethanol (E), B) Pair-Fed (PF) and C) Control (C) dams. The points represent individual rats. The sigmoid curve was fitted using a three-parameter model.

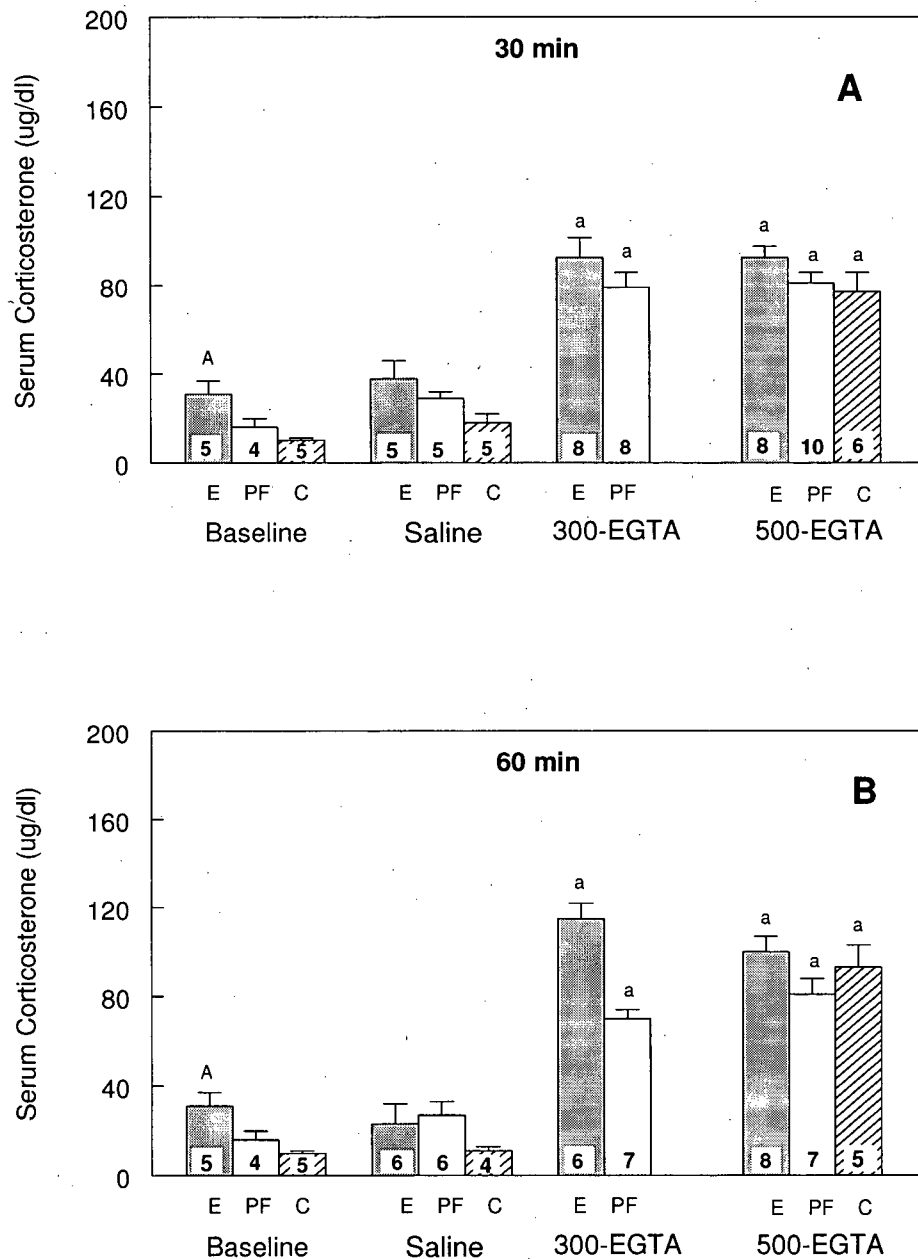


Figure II-5. Serum corticosterone levels in Ethanol (E), Pair-Fed (PF) and Control (C) dams at baseline, and at A) 30 min and B) 60 min after injection of saline or EGTA. Values are mean \pm SE. Values for n are given in the bars. The upper case letter represents significant differences ($p < 0.05$) between diets at baseline: A, significantly higher than PF and C. The lower case letters represent significant differences ($p < 0.05$) within the same diet: a, significantly different from Saline. Overall, corticosterone levels were significantly ($p < 0.05$) higher in E vs. C rats after 500-EGTA treatment, and in E vs. PF rats after 300-EGTA treatment.

