MYOCARDIAL BUFFERING CAPACITY
&
HYDROGEN ION ACCUMULATION DURING GLOBAL ISCHEMIA:
ARE THERE GENDER DIFFERENCES?

by

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ABSTRACT

Myocardial Buffering Capacity & Hydrogen Ion Accumulation: Are there gender differences?
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Despite a 2-fold higher LV BC in females compared to males, during global ischemia, hydrogen ion accumulation ($\Delta[H^+]$) appeared to be higher. In addition, in females, lactate levels (30 min. ischemia) were 11% less in the LV and 65% less in the RV, than males. The ovarian stages estrus and metestrus demonstrated a 50% lower BC compared to proestrus, with the lowest $\Delta[H^+]$ levels in estrus. Within ventricles, females displayed exaggerated LV-RV differences with respect to $\Delta[H^+]$ and lactate levels (30 minutes ischemia). Female hearts may be at a greater risk of ischemic injury in the LV which may also depend on the ovarian stage of the cycle.

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Preface

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Abstracts

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2. **Boscarino C**, Wallen WJ, Belanger MP, Wittnich C. Does Ovarian Cycling Affect Development and Tolerance of Acidosis During Ischemia In The Heart? FASEB J., 2000:14(4):A572.

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List of Abbreviations

A	
A-	anion or conjugate base
acetyl Co-A	acetyl coenzyme A
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
B	
BC	buffering capacity
C	
Ca^{2+}	calcium
$[Ca^{2+}]$	concentration of calcium
CO_2	carbon dioxide
CK	creatine kinase
CrP	creatine phosphate
D	
DHEA	dehydroepiandrosterone
DNA	deoxyribose nucleic acid
E	
ECF	extracellular fluid
ER	estrogen receptor
F	
FSH	follicular stimulating hormone
E	
EtOH	ethanol
H	
H_2CO_3	carbonic acid
HCO_3^-	bicarbonate
H^+	hydrogen ion
HPO_4^{2-}	hydrogen phosphate
$[H^+]$	hydrogen ion concentration
H_2O	water
$H_2PO_4^-$	dihydrogen phosphate
HA	weak acid
HCl	hydrochloric acid
HK	hexokinase
I	
IAA	iodoacetic acid
ICF	intracellular fluid
K	
$KHCO_3$	potassium bicarbonate

L

LDH	lactate dehydrogenase
LDL	low density lipoproteins
LH	luteinizing hormone
LV	left ventricle

M

MI	myocardial infarction
mRNA	messenger ribonucleic acid

N

n	sample size
NaOH	sodium hydroxide
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)

O

OH^-	hydroxyl ion
pO_2	partial pressure of oxygen

P

P_i^{2-}	inorganic phosphate
pCO_2	partial pressure of carbon dioxide
PFK	phosphofructokinase
pH	negative logarithm of the hydrogen ion concentration of a solution
pH_i	intracellular pH
pKa	negative logarithm of the dissociation constant (k) for an acid (a)

R

RV	right ventricle

S

SD	standard deviation

U

UDP	uridine diphosphate
UTP	uridine triphosphate

V

V_1	alpha-alpha homodimer of myosin heavy chain (myosin isoenzyme)
V_3	beta-beta homodimer of myosin heavy chain (myosin isoenzyme)

W

WKY	wistar kyoto
wt	weight

Symbols and Units

β	beta
a	alpha
°C	degrees Celsius
Δ [H^-]	hydrogen ion accumulation
Δ lactate	lactate accumulation
g	grams
g/dl	grams per deciliter
IU	international units
kJ/mol	kilojoules per mole
M	molar
mg/day	milligrams per day
mg/kg	milligram per kilogram
mmol	millimoles
mm/Hg	millimeters of mercury
mol/L	moles per liter
ng/ml	nanogram per milliliter
nmol	nanomoles
pg/ml	picogram per milliliter
µL	micro liters
µmol	micro moles
µmol/g dry wt	micro moles per gram dry weight

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Rationale

Cardiovascular disease is the number one cause of death in men and women in the United States and Canada (Lerner & Kannel, 1985; Eysmann *et al.*, 1992 and Vaccarino *et al.*, 1999). One type of cardiovascular disease is Ischemic Heart Disease (IHD). This disease is a condition of insufficient myocardial perfusion and is most commonly due to obstruction of the coronary arteries, resulting in a myocardial infarction (MI), better known as a heart attack. IHD is the leading cause of death among the U.S. population, accounting for more than 600,000 deaths annually (Lilly, 1998). Statistics Canada reported a gender difference in the incidence of MI in men and women of 32% and 24%, respectively (Statistics Canada, 1995). This has led researchers to focus on studying sex-based differences in the incidence of a myocardial infarction. However, conflicting studies exist as to whether short-term mortality after an MI is higher in women than men (Vaccarino *et al.*, 1999). A twenty-six year follow-up of the Framingham population examined 5127 people and followed them biennially for 26 years. As for death during a coronary attack, women demonstrated an excess death risk over men between the ages of 35 and 84 with case fatality rates of .32 and .27, for women and men, respectively (Lerner & Kannel, 1985). Maynard et al. (1997) examined 4255 women and 8076 men who developed an acute MI. These women were found to be eight years older and more often had a history of congestive heart failure, hypertension or diabetes mellitus. After adjustment for the above factors, women were found to have a higher hospital mortality rate than men,13.7% vs. 7.8% (P=.004), respectively. The Global Utilization of Streptokinase and Tissue Plasminogen Activation for Occluded Coronary Arteries (GUSTO-I) examined the largest number of women (n=10,315) with confirmed MI. The mean age of women was 66 and 59 for men (n=30,653).

The unadjusted mortality rate for women was found to be two-fold higher compared to men. Once baseline characteristics, age, diabetes, heart rate, blood pressure, weight and height were adjusted, women still demonstrated a higher mortality rate, with odds 15% higher compared to men (Weaver *et al.*, 1996). Vaccarino et al. (1999) analyzed 155,565 women and 229,313 men between the ages of 30 and 89 years of age, enrolled in the National Registry of Myocardial Infarction 2. This study found sex-based differences varied according to age. In those patients less than 50 years of age, women demonstrated a significantly ($p<.001$) two-fold higher mortality rate during hospitalization, even after adjustment for all variables. There were no differences in mortality after the age of 75. Bueno et al. (1995) also found similar results in patients over 75 years of age. Additional studies found similar results to the above studies. For example Gorodeski (1994) found mortality rates after an acute MI to be 39% and 31% for women and men, respectively. In contrast to the above studies, Fiebach et al. (1990) examined 332 women and 790 men with acute MI. Crude in-hospital mortality rates were found to be higher in women compared to men, 14% vs. 9%, respectively. However, when variables which affect outcome were adjusted, gender did not play a role on in-hospital mortality rates. The authors conclude that female gender is not an independent predictor for early mortality. Malacrida et al. (1998) found only a small independent association between female gender and mortality after an acute MI. This study examined 9600 women and 26,480 men in The Third International Study of Infarct Survival (ISIS-3). After adjustment for age and baseline characteristics, sex appeared to play a small independent effect on early mortality. Clearly, controversy remains as to whether or not gender affects mortality rate after an acute MI.

During myocardial ischemia, oxygen availability is impaired due to an obstruction in one or more of the coronary arteries (Jennings *et al.*, 1986 and Ganong, 1997). As a result, the cells

affected revert to anaerobic glycolysis for energy while at the same time during this pathway produce the following byproducts, hydrogen ions (H^+) and lactate (Jennings *et al.*, 1986 and Ganong, 1997). During ischemia, there are three sources of hydrogen ion production, which lead to the development of acidosis. The major sources are ATP degradation and anaerobic glycolysis. The turnover of glycogen is a minor source of hydrogen ions (Dennis *et al.*,1991 and Schussheim & Radda, 1993). Intracellular acidosis has been shown for over a century to exert a negative inotropic effect in heart muscle (Poole-Wilson, 1989; Harrison *et al.*, 1992 and Schussheim & Radda, 1993). An estimated 50% of the negative inotropic effect of ischemia in the heart is due to intracellular acidosis (Jacobus *et al.*, 1982). In isolated ventricular rat myocytes, a rapid decrease in intracellular pH caused a parallel decrease in contractile activity (Harrison *et al.*, 1992). Intracellular acidosis is also a contributing factor to the development of cell necrosis (Poole-Wilson, 1989). The ability to resist changes in pH is known as the buffering capacity of the cell. The cell attempts to minimize the development of acidosis via various mechanisms. Such mechanisms include a combination of transmembrane transporters and intrinsic cellular components such as bicarbonate, phosphates and proteins (Lowenstein, 1993; Halperin, 1993 and Abelow, 1998). However, due to the lack of blood flow during ischemia, transmembrane transporters no longer function, hence the cell relies upon the intracellular components to minimize acidosis (Madshus, 1988). An increased buffering capacity has been shown to limit the degree of acidosis during ischemia (Kida *et al.*, 1991; Takeuchi *et al.*, 1995 and Pisarenko, 1996). When Garlick et al., (1979) added 100mM- Hepes (a buffer solution containing histidine) to perfused rat hearts prior to 12 minutes of ischemia, both the buffering capacity and pH were higher compared to control. Numerous studies using piglets, rabbits, canines and rats, demonstrated a reduction in the degree of acidosis as well as improved myocardial function upon

reperfusion when histidine was added as a buffer prior to an ischemic event (Hachida *et al.*, 1993; Ohkado *et al.*, 1993; Takeuchi *et al.*, 1995, 1996, 1997). This is because histidine is an effective buffer due to the pKa of the imidazole group. Thus hearts with greater buffering capacity may limit the degree of intracellular acidosis during ischemia and therefore potentially protect the heart from ischemic injury.

Glycogen is the endogenous substrate for the cell during anaerobic glycolysis (Ganong, 1997). Studies suggest a reduction in glycogen content prior to ischemia will reduce the development of acidosis, and an increase in glycogen content will increase the development of acidosis (Murry *et al.*, 1990; Weiss *et al.*, 1993; Schaefer *et al.*, 1995; Lavanchy *et al.*, 1996 and Depré *et al.*, 1998). McNulty et al. (1996) studied the effects of glycogen depletion prior to ischemia on infarct size. The left coronary artery was ligated in adult male Sprague-Dawley rats and placed into two groups: a preconditioned group where three 3 minute coronary occlusions were conducted prior to 45 minutes of ischemia and a control group, consisting of 45 minutes of ischemia with no preconditioning. The concentration of glycogen prior to ischemia in the preconditioned group was significantly lower, (12.5 ± 1.8 μmol/g wet wt) compared to control (24.9 ± 2.5 μmol/g wet wt). Furthermore, the accumulation of lactate during ischemia was also significantly lower in the preconditioned group. When infarct size was noted, the preconditioned group was found to have a 66% reduction in infarct size relative to controls. The authors conclude that myocardial glycogen depletion may be beneficial in reducing infarct size. Reduced acidosis was also found to reduce infarct size in pigs subjected to preconditioning (Kida *et al.*, 1991). Neely and Grotyohann, (1986) found glycogen depletion in rat hearts prior to ischemia resulted in less tissue lactate accumulation compared to controls. The accumulation of lactate was also found to be inversely related to recovery of ventricular function. Murry et al. (1990)

also noted a reduced proton production and lactate levels via reduced rate of glycogen breakdown and anaerobic glycolysis during ischemia in preconditioned dog hearts compared to controls. The study also demonstrated delayed ischemic cell death.

Gender based differences in myocardial metabolism have recently been discovered. A two-fold higher glycogen content has been found in adult SHR hypertensive female rats compared to hypertensive males (Wallen *et al.*, 1999). Furthermore, the enzyme phosphofructokinase (PFK) has also been shown to have a two-fold higher activity in WKY normotensive female rats compared to normotensive males (Wallen *et al.*, 1999). If normotensive females also contain a two-fold higher myocardial glycogen content, then a higher PFK activity in addition to an increased substrate level may potentially translate into a higher degree of acidosis during ischemia in females compared to males. If an increase in acidosis occurs in females, this may potentially lead to an increase in ischemic injury.