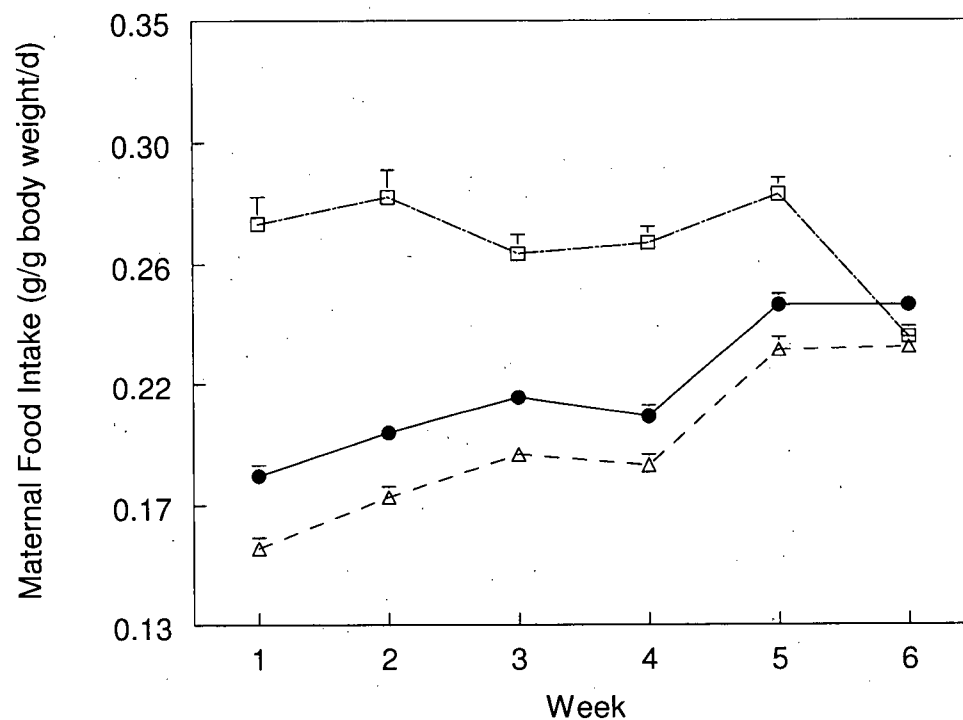


Figure II-6. Mean daily food intakes during each week of the experimental period for the Ethanol (●), Pair-Fed (Δ) and Control (□) dams. Week 1-3 were prior to breeding and weeks 4-6 were during gestation. Values are mean \pm SE. Over the gestation period, $C > E > PF$, p 's > 0.001 .

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CHAPTER III

3.1 GENERAL DISCUSSION

In spite of warnings to the contrary, the prevalence of women that have one or more drink of ethanol during pregnancy is high (~20% in Canada and the U.S., U.S department of Health and Human Services, 1991; Roberts and Nanson, 2000), and approximately 2% of pregnant women consume ethanol at high levels (Ebrahim, et al., 1998; Flynn et al., 2003). Ethanol intake during pregnancy has many adverse effects on the offspring, including increased neonatal morbidity and mortality (Abel, 1982) and retarded growth and skeletal development (Jones et al., 1973; Weinberg et al., 1990; Jacobson et al., 1994; Keiver et al., 1996, 1997; Habbick et al., 1998; Day et al., 2002, Keiver and Weinberg 2004; Simpson et al., 2005). In contrast to the effects on the offspring, the effects of drinking during pregnancy on the health of the mother have not been well studied. We (Keiver et al., 1996; Keiver and Weinberg, 2003) and others (Baran et al., 1982) have shown that chronic ethanol intake during pregnancy in the rat results in maternal hypocalcemia, and decreased mineral content (Keiver et al., 1996; Keiver and Weinberg, 2003) and mineral density (Ronis et al., 2003) of maternal bone. Although the mechanisms by which ethanol affects maternal bone are unknown, disruptions in Ca homeostasis are often associated with negative effects on bone, and thus ethanol's effects on Ca regulation could contribute to its effects on maternal bone.

It is becoming increasingly evident that ethanol alters the relationship between PTH and blood Ca, resulting in levels of PTH that are low relative to Ca levels. The purpose of this thesis was to investigate if the lack of response of serum PTH to the ethanol-induced hypocalcemia represents an inability to raise PTH to regulate blood iCa, or if the ability to regulate iCa is intact, but ethanol lowers the levels of iCa that is maintained. Specifically, we

determined if chronic ethanol consumption impaired the ability of the pregnant rat to increase her serum levels of PTH in response to a severe acute hypocalcemic challenge, which is a test of parathyroid gland function.

The results presented in this thesis suggest that ethanol does not impair the ability of the pregnant rat to raise her serum PTH levels in response to an acute hypocalcemic challenge, but may decrease the maximal PTH levels achievable in blood, and thus alter the relationship between PTH and iCa. Although the mechanism by which ethanol may decrease maximal PTH levels cannot be determined from this study, it may reflect a decrease in the amount of PTH stored in the parathyroid glands (Habener et al., 1975; Fox, 1991). The altered PTH-iCa relationship is not likely to affect the dam's ability to respond to small fluctuations in blood iCa levels, but could be of concern in situations of severe or chronic hypocalcemia.

Severe hypocalcemia might be more likely to occur during a binge pattern of drinking, rather than during more continuous chronic drinking. The magnitude of hypocalcemia increases with BEC or dose of ethanol (Krishnamra and Limlomwongse, 1983; Krishnamra et al., 1987; Laitinen et al., 1994; Petroianu et al., 1991; Keiver and Weinberg, 2003, Keiver et al., in press), and peak BECs achieved with a binge drinking pattern are higher than those achieved with a more continuous drinking pattern (Maier and West, 2001). Moreover, a decreased ability to secrete maximal levels of PTH could potentially impair the ability of the pregnant female to respond to chronic hypocalcemia, as might occur during consumption of a low Ca diet. A significant number of people consume very low levels of Ca in North America (Institute of Medicine, 1997), and this could be exacerbated by the inadequate diets consumed by many alcoholics.