The question whether there are gender differences in the ability of the heart to handle acidosis (buffering capacity), as well as the development of acidosis during global ischemia has not yet been answered and, therefore formed the foundation of this thesis.

INTRODUCTION

Part 1: Myocardial Ischemia

A. What is Myocardial Ischemia?

Myocardial ischemia is defined as the lack of blood flow to the heart (Jennings *et al.*, 1986 and Ferrari, 1995). Ischemia may affect the entire ventricle of the heart, termed 'global' ischemia, or a fraction of the ventricle, 'regional' ischemia (Berne & Levy, 1997).

Normally, in the presence of blood flow and provided availability is not an issue, the adult human heart derives its energy primarily via the oxidation of fatty acids (Berne & Levy, 1997). However during ischemia, due to the lack of oxygen availability, within approximately 15 to 30 seconds the heart relies upon anaerobic glycolysis for energy (Jennings *et al.*, 1986; Hearse *et al.*, 1981 and Berne & Levy, 1997). At the same time energy is being produced via anaerobic glycolysis, the by-products H^+ and lactate are also produced, which contribute to intracellular acidosis during ischemia. Studies have shown the accumulation of H^+ and lactate to exert deleterious effects in the heart (Jennings *et al.*, 1986; Hearse *et al.*, 1981; Ferrari, 1995 and Berne & Levy, 1997). Thus within seconds after the onset of myocardial ischemia, intracellular acidosis develops, a breakdown in high energy phosphate occurs, where creatine phosphate declines earlier than ATP levels. After approximately one minute, lactate levels quadruple and creatine phosphate levels decrease by approximately 80% (Jennings *et al.*, 1986; Hearse *et al.*, 1981 and Ferrari, 1995). The production of H^+, hence development of acidosis, results from the breakdown of ATP and anaerobic glycolysis (Gevers, 1977 and Dennis *et al.*, 1991). Glycogen turnover, which is known to exist during ischemia (Fraser *et al.*,1998), acts as a minor source of H^+ (Gevers, 1977 and Dennis *et al.*, 1991).

B. Production of H^+ During Ischemia

1. ATP Degradation

Adenosine triphosphate (ATP) is the principle source of chemical energy used to drive a wide variety of biochemical reactions in the cell (Lehninger *et al.*, 1993). The structure of ATP consists of a series of three phosphate groups covalently linked at the 5' hydroxyl of ribose with adenine as its base (Figure 1) (Lehninger *et al.*, 1993).

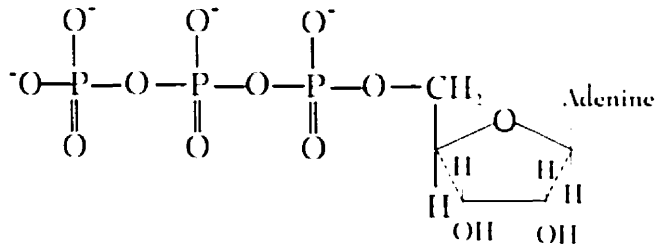

Figure 1: Molecular structure of an ATP molecule. Modified from Lehninger *et al.*, 1993.

ATP hydrolysis is an energy yielding reaction via the release of a terminal phosphate group, yielding approximately 30 kJ/mol. Since the hydrolysis is an exergonic reaction, the standard free energy ($\Delta G^{o'}$) is -30 kJ/mol (Figure 2) (Lehninger *et al.*, 1993).

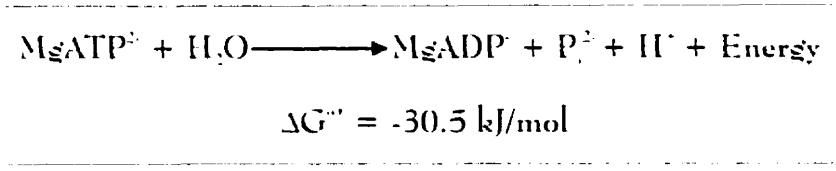

Figure 2: ATP hydrolysis. Modified from Lehninger *et al.*, 1993.

Baseline ATP levels in the rat heart are approximately 24 µmol/g dry wt and decrease to levels as

low as 2.5 μmol/g dry wt during a thirty-minute global ischemic period (Schaeffer *et al.*, 1995; Cave *et al.*, 1997 and Green *et al.*, 1998). Inorganic phosphate levels at baseline are approximately 3 mM and in a linear manner, reach levels as high as 30 mM by 30 minutes of global ischemia (Cross *et al.*, 1996 and Lavanchy *et al.*, 1996). Gevers (1977) and Dennis et al. (1991) have proposed the degradation of ATP (Figure 3) as a major source of H^+ during ischemia and studies have shown an increase in pH at end ischemia concomitant with a slower rate of ATP degradation during ischemia (Murray *et al.*, 1990; Kida *et al.*, 1991 and Schaefer *et al.*, 1995).

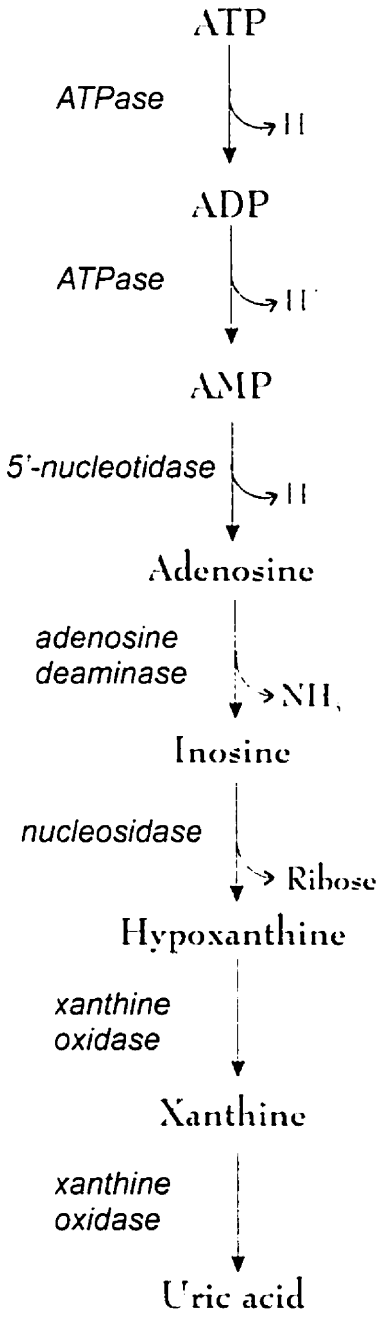

Figure 3: ATP degradation. Modified from Lehninger *et al.*,1993.

2. Glycolysis

Glycolysis is the breakdown of a molecule of glucose involving a series of enzymatic reactions to yield two molecules of pyruvate and ATP (Figure 4) (Lehninger *et al.*, 1993). The pathway begins with the phosphorylation of glucose, an irreversible reaction catalyzed by hexokinase (HK) (Figure 4, Step 1). The next step (Figure 4, Step 2), involves the isomerization of glucose-6-phosphate, an aldose to fructose-6-phosphate, a ketose. The next step, Step 3, is the irreversible conversion of fructose-6-phosphate to fructose 1,6-bisphosphate via PFK-1. The notation PFK-1 is used to distinguish the enzyme from PFK-2, which catalyzes the formation of fructose-2,6-biphosphate from fructose-6-phosphate. This step is also the major point of regulation in glycolysis. PFK-1 is allosterically inhibited by a high ATP content thus reducing the affinity of PFK for fructose-6-phosphate. A high concentration of AMP and ADP allosterically relieve this inhibition. Thus the need for ATP signals glucose breakdown as determined by the ATP/AMP ratio. A 15% decrease in ATP results in a 2-fold increase in ADP and a 5 -fold increase in AMP. Thus a small decrease in ATP markedly stimulates glycolysis (Lehninger *et al.*, 1993 and King & Opie, 1998). Step 4 involves the cleavage of fructose-1,6-bisphosphate via aldolase to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate in which the latter is rapidly converted to the former molecule via triose phosphate isomerase (Step 5). In a reversible reaction (Step 6), glyceraldehyde-3-phosphate is converted to 1,3-bisphosphoglycerate. During the latter the coenzyme NAD^+ is reduced to $NADH + H^+$. An ATP molecule is formed when 1,3-bisphosphoglycerate transfers a phosphate group to ADP forming 3-phosphoglycerate + ATP (Step 7). The next two steps (Steps 8 & 9) involve a reversible shift in the phosphate group of 3-phosphoglycerate and the removal of water forming phosphoenolpyruvate. The final step (Step 10) is the transfer of the phosphate group from the latter product to ADP via pyruvate kinase,

ultimately resulting in ATP and pyruvate (Lehninger *et al.*, 1991 and King & Opie, 1998). Thus pyruvate is the major end-product of glycolysis and is the junction point of carbohydrate metabolism. The fate of this molecule is dependent on the presence or lack of oxygen (Lehninger *et al.*, 1998). Under aerobic conditions, pyruvate is converted to acetyl CoA via the enzyme pyruvate dehydrogenase (PDH) and then enters the Citric Acid Cycle (Krebs cycle) (Figure 4, Step 10a). Under aerobic conditions, H^+ production from the metabolism of glucose ultimately enters oxidative phosphorylation in the mitochondria and thus net H^+ production is zero (Lehninger *et al.*, 1993 and King & Opie, 1998). However under anaerobic conditions, oxidative phosphorylation is inhibited and as a result, pyruvate is converted to lactate by the enzyme lactate dehydrogenase (LDH) (Figure 4, Step 10b). This latter step permits the regeneration of NAD^+ and hence the continuation of glycolysis (Lehninger *et al.*, 1993; Depré *et al.*, 1998 and King & Opie, 1998). With the continuation of anaerobic glycolysis, lactate is produced (Figure 4, Step 10b) and accumulates within the cytosol (Dennis *et al.*, 1991; King & Opie, 1998 and Dyck & Lopaschuk, 1998). With an increase in H^+ and lactate, the activity of PFK-1 is inhibited, ultimately halting glycolysis (King & Opie, 1998). The accumulation of H^+ and lactate during anaerobic glycolysis, have been shown to pose deleterious effects in the heart (King & Opie, 1998) manifested as ischemic contracture as well as cell necrosis (Ferrari, 1995; Berne & Levy, 1997; King & Opie, 1998 and Dyck & Lopaschuk, 1998).

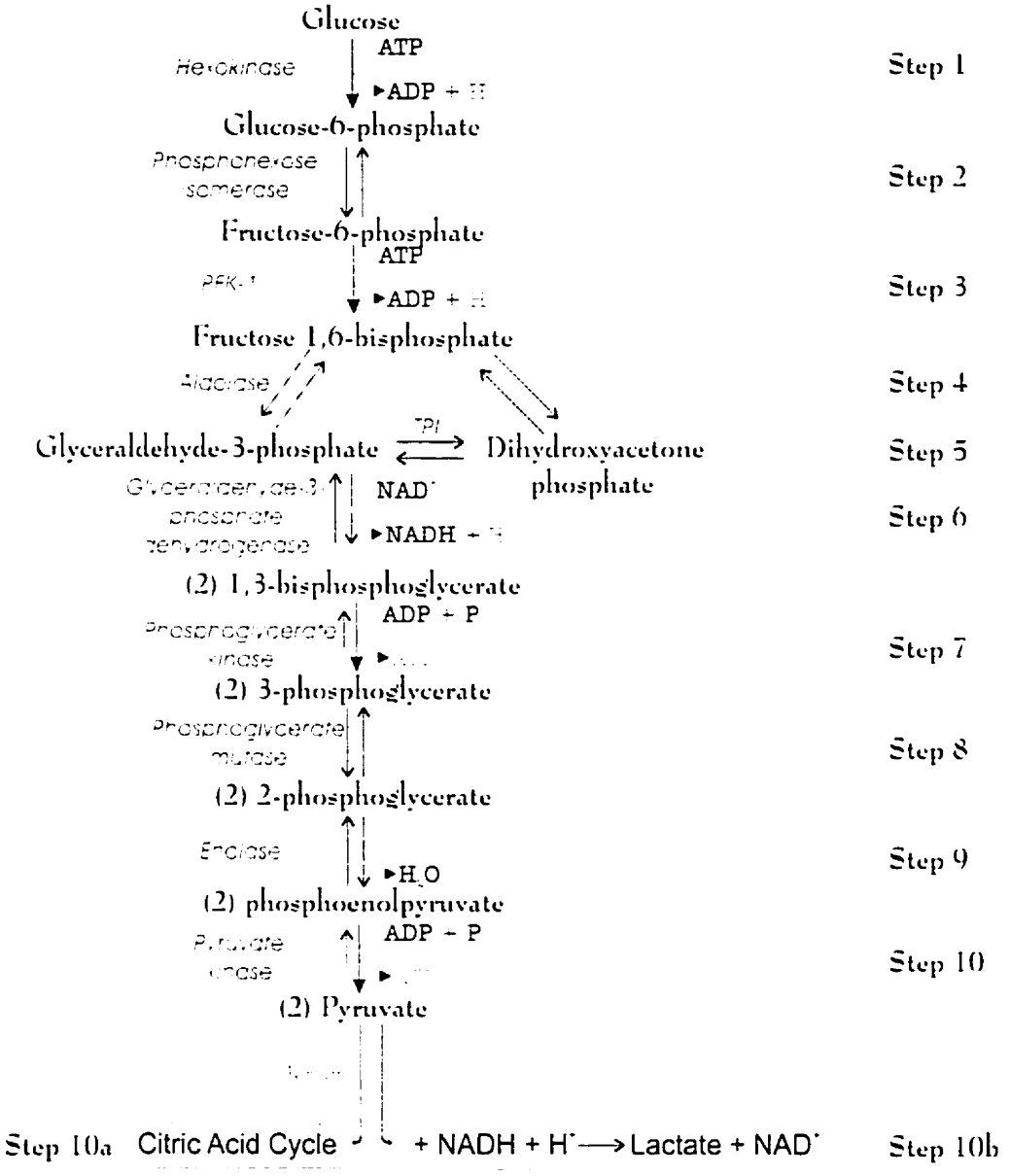

Figure 4: The Glycolytic pathway. Pyruvate, the end-product, may take one of two routes; aerobic route or anaerobic route, Step 10a and 10b, respectively. Hydrogen ions produced during ischemia are shown in red. Enzymes are shown in purple. (TPI = triose phosphate isomerase) (Modified by Lehninger *et al.*, 1993).

3. Glycogenolysis

Glycogen, a polysaccharide consisting of a polymer of glucose subunits, is an endogenous glycolytic substrate. Through a series of enzymatic reactions, glycogen may enter the glycolytic pathway as glucose-6-phosphate (Lehninger *et al.*, 1991; Ganong, 1997; Hashimoto *et al.*, 1997 and King & Opie, 1998). Glycogen phosphorylase, the enzyme controlling glycogenolysis (Figure 5A) occurs in two forms; an active form, phosphorylase *a* and a less active form, phosphorylase *b*. (Lehninger *et al.*, 1991 and Lavanchy *et al.*, 1996). The ratio of the latter two forms predicts the rate of glycogen breakdown. Phosphorylase *a* which initiates glycogen breakdown, is active once the serine (Ser) residues are phosphorylated via the hydrolysis of ATP (Figure 5A) (Lehninger *et al.*, 1991 and Depré *et al.*, 1998). Phosphorylase *a* may now convert a glucose residue from the glycogen branch, with the addition of a phosphate, to form glucose-1-phosphate (Figure 5B). Phosphoglucomutase then converts glucose-1-phosphate to glucose-6-phosphate in a reversible reaction (Figure 5B) (Lehninger *et al.*, 1991). Glucose-6-phosphate may now enter the glycolytic pathway.

The simultaneous synthesis and degradation of glycogen, known as glycogen turnover, exists under aerobic conditions. However under anaerobic conditions AMP concentrations increase and allosterically act on phosphorylase *b* to activate phosphorylase *a* (Lehninger *et al.*, 1991and Lavanchy *et al.*, 1996). Studies have shown a higher activity of the latter enzyme during no-flow ischemia compared to low-flow ischemia (Lavanchy *et al.*, 1996). Therefore during ischemia, glycogen degradation predominates over glycogen synthesis (Dennis *et al.*, 1991 and Fraser *et al.*, 1998). It is during glycogen synthesis in which one H^+ is produced and therefore glycogen turnover during ischemia is a minor source of H^+ during ischemia (Figure 6) (Gevers *et al.*, 1979 and Dennis *et al.*, 1991).

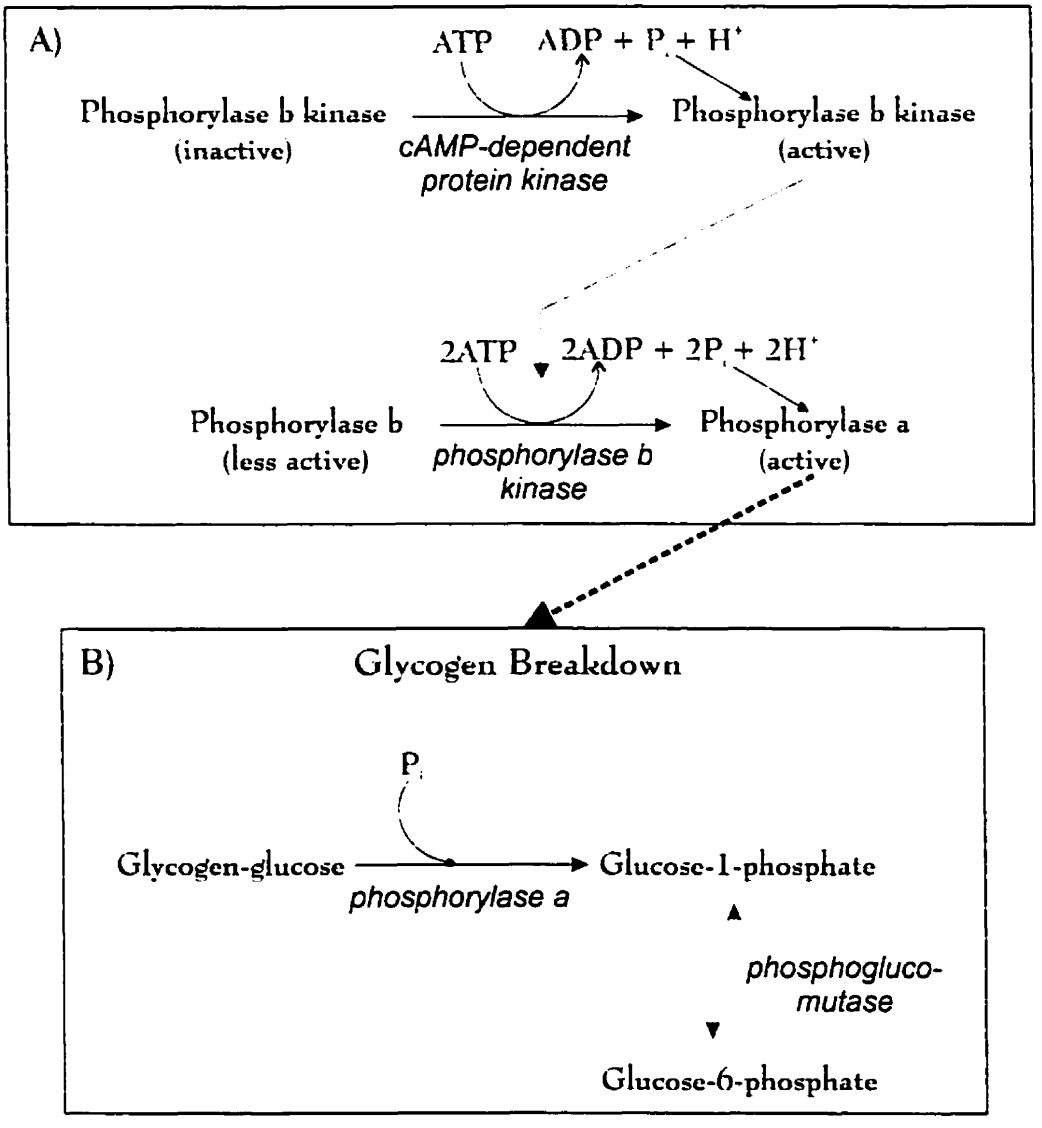

Figure 5: A) Series of enzymatic activations prior to glycogen breakdown. B)Glycogen breakdown. Glucose-6-phosphate may enter glycolysis. Modified from Lehninger *et al.*, 1991.

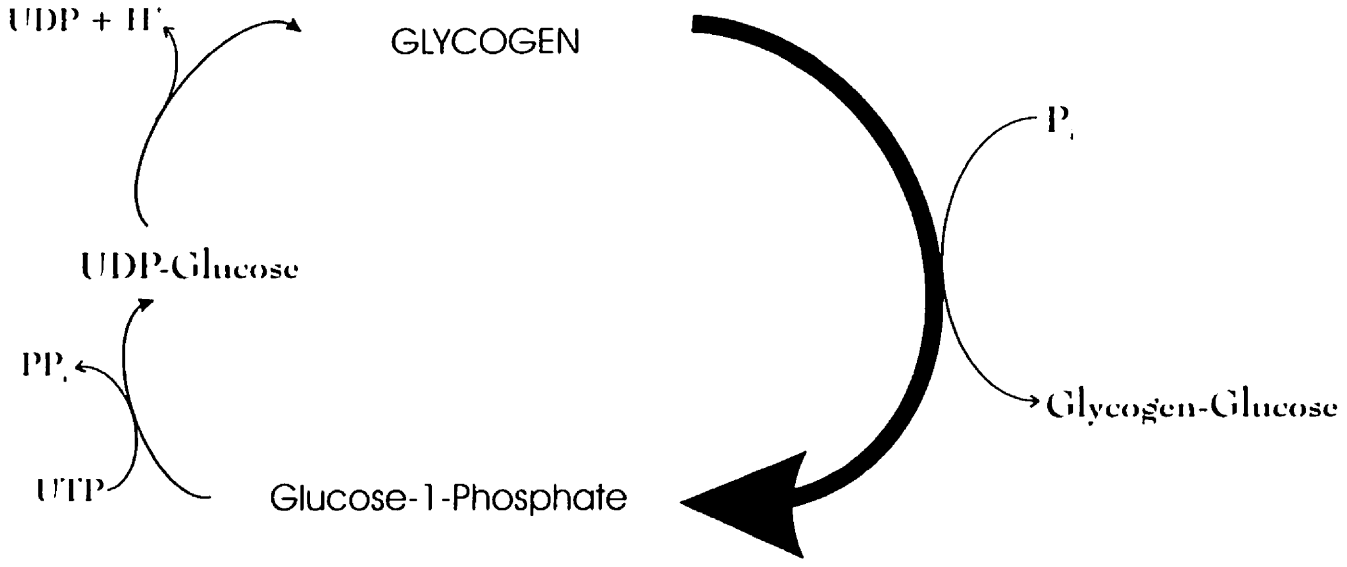

Figure 6: Glycogen breakdown predominates over glycogen synthesis during ischemia (Fraser *et al.*, 1998).

The Glycogen-Acidosis Issue

Much evidence suggests a reduced glycogen content prior to ischemia is beneficial to the integrity and function of the myocardium. A reduced glycogen content is believed to be associated with a decrease in lactate and H^+ production, hence a decrease in the detrimental effects associated with the latter by-products. On the contrary, several studies believe a high glycogen level may be beneficial to the ischemic myocardium by providing more ATP (Lavanchy *et al.*, 1996 and Depré *et al.*, 1998). However, controversy in the literature remains as to whether a high vs. a low glycogen content during ischemia is beneficial to the heart.