Disruptions in Ca homeostasis are often associated with negative effects on bone, and thus ethanol's effects on the relationship between PTH and iCa could contribute to negative effects on the long term bone health of both mother and offspring. An inability to maintain bone mass during pregnancy, as a result of impaired Ca homeostasis, could affect the achievement or maintenance of peak bone mass and increase the risk for osteoporosis in later life (Heaney et al., 2000). Moreover, maternal (and fetal) hypocalcemia is associated with decreased fetal bone mineralization, decreased birth weight, and increased neonatal morbidity and mortality (Jones et al., 1973; Abel, 1982; Habbick et al., 1998), thus ethanol-induced disruptions in Ca homeostasis may underlie some of ethanol's effects in fetal development. Further, increased fetal PTH/PTHrP activity, resulting from altered maternal Ca homeostasis, has been hypothesized to be important in programming of bone during prenatal life (Tobias and Cooper, 2004), and so ethanol-induced alterations in maternal/fetal Ca homeostasis may have negative effects on bone cell function in adulthood. Thus, the effect of ethanol on the ability to maintain Ca homeostasis is an important area to study.

3.2 LIMITATIONS

3.2.1 Variable Response of Blood iCa Levels

In the present study, the response of blood iCa to a given dose of EGTA varied considerably among individual rats. This made it difficult to assess the effects of ethanol on the sensitivity of response of PTH to changes in iCa from the group data by ANOVA. The variability of the response could be decreased by using a hypocalcemic clamp technique, which lowers blood iCa levels by continuous intravenous infusion of EGTA or other hypocalcemic agents, and maintains blood iCa at a constant level (Fox, 1991). Likewise,

hypercalcemia can be induced by a Ca clamp technique, where high blood Ca levels can be induced by Ca infusion and maintained over a set period of time. The Ca clamp technique allows more accurate determinations of curve parameters to be made, which would facilitate group comparisons. However, this procedure requires the placement of indwelling catheters in a femoral artery and vein of the rat, and, due to stress on the mother and fetus, would be impractical in the pregnant rat model.

3.2.2 Variability in Food Intake Among Groups

Rats receiving ethanol typically reduce their food intake, especially when ethanol is first introduced. To control for the reduced nutrient intake associated with ethanol consumption, a PF control group was included and fed an isocaloric liquid diet in amounts equivalent to that consumed by the ethanol animals (DeCarli and Lieber, 1967; Weiner, 1980). Thus, differences between the E and PF group could be attributed to the effects of ethanol.

In the present study, despite careful pair-feeding (no errors in diet preparation or feeding calculations), mean food intakes (Figure II-6) and associated Ca intakes during the experimental period were slightly, but significantly, higher in the E, compared with PF, rats. The reason for this discrepancy is not known. Calculations involved in pair feeding include calculating the per gram body weight intake of liquid diet of the E rat (factor-A), multiplying this by the PF rat's body weight to obtain the theoretical amount of pair-fed liquid diet (factor-B), and the addition of 15 g of diet (factor-C). Factor-C has been determined experimentally from our previous studies, and is included to adjust for two things: 1) Pair-fed rats cannot completely empty the diet bottles and obtain all the diet offered, and 2) the

ethanol diet density is slightly less than the density of the pair-fed diet. The PF and E rats are fed an equivalent weight of diet, but the diets are isocaloric on a volume basis. It is possible that the factor-C used in pair-feeding calculations needs to be adjusted for the diet used in the present study (Dyets Inc). Although the adequacy of this factor for the Dyets' diet was piloted, our previous studies have used diet from BioServ Inc. (Frenchtown, NJ), and so it is possible that more extensive piloting is required for determination of this factor. However, as the PF rats had lower intake of food (and Ca) than the E rats, the difference in PTH response between the E and PF rats cannot be attributed to an ethanol-induced decrease in nutrient intake.

3.2.3 Incomplete PTH-iCa Curves

Our study tested parathyroid gland function (i.e. given a strong stimulus, would PTH be able to respond) and was not designed to generate curves that fully describe the relationship between PTH and iCa. Thus, we did not administer Ca to the rats and increase their iCa levels in order to examine minimal PTH levels, which is required for set-point estimations. Although visual examination of the curves suggests that the minimal PTH levels were achieved or close to being achieved, the actual set-point for the PTH-iCa relationships may be slightly higher than estimated from the present study. Moreover, due to the number of time points of blood collection (30 and 60 min) and treatment groups (Baseline, Saline, 300-EGTA, and 500-EGTA) in this study, a substantial number of rats were required. To conserve animals, the C rats were not administered the 300-EGTA treatment and, thus, insufficient data were collected to generate a PTH-iCa response curve for this group.

Attainment of more complete PTH-iCa response curves would have allowed us to more fully examine the effects of ethanol on the PTH-iCa relationship.