Using a Langendorff perfusion apparatus, Garlick et al. (1979) examined the relationship between glycogen content and acidosis during ischemia in glycogen depleted isolated rat hearts. By 14 minutes of global ischemia, the pH in glycogen depleted hearts dropped to 6.6 ± 0.08 compared to control hearts of 6.2 ± 0.06. When ATP content was measured in glycogen depleted hearts and compared to pH changes, the authors found a leveling off of pH with ATP depletion. They concluded a relationship exists between final ischemic pH and glycogen content (Garlick *et al.*, 1979). In glycogen depleted hearts exposed to global ischemia, Neely and Grotyohann (1984), found an improved recovery of function upon reperfusion which correlated with reduced lactate accumulation (Neely and Grotyohann, 1984). McNultly et al. (1996) also found significantly higher lactate levels after 20 minutes of total global ischemia in glycogen-loaded rat hearts compared to glycogen-depleted hearts. This is probably due to the higher rate of glycogen consumption observed in glycogen-loaded hearts compared to glycogen-depleted hearts within the first few minutes of ischemia (McNulty *et al.*, 1996). Therefore, according to the above studies, a depletion in myocardial glycogen content prior to ischemia enhances recovery of function due to a decrease in the accumulation of lactate and H^+.

Although the above and additional studies link a beneficial effect of glycogen-depletion prior to myocardial ischemia due to a decrease in metabolic end-products, controversy still remains. Cross et al. (1996) suggest the extent of glycogen depletion determines whether the advantage of increased glycolysis outweighs the detrimental accumulation of lactate and H^+. Therefore, Cross et al. (1996) examined the effects of high glycogen on ischemic myocardium using Langendorff-perfused rat hearts. Hearts were either depleted or loaded with glycogen prior to 12 and 32 minutes of low-flow (0.5 ml/min) ischemia. The authors found tissue lactate levels to be higher in the high-glycogen hearts. It was also observed that during brief low-flow ischemia, a high endogenous glycogen content maintained glycolysis and outweighed any detrimental effect of the increased [H^+]. However, during prolonged low-flow ischemia, glycogen was fully depleted and myocardial injury occurred due to the lower pH (6.6) in glycogen-loaded hearts (Cross *et al.*, 1996). Supporting studies indicate a sustained glycolytic metabolism may preserve cellular viability during prolonged periods of ischemia, thus potentially delaying the onset of contracture. Vanoverschelde et al. (1994) found a modest drop in ATP levels after a 60 minute low-flow ischemia in an isolated rabbit heart model in which glycogen had been increased prior to ischemia. The study also found an inverse relationship between ischemic contracture and tissue ATP levels, that is, hearts with increased glycogen reduced the time to ischemic contracture. Thus, the authors conclude a positive effect of the maintenance of high rates of glycolysis during ischemia.

Several issues may explain the differences among studies. For example, in experiments where glycogen was found to be beneficial, perhaps the glycogen was not fully depleted at end ischemia. Furthermore, studies that employed an ischemic flow rate may actually have alleviated the detrimental effects of a high glycogen content by removal of by-products, even if glycogen

were fully depleted at end ischemia (Cross *et al.*, 1996 and King & Opie, 1998). In addition to the latter, studies have shown the rate of glycogenolysis is higher under no-flow ischemia vs. low-flow ischemia, suggestive of an enhanced breakdown of glycogen under no-flow ischemia. (Vanoverschelde *et al.*, 1994 and Lavanchy *et al.*, 1996). Another contribution to the controversy may be species-specific differences in the ability to utilize glycogen. For example, under identical protocols, ischemic glycogen depletion is less in rabbits compared to rats (Cross *et al.*, 1996). Thus, a number of key factors should be taken into consideration when analyzing the effect of glycogen loading, such as; the experimental protocol, species, source of glycolytic substrate and residual flow (King & Opie, 1998).

In summary, during ischemia, three protons are produced in the degradation of ATP to adenosine. During anaerobic glycolysis, each molecule of glucose produces two H^+, due to ATP turnover and 2 lactate molecules. Thus ATP degradation and anaerobic glycolysis are major sources of H^+ during ischemia. Glycogen synthesis produces one H^+ and during ischemia glycogen degradation predominates over synthesis Therefore glycogen turnover acts as a minor source of H^+ during ischemia. The cell attempts to prevent the development of intracellular acidosis by a variety of mechanisms. The mechanisms involved in maintaining a physiological pH range under normal and ischemic conditions will be discussed next.

PART 2: pH

A. *What is pH and Why is it important?*

The term pH refers to the hydrogen ion concentration [H^+] of a solution. It is defined by the expression pH = -log[H^+]. The pH scale relates a hydrogen ion concentration to a numeric value. It also designates the H^+ and OH^- (hydroxide ion) concentrations. For example, when there are exactly equal concentrations of H^+ and OH^-, pH = 7.0 and the solution is said to be neutral. Whereas solutions that have a pH greater than 7.0 are alkaline or basic and the concentration of OH^- is greater than that of H^+. Conversely, a pH less than 7.0 is acidic and the concentration of H^+ is greater than OH^-. Furthermore, because the scale is a base-10 logarithmic scale, a one-unit change in pH corresponds to a ten-fold change in [H^+] (Lehninger *et al.*, 1993; Ganong, 1997 and Abelow, 1998).

Intracellular (ICF) and extracellular (ECF) pH are maintained at constant levels. In a healthy individual the pH of the ECF is between 7.35 - 7.45 and the ICF is between 6.9 -7.0 (Ganong, 1997). A variety of intracellular processes are pH sensitive and thus maintaining an optimal cellular pH is vital for the cells. For example, the activity of phosphofructokinase (PFK), the rate limiting enzyme of glycolysis, increases with a small increase in pH within the physiological range. The rate of protein synthesis increases from a pH of 6.9 to an optimum at pH 7.4 (Madshus, 1988; Ganong, 1997). Furthermore, muscle contractility in the heart is dramatically reduced upon acidification (Jacobus *et al.*, 1982; Nakanishi *et al.*, 1990; Vanoverschelde *et al.*, 1994; Vanderberg *et al.*, 1994; Palmer & Kentish, 1996; Komukai *et al.*, 1998). Other cellular processes or events that are pH sensitive include, conductivity of ion channels and protein interaction (Madshus, 1988; Lowenstein, 1993; Ganong, 1997 and Abelow,

1998). Therefore, changes in the intracellular pH may be associated with an impairment in cellular processes, leading to disruption in the function and metabolism of the cell, affecting cellular homeostasis and ultimately cell survival (Madshus, 1988; Halperin, 1993 and Ganong, 1997). Thus, due to the vital importance of a normal intracellular pH, the hydrogen ion concentration is constantly under strict regulation via several mechanisms (Lowenstein, 1993; Ganong, 1997 and Abelow, 1998) as detailed below.

B. *pH Regulation*

The regulation of pH is governed by **buffers** and **transmembrane transporters** (Halperin, 1993; Lowenstein, 1993; Grace *et al.*, 1993 and Abelow, 1998).

Buffers:

1. Concepts

All buffers consist of 1) a weak acid (e.g. HA) and 2) the conjugate base of that acid (A^-). Thus when a weak acid is dissolved in water, it dissociates poorly into free protons (H^+) and anions (A^-). At dynamic equilibrium, the reaction rates are equal and can be viewed as:

$$HA \leftrightarrow H^+ + A^- \qquad (2.A.1)$$

Therefore a buffer acts in the following way, when the $[H^+]$ rises, dissolved A^- combines with free protons via the reaction:

$$HA \leftarrow A^- + H^+ \qquad (2.A.2)$$

thereby minimizing the rise in $[H^+]$. Conversely, HA liberates free protons when the $[H^+]$ falls via the reaction:

$$HA \rightleftharpoons A^- + H^+ \qquad (2.A.3)$$

Hence a buffer minimizes or resists changes in pH (Halperin, 1993; Lowenstein, 1993; Ganong, 1997 and Abelow, 1998).

Applying the law of mass action to the equation of a buffer system (1.A.1), derives:

$$\frac{[H^+][A^-]}{[HA]} = K \qquad (2.A.4)$$

where K = dissociation constant. When the above equation is solved for H^+ and put into pH notation, the resulting equation is known as the Henderson-Hasselbach equation:

$$pH = pK + \log[A^-]/[HA] \qquad (2.A.5)$$

According to the above equation, the pK of an acid is the pH at which the acid is half dissociated, i.e.: $[HA] = [A^-]$, or $[A^-]/[HA] = 1$ and therefore pK = pH. Therefore, a buffer is at maximal activity when the pK is close to the pH in which it is operating. Depending on an acid's tendency to dissociate, different acids have different pKs. The higher the pK, the weaker the acid and the lower the pK, the stronger the acid (Lowenstein, 1993; Lehninger, 1993; Ganong,1997 and Abelow, 1998).

The ability of a cell to resist changes in pH is referred to as the Buffering Capacity (BC) (Ganong, 1997 and Abelow, 1998). Experimentally, buffering capacity is defined as the number of moles of a strong acid required to decrease the pH by 1 pH unit in 1 L of solution (de Albuquerque *et al.*, 1995). Two factors determine buffering capacity, 1) the concentration of

buffer and 2) the pK of the buffer. Therefore, a cell with a high buffering capacity requires a substantial amount of strong acid or base to alter the pH by one unit. Likewise, a cell with a low BC, requires a small amount of strong acid or base to alter the pH one unit. An effective buffer is one whose pK is close to the pH of the solution. For example, a weak acid with a pK of 5.0 will act as an effective buffer in a solution with a pH of 5.3, and ineffective in a solution with a pH of 9.0. Hence, the higher the buffer concentration and the closer pH to pK, the greater the BC (Lowenstein, 1993; Halperin, 1993; Ganong, 1997 and Abelow, 1998).

2. *Buffer Systems*

Buffers systems in our body, either the ICF or ECF, include, bicarbonate, hemoglobin, phosphates and proteins (Lowenstein, 1993; Halperin, 1993; Ganong, 1997 and Abelow, 1998). Each is discussed below.

Bicarbonate Buffer System

The most powerful and important buffer of the ECF is the bicarbonate buffer system:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \qquad (2.A.6)$$

where: CO_2 = carbon dioxide (the weak acid), H_2O = water, H_2CO_3 = carbonic acid and HCO_3^- = bicarbonate (conjugate base). Therefore a rise in $[H^+]$ will drive the equation to the left, thus consuming protons and liberating $CO_2 + H_2O$. Likewise, a fall in $[H^+]$ will pull the equilibrium to the right, releasing protons and bicarbonate and consuming CO_2 and H_2O (Lowenstein, 1993; Halperin, 1993; Ganong, 1997 and Abelow, 1998). The reaction rate of:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \qquad (2.A.7)$$

is greatly increased in the presence of an enzyme known as carbonic anhydrase, found in red

blood cells (Lowenstein, 1993 and Ganong, 1997).

The bicarbonate concentration in the ECF is approximately 24 mmol/L. In the ICF the concentration is very low, approximately 8 mmol/L. The pK of the bicarbonate buffer system is 6.1 and recall the pH of the ECF is approximately 7.4 (Lowenstein, 1993 and Abelow, 1998). According to the concept of pK and BC, the bicarbonate buffer system is not an effective buffer. However, a combination of factors explain it's effectiveness. First, the total concentration of $[CO_2] + [HCO_3^-]$ is high, approximately 26 mmol/L. Second, alveolar ventilation constantly maintains the concentration of CO_2 in the arterial blood at 1.2 mmol/L. Thus when the $[CO_2]$ increases, alveolar ventilation increases thus releasing CO_2 and vice-versa. Therefore, this "open system" enhances the efficiency of the bicarbonate buffer system tremendously. Finally, HCO_3^- lost during the buffering process is replaced by the kidneys, thus assisting in maintaining a normal pH (Lowenstein, 1993; Halperin, 1993; Ganong, 1997 and Abelow, 1998).

Therefore, the bicarbonate buffer system is the most powerful and dynamic buffer system in the ECF, explained by its high concentration and physiological regulation of $_pCO_2$ and $[HCO_3^-]$ by the lungs and kidneys, respectively.

Hemoglobin

Hemoglobin, a protein found inside red blood cells, is the major buffer in the ECF. The effectiveness of this buffer is due to a residue on the protein known as histidine. Histidine contains an imidazole side group whose pK varies depending on its position in the protein chain. The average pK is approximately is 6.5, hence an excellent ICF and ECF buffer (Abelow, 1998).

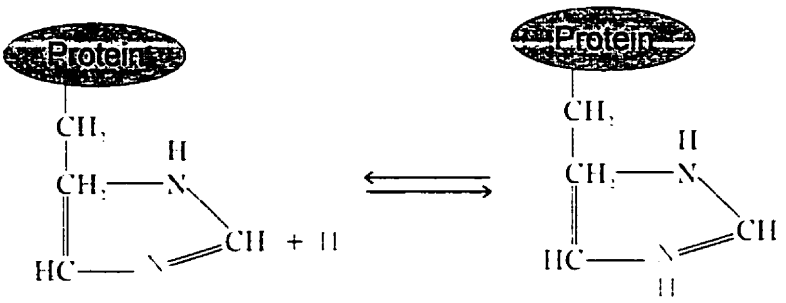

Figure 7: The imidazole group of histidine-rich proteins mitigates changes in pH via protonating a nitrogen (N). Modified from Lehninger *et al.*, 1993.

Thus when [H^+] rises, the equilibrium shifts to the right, binding H^+. Likewise, when [H^+] falls, the equilibrium shifts to the left, (Ganong, 1997 and Abelow, 1998). A hemoglobin molecule contains 38 histidine residues, and in combination with a high concentration in the blood, approximately 15 g/dL, it is an efficient ECF buffer (Ganong, 1997 and Abelow, 1998). Myoglobin, which acts as a oxygen reservoir for the heart, also contains histidine and in combination with its high concentration, .2mM, contributes greatly to intracellular buffering (Ganong, 1997 and Shimouchi, 1985).

Phosphates

In order for phosphates to act as buffers they must exist in their free inorganic form (Pi). Unlike the bicarbonate buffer system, once a phosphate buffers H^+, it is retained. The pK of the dissociation:

$$H_2PO_4^- \leftrightarrow H^+ + HPO_4^{2-} \qquad (2.A.8)$$

is 6.86 and exerts a maximal buffering in the pH range of 6.4 - 7.6 (Lowenstein, 1993; Do *et al.*, 1995; Ganong, 1997 and Abelow, 1998). Therefore, $H_2PO_4^-$ acts as the weak acid and HPO_4^{2-} acts as the conjugate base. Phosphates bound in organic compounds such as ATP and CrP act as

buffers as well (Wolfe *et al.*, 1988; Do *et al.*, 1995 and Abelow, 1998). In the conversion of CrP to creatine and ATP, a process catalyzed by the enzyme creatine kinase, creatine consumes a H^+ in the process (Equation 2.A.9). During the early period of ischemia, the latter process is associated with short, transient intracellular alkalinization (Portman & Eyster, 1994). The concentration of phosphates in the ECF is too low, approximately 1 mmol/L, to act as an important buffer. However, inorganic phosphates exist in high concentrations in the ICF, hence play a vital role in intracellular buffering (Lowenstein, 1997 and Abelow, 1998).

$$CrP + ADP + H^+ \leftrightarrow Creatine + ATP \qquad (2.A.9)$$

Proteins

As discussed, proteins with histidine side groups are excellent buffers. As in hemoglobin, the pK of histidine is 6.5 - 7.0. A protein consists of hundreds of linked amino acids including a dozen or more histidines. Therefore a single protein may have many buffer sites, in which each histidine site acts independently (Figure 7) (Lowenstein, 1997 and Abelow, 1998).

Proteins contribute little to buffering in the ECF because of the their low concentraiton <10mmol (7 g/dl), but are the most effective intracellular buffers due to their high concentration in the ICF, 400 nmol (20 g/ dl) (Halperin, 1993 and Lowenstein, 1997). As with phosphates, once a protein buffers a H^+, it is retained (Halperin, 1993 and Lowenstein, 1997).

3. *The Buffers Systems in the ECF and ICF*

As discussed, there is considerable difference in the composition of buffers in the ECF

and ICF. The ECF is divided into blood and interstitial fluid. The major buffers in the blood are: 1. Hemoglobin 2. Bicarbonate and 3. Proteins. The major buffer in the interstitial fluid is bicarbonate. In the ICF, the major buffers are phosphates (e.g. from the breakdown of ATP and CrP) and proteins rich in histidine (Lowenstein, 1993; Ganong, 1997 and Abelow, 1998).

Cellular buffers minimize changes in $[H^+]$ by binding H^+. However the maintenance of a physiological pH_i requires the removal of the buffered H^+, thus re-establishing cellular buffers. This is accomplished by transmembrane transporters (Lowenstein, 1993).

Transmembrane Transporters:

In addition to intracellular buffers, pH_i is governed by the balance of several sarcolemmal transporters (Lagadic-Gossmann *et al.*, 1996; Pucéat *et al.*, 1998; Meiltz *et al.*, 1998 and Leem *et al.*, 1999). Each transporter displays a unique function and role in regulating pH_i (Ng *et al.*, 1993; Lowenstein, 1993 and Leem *et al.*, 1999).

Alkalosis

Chloride (Cl^-) - Bicarbonate (HCO_3^-) Antiport

Under physiological conditions, the Cl^-/HCO_3^- exchanger extrudes intracellular HCO_3^- in exchange for extracellular Cl^- (Madshus, 1988) (Figure 8). This transporter is activated further during cytosolic alkalinization (Lazdunski *et al.*, 1985 and Madshus, 1988). It is modestly activated up to a pH of 7.15, after which the activity increases steeply with pH_i (Leem *et al.*, 1999). This steep pH sensitivity indicates that it is the major system for pH_i regulation from an alkali overload (Leem *et al.*, 1999). However, the activity of the Cl^-/HCO_3^- antiport decreases to approximately zero at pH of 6.70 (Leem *et al.*, 1999).

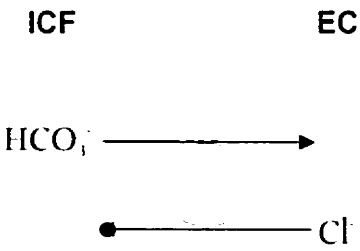

Figure 8: The Cl^- – HCO_3^- Antiport (CBA). Modified from Madshus, 1988.

Chloride (Cl^-) – Hydroxide (OH^-) Exchanger

This transporter is Cl^- dependent and displays a modest, near-linear increase in activity over the pH range of 7.05 - 7.50 (Leem *et al.*, 1999). Hydrogen ions are transported into the cell with Cl^- in exchange for an intracellular hydroxide ion (Vaughan- Jones, 1988) (Figure 9). The activity of the Cl^--OH exchanger also decreases to approximately zero at pH 6.70 (Leem *et al.*, 1999).

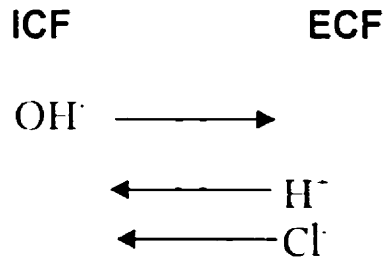

Figure 9: The Cl^--OH^- Exchanger (CHE). Modified from Madshus, 1988.

Acidosis

Sodium (Na^+) - Hydrogen (H^+) Exchanger (NHE)

Another type of hydrogen ion transport protein found in the plasma membrane is the Na^+/H^+ exchanger. It is the major pathway for pH_i regulation during cytosolic acidification (pH_i

6.6), however not the major mechanism under normal physiological conditions (Roos & Boron, 1981; Frelin *et al.*, 1985 and Lazdunski *et al.*, 1985). The Na^+/H^+ exchanger, as stated, transports one H^+ in exchange for one Na^+, thus it is an electroneutral process (Figure 10). The transport of H^+ utilizes the energy gradient established by the Na^+-K^+ ATPase (Lowenstein, 1993; Hirst, 1994 and Chow, 1999). This exchanger is active at resting pH_i and activated further as pH_i decreases. The greatest activation occurs once the pH_i falls below 6.90 and the activity is said to increase 30-fold up to pH_i of 6.55 (Leem *et al.*, 1999). However, during ischemia the Na^+/H^+ exchanger becomes inactive due to a combination of a build up of extracellular $[H^+]$ and a possible metabolic inhibition due to intracellular acidosis (Park *et al.*, 1999).

Six mammalian Na^+/H^+ exchanger isoforms have been identified to date. Isoform 1, also known as NHE1 is ubiquitously expressed and considered to be the "housekeeping" isoform due to its primary responsibility of pH_i homeostasis (Tse *et al.*, 1994 and Chow, 1999). The site of regulation, which determines the activity of the exchanger, is a cytoplasmic carboxy-terminal sequence. An increase in the intracellular H^+ concentration accelerates the exchanger's activity, and vice-versa (Lowenstein, 1993; Bianchini & Pouysségur, 1994 Leem *et al.*, 1999 and Chow, 1999). The extracellular portion of the exchanger is relatively insensitive to changes in H^+ concentration. It is hypothesized that two separate and independent proton-binding sites are located on the cytoplasmic side. One site is thought to act as the modulator, while the other acts as the H^+ transport site (Madshus, 1988; Bianchini & Pouysségur, 1994 and Tse *et al.*, 1994).

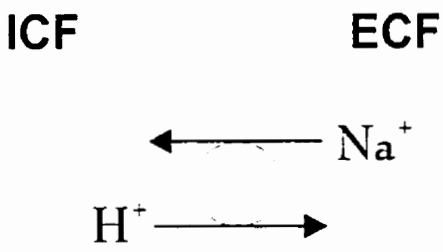

Figure 10: The Na^+-H^+ Exchanger. Modified from Madshus, 1988.

Na^+-dependent -Chloride (Cl^-) - Bicarbonate (HCO_3^-) Exchanger

This transporter is more active at resting pH_i than is the Na^+/H^+ exchanger and is further stimulated upon acidification. The Na^+-dependent Cl^-- HCO_3^- exchanger exchanges one Na^+ and one HCO_3^- for one Cl^- and one H^+ (Liu *et al.*, 1990; Grace *et al.*, 1993 and Ng *et al.*, 1993) (Figure 11). Furthermore, this system is driven by the sodium gradient (Madshus, 1988; Lowenstein, 1993).

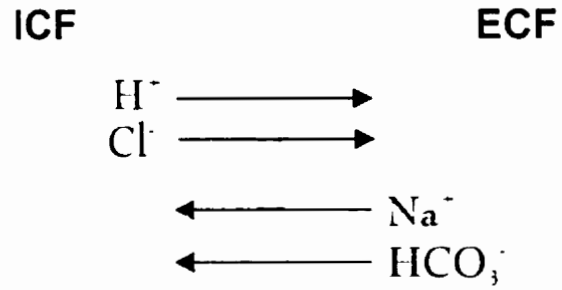

Figure 11: The Na^+-dependent Cl^-- HCO_3^- Exchanger. Modified from Madshus, 1988.

Na^+- Bicarbonate Cotransporter (NBC)

In contrast to the Na^+/H^+ exchanger, the sodium bicarbonate cotransporter is activated roughly linearly with decreasing pH_i and contributes one-third of the recovery from intracellular acidosis whereas the Na^+/H^+ exchanger contributes two-thirds (Grace *et al.*, 1993; Ng *et al.*, 1993

and Leem *et al.*, 1999). This cotransporter is involved with the influx of 2 HCO_3^- ions per Na^+ ion into the cytosol (Figure 12) (Aiello *et al.*, 1998). The NBC family includes pNBC1, kNBC1, NBC2 and mNBC3, which the latter is uniquely expressed in skeletal and heart muscle (Pushkin *et al.*, 1999).

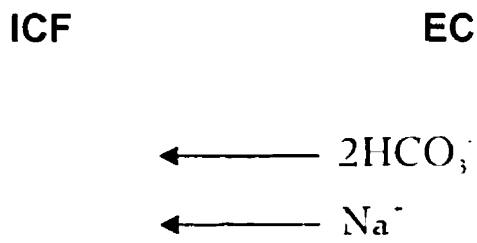

Figure 12: The Na^+- HCO_3^- Cotransporter. Modified from Madshus, 1988.

During the steady state of pH_i, that is 6.95 - 7.25, the above transporters are activated to the same extent. Thus pH_i control is achieved by the balance among the above transporters (Vaughan-Jones, 1988; Grace *et al.*, 1993 and Leem *et al.*, 1999) (Figure 14, pg.32). In addition to the above mentioned transporters, other transporters exist and also regulate pH_i to a certain extent. For example, during periods of high glycolytic flux, a fifth carrier known as the H^+-Lactate cotransporter is recruited (Vaughan-Jones, 1988).