3.3 FUTURE DIRECTIONS

3.3.1 Determine if Ethanol Impairs the Ability of the Dam to Maintain PTH levels in Response to a More Chronic Hypocalcemic Challenge

The present study was not designed to mimic an *in vivo* situation, but was a test of PTH response to an acute hypocalcemic stimulus. It will be important to determine if ethanol impairs the ability of the pregnant dam to respond to a more physiologically relevant Ca challenge than that imposed in this study, such as might be induced by chronic consumption of a low Ca diet. A significant number of people consume very low levels of Ca in North America (Institute of Medicine, 1997), and this could be exacerbated by the inadequate diets consumed by many alcoholics. If ethanol impairs the response of PTH to consumption of a low Ca diet during pregnancy, this could have implications for both maternal and fetal health.

3.3.2 Determine if Ethanol Impairs the Ability of the Dam to Maintain 1, 25(OH)₂D Levels

The acute regulation of Ca is accomplished by the release of PTH from the parathyroids glands, which causes Ca resorption from bone and also enhances renal reabsorption of Ca. However, in a more chronic model of hypocalcemia such as that in which dams are placed on a low Ca diet, long term adjustments in blood Ca may be accomplished by altering the absorption of dietary Ca, thus preventing a significant drain on the Ca of the skeleton. This increased efficiency in intestinal Ca absorption is mediated by increased blood 1,25(OH)₂D levels. Studies with chronic ethanol consumption have shown

that, in addition to impairments in PTH, the response of $1,25(\text{OH})_2\text{D}$ to the ethanol-induced decrease in blood Ca is also impaired (Keiver et al, 1996; Sampson, 1997; Bjorneboe et al., 1986). It is unknown if the lack of response of $1,25(\text{OH})_2\text{D}$ is related to impairments of the response of PTH. $1,25(\text{OH})_2\text{D}$ acts by binding to intracellular receptor proteins in the cell nucleus of the intestine, bone, and kidney (Goff et al., 1990) and regulating mRNA synthesis. Because of this genomic mode of action, the effects of $1,25(\text{OH})_2\text{D}$ on blood Ca levels occur several hours to days after PTH stimulation and would not be expected to respond to the additional hypocalcemia induced by EGTA injection in the timeframe of the present study. However, in a more chronic model of hypocalcemia, it would be important to measure $1,25(\text{OH})_2\text{D}$ levels in order to examine ethanol's effects on the entire Ca-regulatory system.

3.3.3 Determine if Ethanol Causes a Shift of iCa from the Extracellular to Intracellular Compartments in the Parathyroid Cells

Our study suggests that ethanol may interfere with the response of PTH to ethanol-induced hypocalcemia, but not to hypocalcemia induced by EGTA. Peng et al. (1972) proposed that ethanol causes a shift of iCa from the extracellular to the intracellular compartments. If correct, the subsequent rise of intracellular Ca levels in the parathyroid cells would decrease the sensitivity of PTH secretion to the ambient extracellular iCa level, and could explain the observed lack of increase in serum PTH levels to the ethanol-induced hypocalcemia in this and other (Thomas et al, 1990; Laitinen et al., 1994; Keiver and Weinberg, 2003) studies. To test this hypothesis, *in vitro* studies could be performed to investigate the effects of ethanol on intracellular iCa levels in parathyroid cells using fluorescent dye technology. This dye alters its fluorescence properties when it binds to Ca,

and can be used to detect intracellular Ca levels and the hypothesized influx of Ca within the parathyroid cells in response to ethanol.

The effect of ethanol on PTH secretion *in vivo* might also be predicted to vary with the degree of ethanol-induced hypocalcemia and thus the degree of the shift of iCa from the extracellular compartment. To investigate this, acute intraperitoneal injections of ethanol at various doses could be administered to subjects, and the blood iCa and PTH responses measured.

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Appendix I:

Pilot Studies on Dose and Time Course Response of Blood iCa Levels in Pregnant Rats

AI.1 Overall Purpose of Pilot Studies

Prior to the main study, pilot studies were performed to determine: 1) the dose of EGTA that would decrease blood iCa concentrations sufficiently to result in maximal PTH secretion (decrease in iCa of 0.2-0.3 mM iCa decrease, Fox, 1991; Imanishi et al., 2002) and submaximal stimulation of PTH levels, 2) the time course of the EGTA-induced decrease in blood iCa levels to optimize the time of blood collection, and 3) the effects of saline injection and method of blood sampling on blood iCa levels. Since PTH responds almost immediately to a decrease in blood iCa (Yamamoto et al., 1989; Brown, 2000), we wanted to collect blood for PTH analysis when blood iCa was at its lowest point. In addition, we wanted to collect blood during the recovery phase to determine if ethanol delayed recovery of iCa levels. Saline was administered to the rats to determine the response of blood iCa levels to the injection procedure, in order to establish the number of rats that needed to be in the sham treatment. Further, different blood collection methods (tail nick vs. cardiac puncture under ether anesthetic) were to be used in the pilot studies (tail nick) compared to the main study (cardiac puncture), and we wanted to determine if there was a difference in blood iCa levels obtained using these different methods. Cardiac puncture ensures that an adequate amount of blood can be collected (for determining blood iCa, PTH, calcitonin and blood ethanol concentration) under minimal stress.