H^+-Lactate Cotransporter

During myocardial ischemia the rate of glycolysis increases enormously as does the production of lactic acid as a consequence (Halestrap *et al.*, 1997). The pK of lactic acid is 3.8 and at physiological pH, lactic acid is dissociated almost entirely to the lactate anion and H^+ (Halestrap & Price, 1999 and Bonen, 2000). The increase in the latter two ions stimulates efflux

of lactic acid through a proton-linked monocarboxylate transporter (MTC). The latter transporter catalyzes facilitated diffusion of lactate with a proton in an electroneutral manner using energy provided from the concentration gradients of lactate and protons (Figure 13) (Halestrap & Price, 1999 and Bonen, 2000). A family of seven MCT transporters have been identified, MCT1 - MCT7 (Bonen, 2000). MCT1 is expressed in human and rat heart and is abundantly expressed. Many MCT isoforms have been found to exist in the same tissue, as well, several isoforms are species specific (Bonen, 2000). It has recently been shown that the proton-lactate cotransporter is the major route for the extrusion of H^+, more so than the Na^+/H^+ exchanger (Bonen, 2000). However during ischemia, the lactic acid which is removed is unable to be carried away due to the lack of blood flow. As a result, lactic acid accumulates within the cell resulting in acidosis (Halestrap *et al.*, 1997).

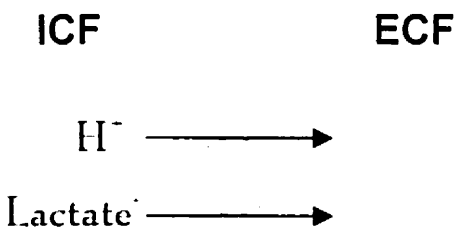

Figure 13: The H^+-Lactate Cotransporter. Modified from Madshus, 1988.

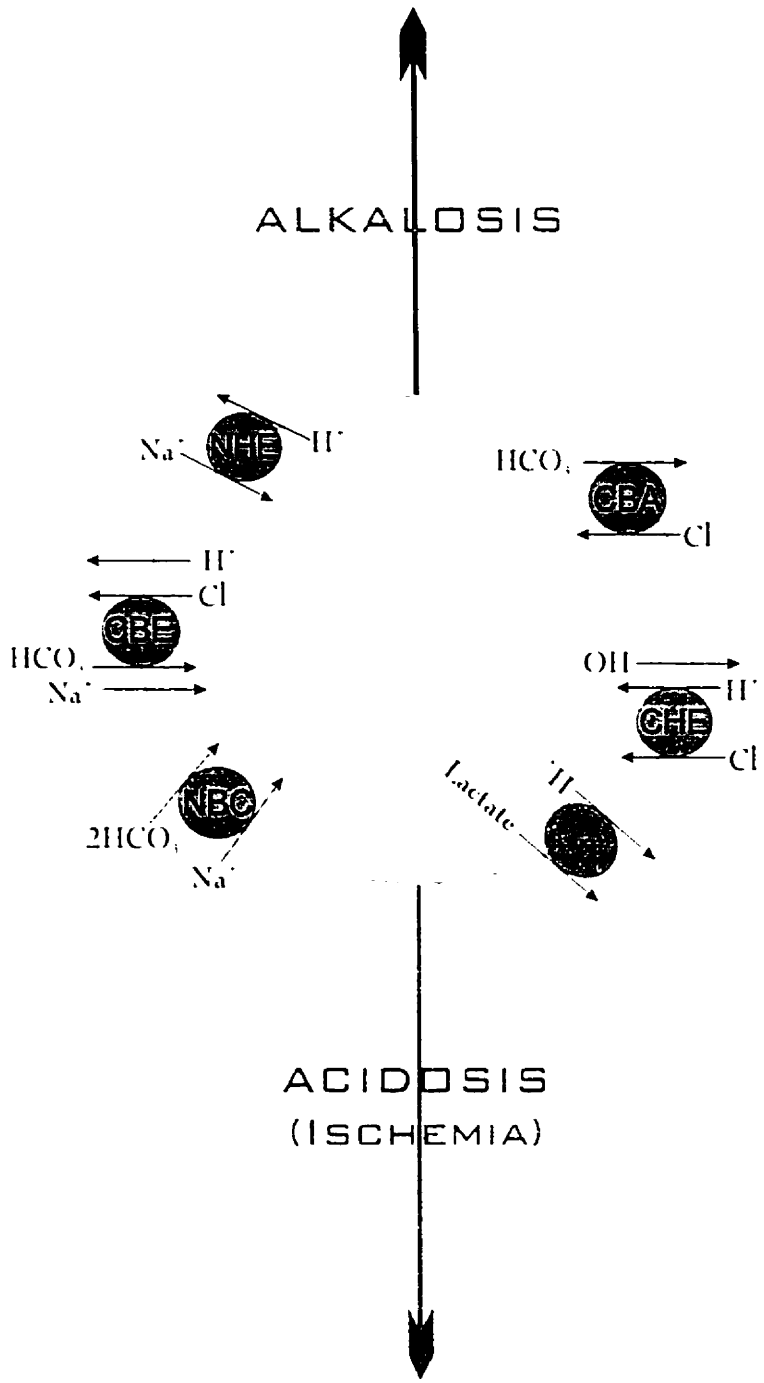

Figure 14: Intracellular pH (pH$_i$) regulatory mechanisms under resting pH$_i$, alkalosis and ischemic acidosis. NHE = Na^+/H^+ exchanger; CBE = Na^+ dependent Cl^-/HCO_3^- exchanger; NBC = Na^+/HCO_3^- cotransporter; CHE = Cl^-/OH^- exchanger; CBA = Cl^-/HCO_3^- antiporter.

In summary, a number of intracellular process are sensitive to pH changes, thus maintaining an optimal cellular pH, which is vital to cellular life. The regulation of pH is a dynamic and intricate process governed by many different specialized systems. The combination of intracellular buffers and transmembrane transporters regulates intracellular pH. Transmembrane transporter activities may increase dramatically or linearly upon acidification or alkalinization, depending on the type of transporter. However, during ischemia when blood flow is impaired transmembrane transporters are no longer effective due to the accumulation of H^+ in the ECF and thus the lack of a H^+ gradient. As a result, intracellular buffers such as phosphates and histidine-rich proteins play a major role in buffering or resisting acidosis. Extracellular pH is stabilized by (in descending order of importance), hemoglobin, bicarbonate, plasma proteins and phosphate.

Part 3: Ovarian Cycling

Due to rapidly evolving studies involving estrogen and the heart, studies are only recently taking into consideration the phase of the menstrual cycle in women when assessing myocardial risks. For example, Methot et al. (2000) found significantly more women had an acute MI within five days after the onset of menstruation, which is the early follicular phase. Lloyd et al. (2000) found myocardial ischemia was easily induced during the early follicular phase in women with established coronary artery disease. Experimentally, Smith et al (2000) suggest at the time of an MI or early post-MI, high estrogen levels can be detrimental, resulting in increased infarct size. Therefore, recent data suggest an ovarian cycling effect with respect to a myocardial infarction, which may be an effect imposed by estrogen. The role of progesterone has not been explored, but may play a role.

This part of the introduction will therefore focus on the main female hormones estrogen and progesterone.

A. Synthesis of...

Cholesterol is the initial precursor for steroid biosynthesis. At the ovary, cholesterol, which is associated with low-density lipoproteins (LDL) in the bloodstream, binds to specific receptors in the plasma membrane. Once the LDL-receptor complex is internalized, it is hydrolyzed within lysosomes and cholesterol is released (Ganong, 1997 and Griffin & Ojeda, 2000).

Estrogen and Progesterone

Cholesterol is transported into the mitochondria of the Theca interna cells of the ovarian

follicle. In order for steroid biosynthesis to occur, a protein known as steroidogenic acute regulatory protein (StAR) promotes the transfer of cholesterol into the inner mitochondrial membrane. An enzyme known as cholesterol-side-chain cleaving enzyme, converts cholesterol to pregnenolone (Figure 15). By the action of 3β-hydroxysteroid dehydrogenase enzyme, pregnenolone is converted to progesterone (Figure 15). Pregnenolone may also be converted to 17α-hydroxypregnenolone via 17α-hydroxylase. The latter hormone and progesterone may be converted to 17α-hydroxyprogesterone (Figure 15). Androstenediones are derived from 17α-hydroxyprogesterone via 17,20-lyase. Androstenediones are preferentially converted into testosterone via 17β-hydroxysteroid dehydrogenase or to estrone via the enzyme aromatase. Both testosterone and estrone may be converted to form estradiol via aromatase and 17β-hydroxysteroid dehydrogenase, respectively (Figure 15). The metabolic conversions discussed above, starting from pregnenolone, occur in the endoplasmic reticulum (Ganong, 1997 and Griffin & Ojeda, 2000).

The Granulosa cells are supplied with androstenedione from the Theca cells and are the major site of estradiol production (Figure 16). Luteinizing hormone (LH) released from the anterior pituitary, acts via cyclic AMP on the Theca interna cells of the ovarian follicle to increase conversion of cholesterol to androstenedione. Follicle stimulating hormone (FSH) is also released from the anterior pituitary and stimulates the release of estradiol via increasing aromatase activity (Figure 16). LH may also increase estradiol production in mature granulosa cells (Ganong, 1997 and Griffin & Ojeda, 2000).

Thus, estradiol and progesterone are the main steroids produced by the ovaries. Estrone is another estrogen secreted by the ovary and is further metabolized to estriol, a reaction which occurs predominantly in the liver. Between the three estrogens, 17β-estradiol, is the most potent

and estriol, is least potent. The androgens, testosterone and androstenendione are also produced and secreted into the bloodstream by the ovaries, however a significant portion is converted to estradiol. In addition to other androgens present in women, testosterone is the primary physiologically active androgen with a plasma level of 1.0 nmol/L (Ganong, 1997 and Griffin & Ojeda, 2000). Estradiol production rate in men is approximately .05 mg/d with an estradiol plasma level of 70 pmol/L (2 ng/dL) (Ganong, 1997 and Griffin & Ojeda, 2000). The adrenal cortex also secretes steroid hormones. The major steroid hormones secreted are dehydroepiandrosterone (DHEA), androstenedione and to a small degree estrogen (Ganong, 1997).

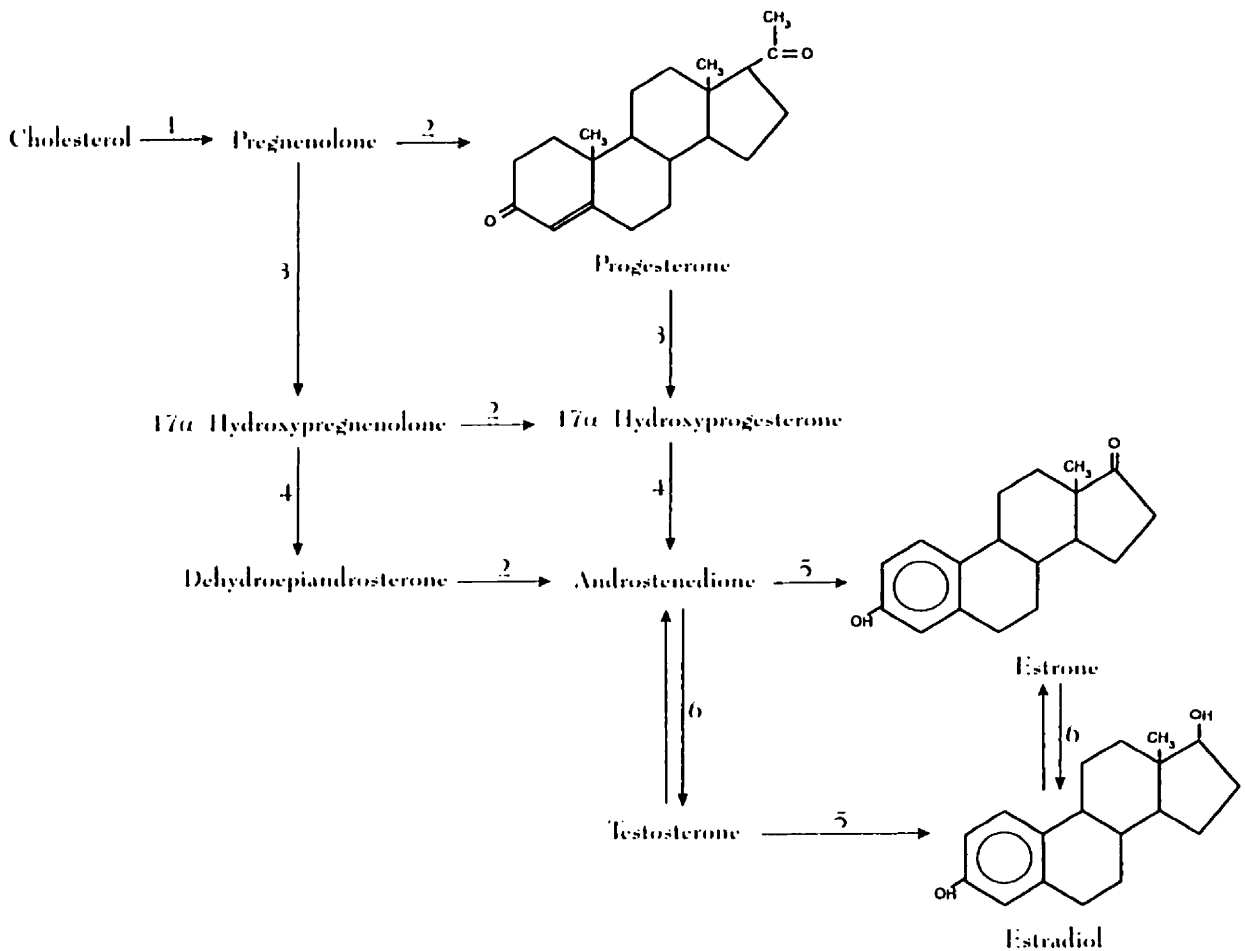

Figure 15: Biosynthesis of estradiol and progesterone in the ovary. Enzymes; 1: Cholesterol side-chain cleavage enzyme complex; 2: 3β- hydroxysteroid dehydrogenase; 3:17α hydroxylase; 4:17,20-lyase; 5: aromatas; 6: 17β hydroxysteroid dehydrogenase. Modified from Guthu & Ojeda, 2000.

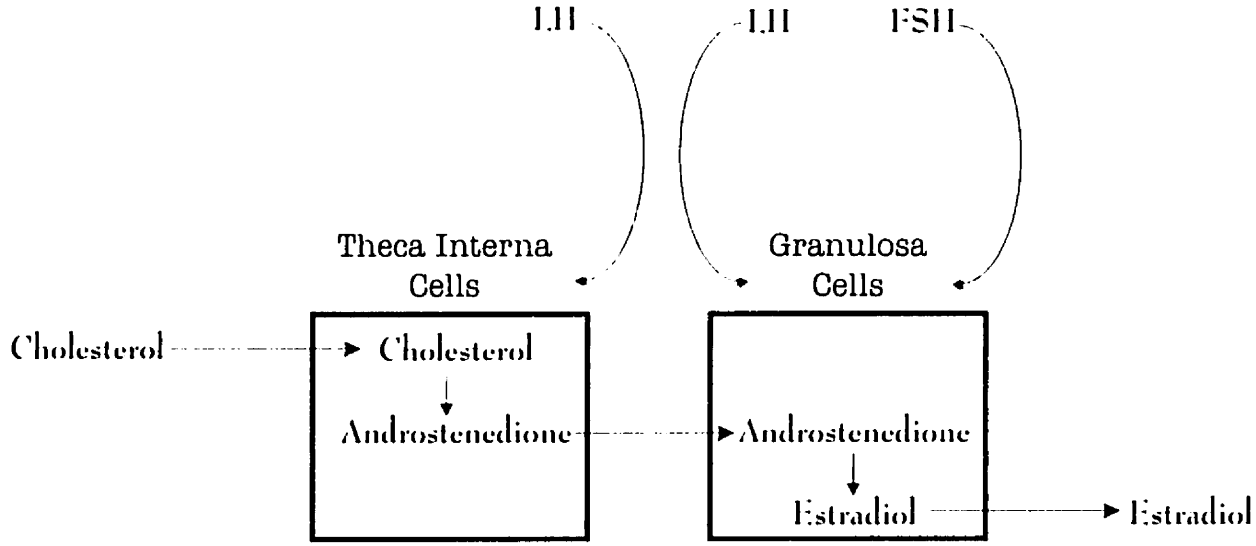

Figure 16: Estradiol synthesis and secretion in the Theca and Granulosa cells in a mature follicle. Modified from Ganong, 1997.

B. Circulation and Metabolism of Estrogen and Progesterone

Steroids circulate in the bloodstream bound to transport proteins. Binding influences the circulation clearance rate. That is, the greater the binding capacity, the slower the clearance rate of the hormone (Griffin & Ojeda, 2000). Therefore hormones which circulate freely or are weakly bound, have a faster disappearance rate from plasma than do bound hormones (Griffin & Ojeda, 2000).

Only 2% of circulating estrogens are free or unbound and >70% are bound. Of those which are bound, approximately 60% are bound to albumin and 38% to sex hormone binding globulin (SHBG). The majority of progesterone also circulates bound to transport proteins, of which 80% is albumin and 18% corticosteroid-binding globulin (CBG), leaving 2% unbound.

In the liver, estrogens are oxidized to glucuronide and sulfate conjugates. Large amounts of the latter metabolites are excreted in the bile and reabsorbed into the bloodstream via the enterohepatic circulation. The remainder are excreted in the urine (Ganong, 1997 and Griffin & Ojeda, 2000). Also in the liver, progesterone is converted into pregnanediol, followed by conjugation to glucuronic acid and finally excreted in the urine (Ganong, 1997 and Griffin & Ojeda, 2000).

C. Mechanism of Steroid Action

Estrogen and Progesterone

Estrogen may enter cells either by simple diffusion or facilitated diffusion. Once inside the cell, estrogen binds to a receptor protein, known as the estrogen receptor (ER) (Figure 17, Step 1). The ER is inactive and complexed with heat shock protein 90 (hsp90) and may be located in the cytoplasm or nucleus. Once estrogen is bound to the ER, the receptor undergoes a

conformational change and forms a dimer. This conformational change allows the dimer to bind to DNA response elements with high-affinity (Figure 17, Step 2). As a result new mRNAs are formed (Figure 17, Step 3), thus eventually new proteins which ultimately modify cell functions (Ganong, 1997 and Kuiper *et al.*, 1998). In the heart, two types of ER exist; ERα and ERβ, each with their own unique function. The latter two ER may form homodimers with themselves (αα or ββ) and/or heterodimers with each other (αβ). The latter possibilities allow for a diverse estrogen signaling pathway, thus expanding the physiological potential of estrogen (Kuiper *et al.*, 1998). Progesterone follows a similar mechanism of action as estrogen (Ganong, 1997).

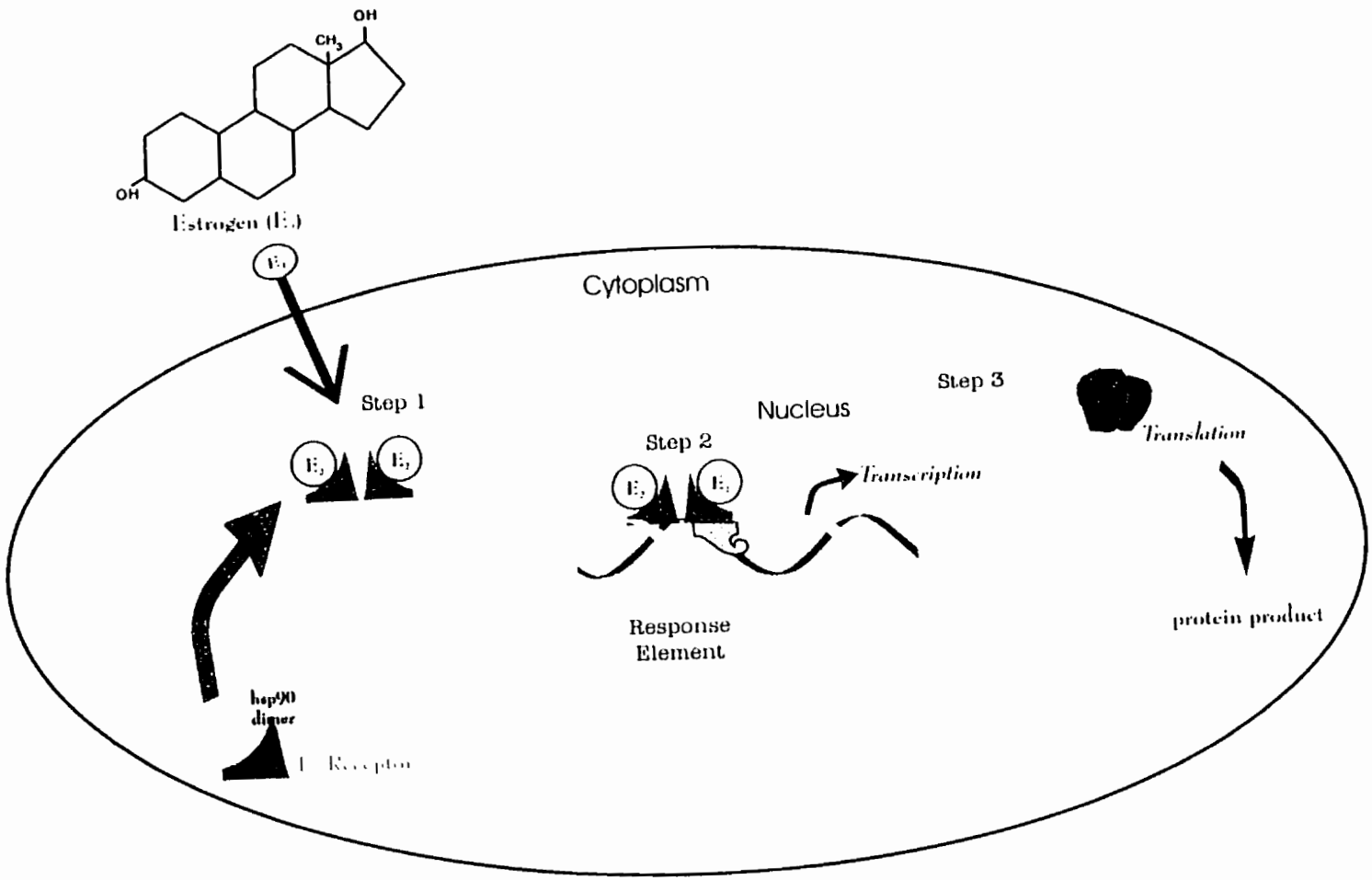

Figure 17: Mechansim of steroid (estrogen, E_2) action. Modified from Kuiper *et al.*, 1998.

D. The Menstrual Cycle and The Estrous Cycle

The Menstrual Cycle

The reproductive system of the adult human female is cyclic and ranges from 25 - 35 days (Figure 18). It is divided into four phases: menstruation (periodic bleeding) a conspicuous feature in humans which lasts 4-5 days; follicular phase (10 -16 days); an ovulatory phase (36 hours) and a luteal phase (14 days) (Ganong, 1997 and Griffin & Ojeda, 2000). 17β-Estradiol remains at a constant low (approximately 50 pg/mL) during day 1-10 of the follicular phase. After day 10, estradiol levels begin to rise, reaching a peak (200 - 250 pg/mL) at day 14 just before ovulation. By day 16 of the luteal phase estradiol levels reach a low of approximately 70 pg/mL, followed by a slight rise to approximately 100 pg/mL on days 21 - 25. After day 25, estradiol levels drop to a low of approximately 50 pg/mL and menstruation soon follows (Ganong, 1997). Progesterone follows a different hormonal pattern, in that levels are constantly low, approximately 1 ng/mL throughout the follicular phase. After this phase, progesterone levels begin to rise, reaching a peak level of approximately 20 ng/mL from day 20 - 24. During the latter days of the luteal phase, progesterone levels dramatically decrease to 1 ng/mL (Ganong, 1997).