AI.2 Pilot-I: Pregnant Female Sprague Dawley rats

METHODS:

Three month old female Sprague Dawley rats (Animal Care Center, University of British Columbia) were weighed (mean body weight = 280 g, range = 240 – 360 g), and

assigned to one of three weight matched groups: an Ethanol (E, $n = 73$) group, which received a liquid diet ad libitum containing 36% ethanol-derived calories; a Pair-Fed (PF, $n = 58$) group, which received an isocaloric liquid diet (with the ethanol calories replaced with maltose-dextrin) in amounts equivalent to that consumed by the E rats; and a Control (C, $n=12$) group, that received ad lib access to the same diet as the PF rats. Diets were supplied by BioServ Inc (Frenchtown, NJ).

After 3 weeks on their respective diets, the rats were bred with Sprague Dawley males and then continued to receive the experimental diets throughout 21 days gestation. Mean time for the rats to breed was 3 days (range = 1-6 days) and day 1 of gestation was marked by the appearance of a vaginal plug. Rats were maintained in a temperature-controlled room with lights on between 0600 and 1800 hr. Diet was given daily at 1700 hr and water given ad libitum.

On day 21 gestation, the rats were divided into weight-matched groups and received an ip injection of EGTA at a dose of $1125 \mu\text{mol/kg}$ body weight (1125-EGTA group, $n=85$) or an equal volume of saline (Saline group, $n=3$). Initially, lower doses of EGTA were tested (based on the study of Imanishi et al., 2002), but were inadequate to decrease blood iCa levels to the desired levels in the pregnant rat. All solutions were microfiltered and adjusted to pH 7.4 prior to injection, and rats were injected between 0800 and 1200 hr.

Multiple observations were made on each rat, with blood collected by tail nick prior to injection (0 min, Baseline) and a maximum of three other time points post-EGTA injection. The times points tested were 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, and 120 min post-injection. The final blood sample was collected by cardiac puncture under light ether anesthesia. In order to determine if a difference in blood iCa levels exists when blood is

collected via tail nick method versus cardiac puncture, 3 rats were sampled by tail nick and then the same rats immediately sampled by cardiac puncture (no treatment). Blood iCa levels and pH were measured in whole blood, collected in capillary tubes (tail nick) or syringes (cardiac puncture) containing Ca-balanced heparin using an ICA1 ionized Ca analyzer (Radiometer, Copenhagen).

RESULTS AND CONCLUSIONS:

There was no significant difference (paired *t* test) in blood iCa levels when blood was collected via tail nick method versus cardiac puncture under ether anesthetic (*n*=3). Blood iCa levels are shown in Figure AI-1, and represent multiple observations made at different time points, from 31 rats. The 1125 $\mu\text{mol/kg}$ body weight dose of EGTA was successful in decreasing iCa levels by ~0.2-0.3 mM in pregnant dams. This dose is higher than that required to produce a similar decrease in blood iCa in the nonpregnant rat (Imanishi et al., 2002), and may reflect the hemodilution, or expanded plasma volume, that occurs during pregnancy.

Figure AI-1 also shows the time course of response for the 1125-EGTA treatment group (and only includes time points that have an *n* \geq 2). The lowest decrease in blood iCa levels were seen at 30 and 45 min post-injection in the E and PF rats, respectively. Blood iCa levels appeared to be recovering at 60 min post-EGTA injection in E rats, and at 120 min in PF rats.

After breeding it became apparent that the intake of ethanol diet (and thus Ca intake) of the pregnant rats was below levels seen in past studies. Since this could have affected the results (i.e. iCa levels obtained), we changed diet companies (to Dyets-Inc., Bethlehem, PA) and re-piloted an additional set of pregnant dams (Pilot-II).

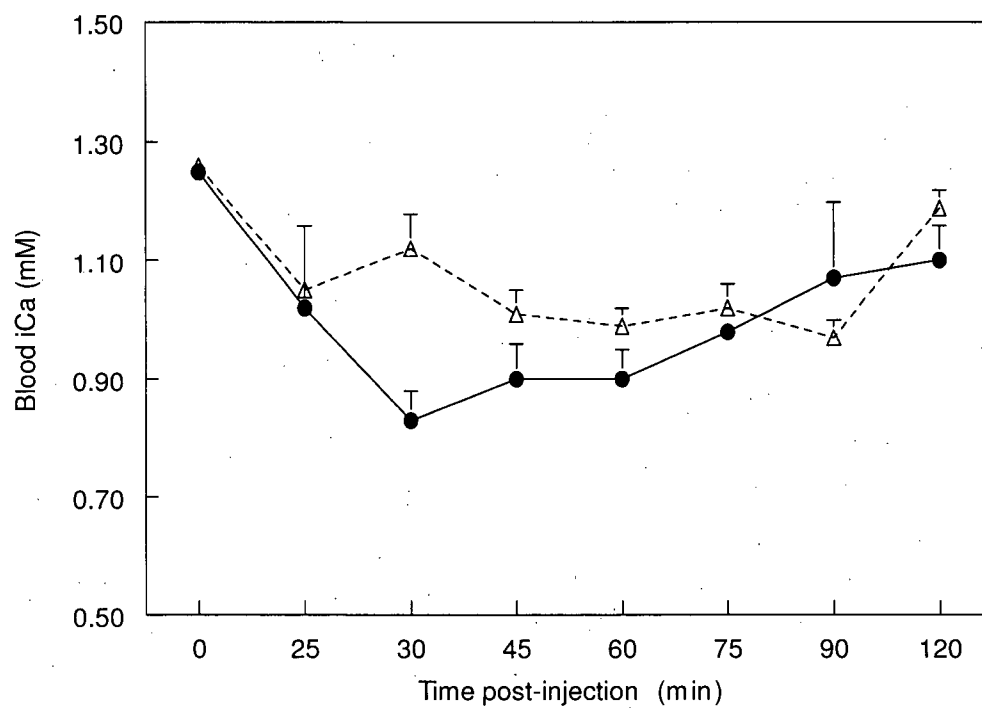


Figure AI-1. Blood iCa levels in pregnant Ethanol (●) and Pair-Fed (Δ) rats at various time points in response to injection of 1125 μmol EGTA/kg body weight. Values are mean \pm SE. Only time points where $n \geq 2$ are included.

AI.3 Pilot-II: Re-pilot Pregnant Female Sprague Dawley rats

PURPOSE:

The goal of this pilot was to establish if the ethanol intake of pregnant rats on the new diet from Dyets Inc. (Bethlehem, PA) was comparable to levels seen in past studies, and to determine if the 1125 $\mu\text{mol/kg}$ body weight EGTA dose was successful in decreasing blood iCa levels by $\sim 0.2\text{--}0.3$ mM in these dams. Moreover, an iCa analyzer that is not affected by the presence of ethanol in blood became available for use (I-Stat Clinical Analyzer, Abbott Laboratories Inc., Mississauga, Ontario), instead of the Radiometer instrument used in previous pilots, which is subject to ethanol interference (Keiver, 2004).

METHODS:

Three month old female Sprague Dawley rats (Animal Care Center, University of British Columbia) were weighed (mean body weight = 280 g, range = 240 – 360 g), and assigned to one of three weight matched groups: Ethanol (E, n=9) group, Pair-Fed (PF, n=5) group, and Control (C, n=1) group, as described in the previous pilot study (Pilot I). Diets were supplied by Dyets Inc. (Bethlehem, PA).

On day 21 gestation, the E, PF and C rats were divided into weight-matched groups and received no injection (Baseline group) or an ip injection of EGTA at a dose of 500 $\mu\text{mol/kg}$ body weight (500-EGTA), 300 $\mu\text{mol/kg}$ body weight (300-EGTA), or equal volume of saline (Saline group). Initially, the 1125 $\mu\text{mol/kg}$ body weight EGTA dose was tested, but it was lethal to the first rat injected. All calculations were checked (no errors were found), new solutions made, and lower doses of EGTA piloted. All solutions were adjusted to pH 7.4 prior to injection and rats were injected between 0800 and 1200 hr. Blood was collected by cardiac puncture under light ether anesthesia at 30 or 60 min post-injection. At

termination, blood was collected into syringes (containing Ca-balanced heparin) and analyzed immediately for iCa and pH using the I-Stat Clinical Analyzer (Abbott Laboratories Inc., Mississauga, Ontario).

RESULTS AND CONCLUSIONS:

Ethanol intake in rats was similar to past studies and greater than our previous pilot study (A1.2), confirming the new diet supplied by Dyets Inc was satisfactory. Blood iCa levels are shown in Table AI-1. A dose of 500 μmol EGTA/kg body weight elicited the desired drop of $\sim 0.2\text{-}0.3$ mM in blood Ca levels at 30 min post-injection, while a dose of 300 $\mu\text{mol/kg}$ body weight decreased iCa to a lesser extent (sufficient to elicit submaximal PTH secretion). These doses were comparable to those used by Imanishi et al. (2002). We found that the EGTA solutions required very vigorous vortexing, to ensure homogeneity after sitting for more than a day or so. We suspect that the pregnant females in the previous pilot (A1.2) required a higher dose of EGTA due to inadequate mixing of the EGTA solution, rather than pregnancy as we had assumed. At 60 min post-injection, the dam receiving the 300 $\mu\text{mol/kg}$ body weight dose of EGTA appeared to be recovering ($n=1$), thus, both time points were chosen to be included in the main study.

Table AI-1. Blood iCa levels in pregnant dams at baseline, or 30 and 60 min after injection of saline or EGTA. Values are mean \pm SE. Values for *n* are given in the brackets.

iCa (mM)					
Time (min)	Diet	Treatment			
		Baseline	Saline	300-EGTA	500-EGTA
0	E	1.18 (n=1)			
	PF	1.34 \pm 0.03 (n=2)			
	C	1.34 (n=1)			
30	E		1.20 (n=1)	1.07 (n=1)	1.00 (n=1)
	PF		1.28 (n=1)	1.05 (n=1)	1.01 (n=1)
	C		-	-	-
60	E		1.20 \pm 0.03 (n=3)	1.19 (n=1)	0.98 (n=1)
	PF		1.27 (n=1)	-	-
	C		-	-	-

Appendix II:

Calcitonin

PURPOSE:

It is possible that the ethanol-induced hypocalcemia in pregnant rats is partly due to an inappropriate response of calcitonin to changes in blood iCa levels. We therefore investigated the effect of ethanol on the response of calcitonin to the decrease in blood iCa levels elicited by EGTA injection.

MATERIALS AND METHODS:

The experimental protocol, diets and treatments are described in Chapter 2. However, as the time course of response of calcitonin to an acute decrease in blood iCa level is unknown, the sampling times (30 and 60 min after EGTA injection) may not be optimal for observation of a response in calcitonin. Serum was analyzed for calcitonin by radioimmunoassay (DiaSorin, Stillwater, MN).

RESULTS AND CONCLUSIONS:

Serum calcitonin levels are shown in Figure AII-1. Injection of EGTA did not result in a decrease in calcitonin in any group, at any time point. This may be because the time frame of sampling was not optimal to detect calcitonin changes. Alternatively, the calcitonin levels of the pregnant rat may be too low to be very sensitive to decreases in blood iCa levels. Although we have previously found an increase in calcitonin in ethanol-exposed pregnant rats despite a decrease in iCa levels (Keiver and Weinberg, 2003), the serum calcitonin levels did not differ significantly with diet at baseline in this study. This may have been due to the low n in the PF group (n=2). However, significant effects of diet were found in the saline-injected (ANOVA, diet $p=0.005$) and EGTA-injected (ANOVA $p's<0.001$) rats. In all analyses, calcitonin levels were lower in PF, compared with E ($p's<0.007$) and C ($p's<0.02$), dams.

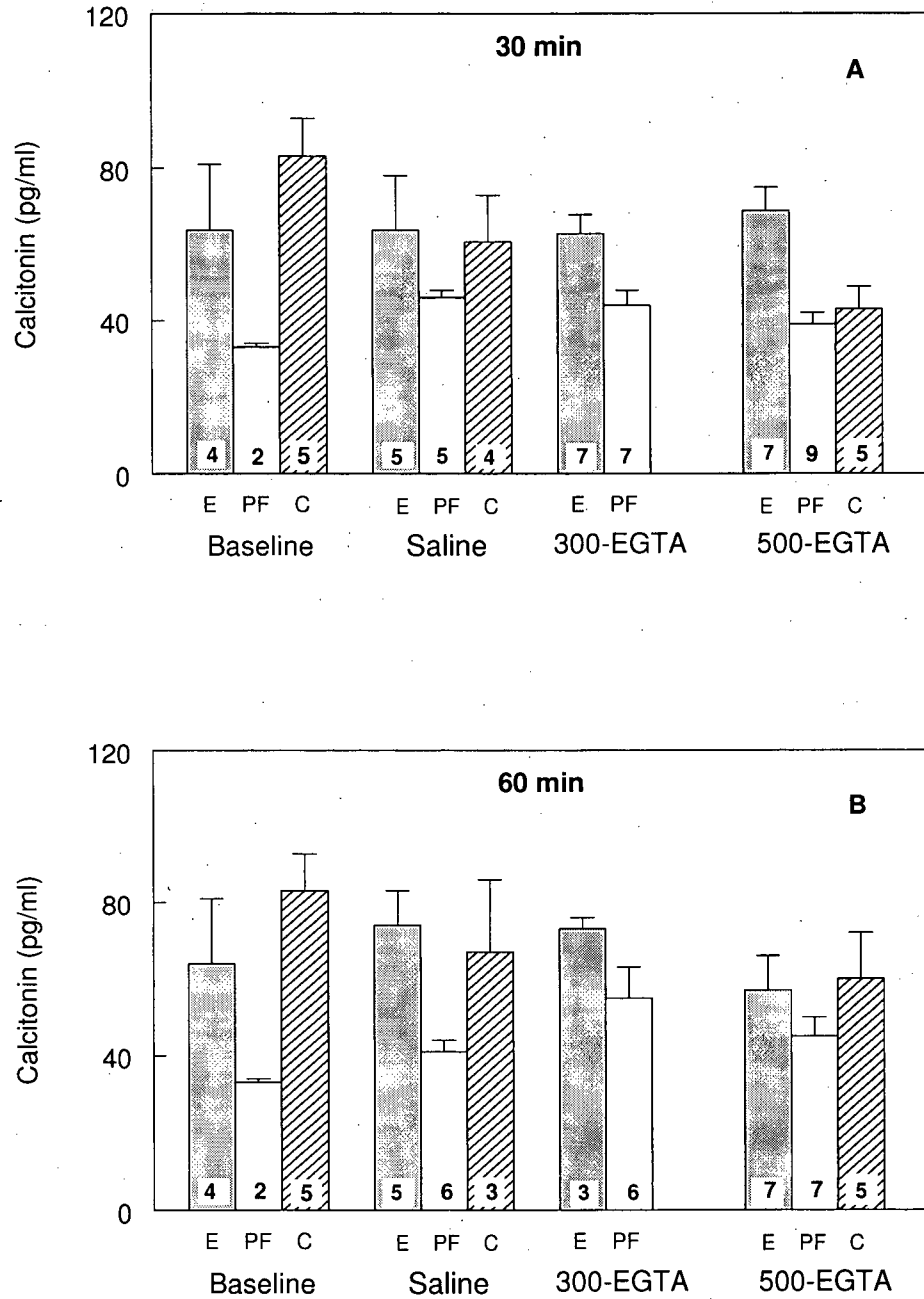


Figure AII-1. Calcitonin levels in pregnant Ethanol (E), Pair-Fed (PF) and Control (C) rats at baseline, and at A) 30 min and B) 60 min after injection of saline or EGTA. Values are mean \pm SE. Values for *n* are given in the bars. Decreasing blood iCa levels by EGTA injection had no effect on calcitonin levels. In all analysis, calcitonin levels were significantly ($p < 0.05$) lower overall in PF, compared with E and C, rats.

Appendix III:
Weight Gain Data

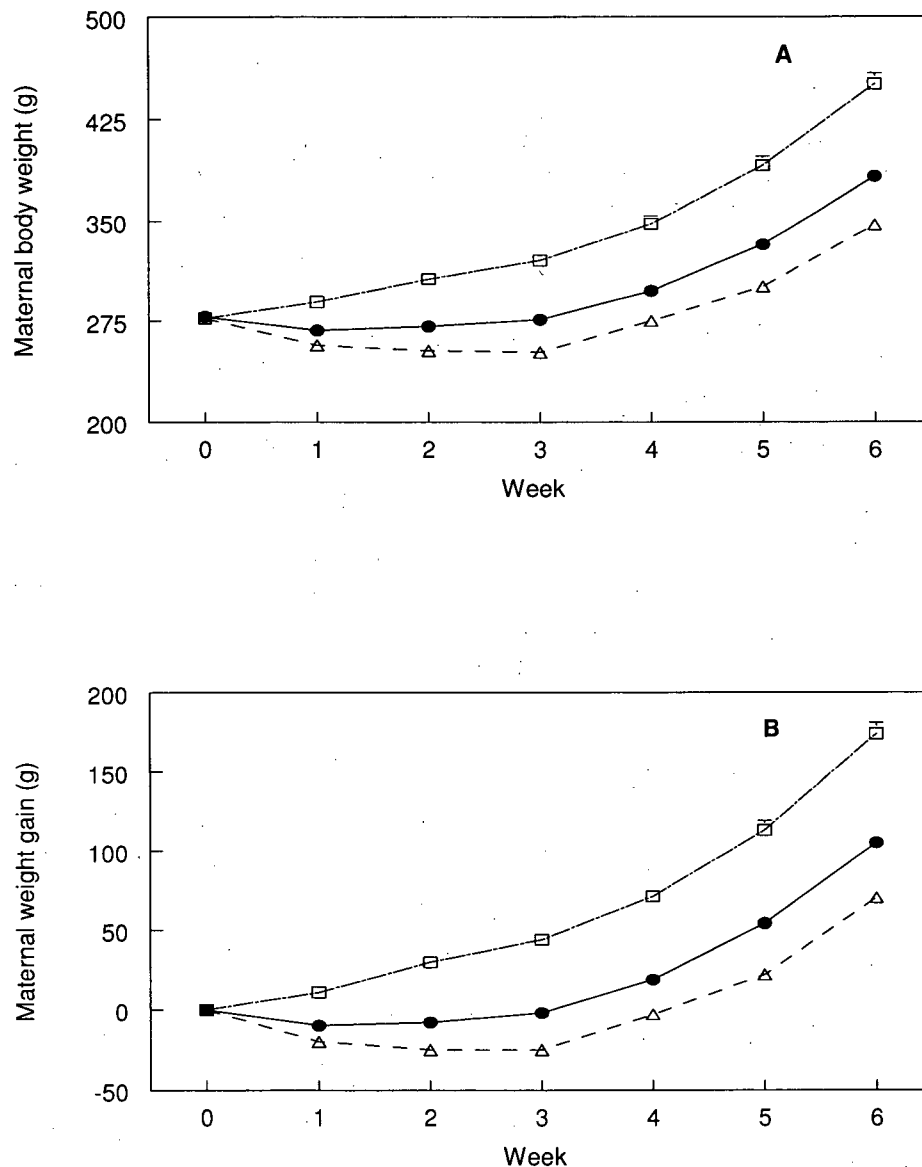


Figure AIII-1. Mean maternal (A) body weight and (B) weight gain (from initial body weight) during each week of the experimental period for the Ethanol (●), Pair-Fed (Δ) and Control (□) dams. Weeks 1-3 were prior to breeding and weeks 4-6 were during gestation. Values are mean \pm SE. Although initial body weights did not differ (Week 0), maternal body weight and weight gain varied significantly with diet (ANOVAs, p 's <0.001) and week (ANOVAs, p 's <0.001) and a diet by week interaction (p 's <0.001) was found.

Appendix IV:

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