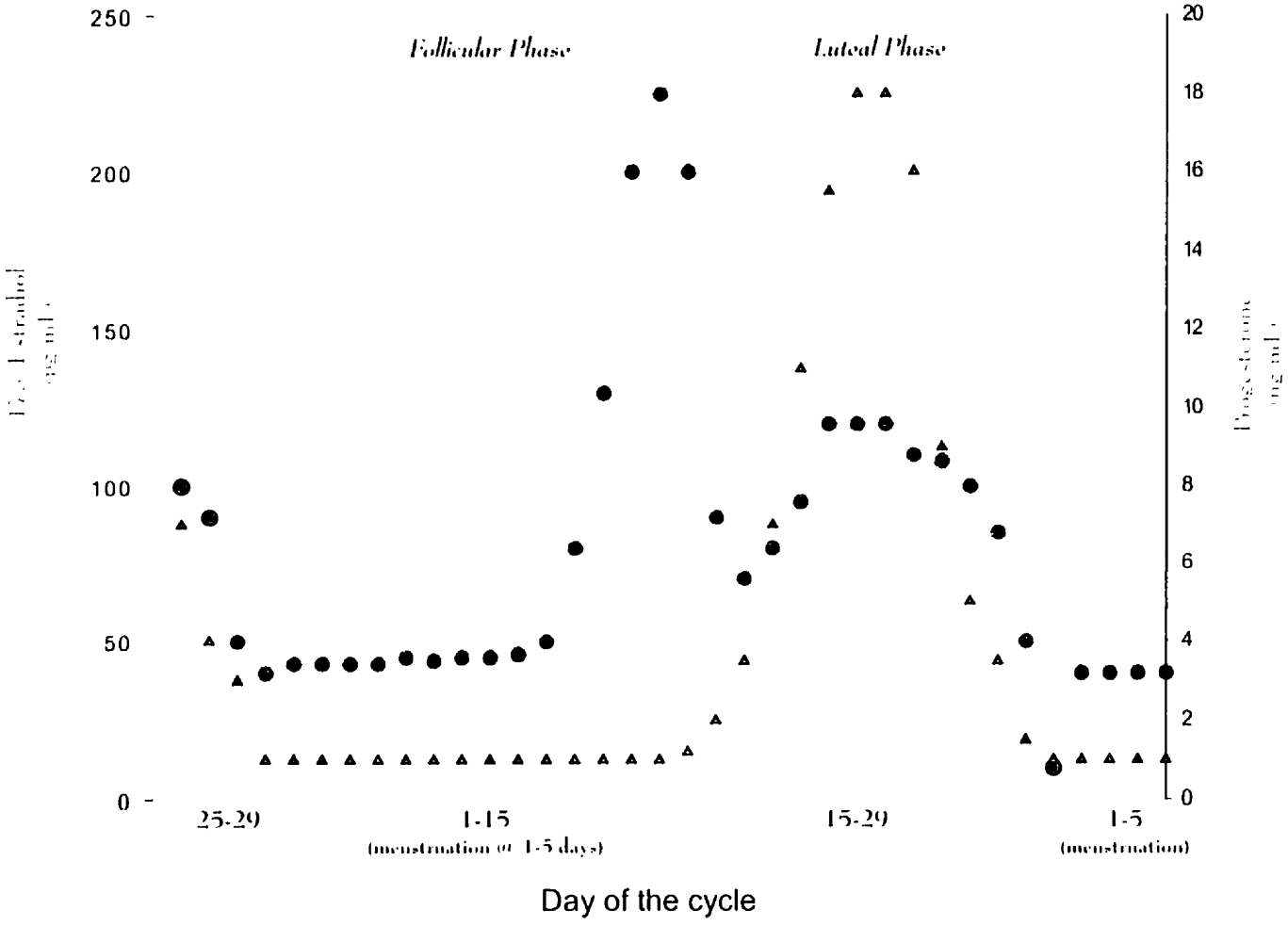

Figure 18: The human menstrual cycle. Modified from Ganong, 1997.

The Estrous Cycle

The estrous cycle is the sexual cycle of mammals other than primates which do not menstruate. It is denoted by a period of sexual interest (estrus) in the female at the time of ovulation (Ganong, 1997 and Freeman, 1994). The underlying endocrine events however, are essentially the same as those in the menstrual cycle (Ganong, 1997).

The estrous cycle is divided into four stages: estrus, metestrus, diestrus and proestrus (Figure 19). During proestrus, which lasts 12 - 14 hours, the animal is 'coming on heat'. During estrus, sexual intercourse may take place and this stage is 25 - 27 hours in length. In the absence of conception, metestrus follows for a brief 6 - 8 hours. Diestrus is the stage at which ovarian secretions prepare the reproductive tract for the fertilized ovum and is between 55 -57 hours in length. In the absence of fertilization the cycle begins again with proestrus. Ovulation occurs every 4 to 5 days.

During estrus, plasma estradiol levels are basal, approximately 10 pg/mL (Figure 20). Plasma levels begin to rise late in metestrus through early diestrus, reaching levels of approximately 20 pg/mL (Figure 21). Estradiol concentrations continue to rise through diestrus to early proestrus, reaching a plateau peak (approximately 40 pg/mL) at midproestrus (Figure 20). Estradiol levels fall rapidly during the early evening, reaching basal levels by the early morning of estrus (Figure 20) (Freeman, 1994).

Progesterone follows the same pattern during the estrous cycle. Both peak during the afternoon of metestrus (20 -30 ng/mL) and progesterone peaks soon after estradiol peaks in the evening of proestrus (40 - 50 ng/mL) (Figure 20). After the above two peaks, progesterone reaches levels between 5 - 10 ng/mL during all other times in the estrous cycle (Freeman, 1994).

It is interesting to note, testosterone demonstrates a similar pattern to estradiol throughout

the estrous cycle. The highest concentration, approximately 179 ng/mL, occurs on the afternoon of proestrus (Dupon and Kim, 1973).

Identification of Estrous Stages

The stages of the estrous cycle can be determined via identification of cell types in the vaginal smear. It is the ratio of the presence of the following three cell types that distinguishes which stage the rat is in: round epithelial cells, cornified epithelial cells and leukocytes (Table 1). During proestrus, the predominant cell type are nucleated epithelial cells. A few cornified epithelial cells may appear and leukocytes are absent. Estrus is identified by a predominance of cornified epithelial cells which appear in large groups, as well as the absence of leukocytes and round epithelial cells. A large number of leukocytes identifies metestrus and diestrus is identified as the presence of leukocytes as well as nucleated epithelium (Freeman, 1994).

Table 1: Identification of the estrous stages via identification of cell types in vaginal smears. (∅ denotes none present)

Stage	Cell Type Present
Proestrus	↑ nucleated epithelium: few cornified; ∅ leukocytes
Estrus	↑cornified epithelial; ∅ nucleated; ∅ leukocytes
Metestrus	↑ leukocytes
Diestrus	↑ leukocytes and ↑ round epithelium

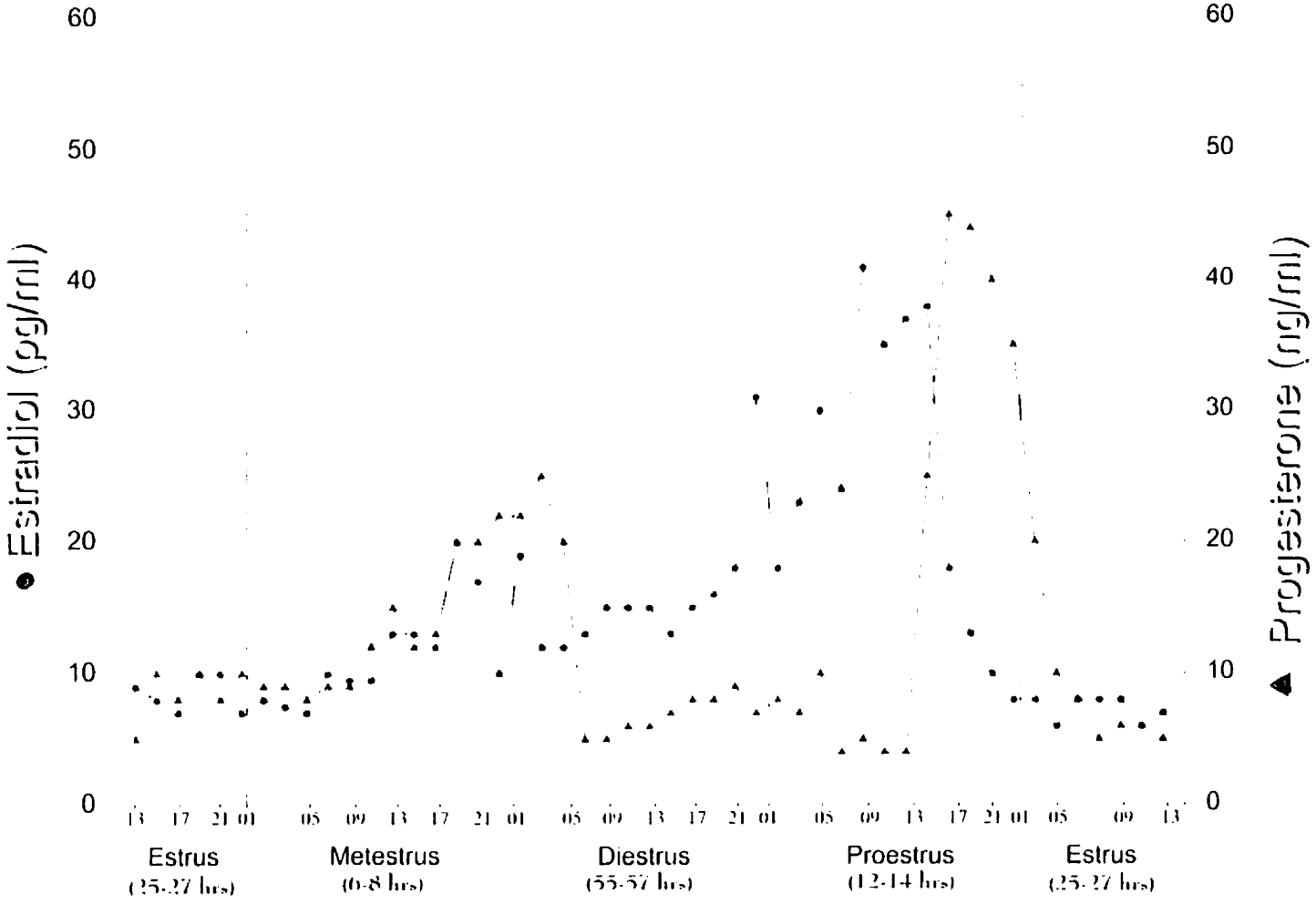

Figure 19: The Estrous Cycle of the adult female rat. Modified from Freeman, 1994.

E. The Hormones: Is there a link to the development of acidosis during ischemia?

As previously mentioned in part 1, during ischemia the cell reverts to anaerobic glycolysis for the production of energy, acting as a major source of H^+ production during ischemia. Glucose and glycogen (endogenous glucose source) are substrates utilized in the anaerobic pathway. Estrogen and progesterone are known to influence glucose metabolism in several ways, which in return may affect the development of acidosis during ischemia. Estrogen is known to increase glucose uptake and utilization in female rat brain (Namba and Sokoloff, 1984; Bishop and Simpkins, 1995 and Zhang *et al.*,1998) an effect that is antagonized by progesterone (Porino *et al.*, 1982; Sutter-Dub, 1986; Leturqué *et al.*, 1989 and Cordoba *et al.*, 1991). Changes in glucose metabolism have also been found to be related to the estrous cycle. In adult female rats, the highest level of cerebral glucose utilization occurred during proestrus and metestrus and lower levels during estrus and diestrus (Nehhlig *et al.*, 1985). In mature female ewes, glucose entry rate was found to be highest during late diestrus. Plasma glucose concentration was also found to increase significantly from estrus to diestrus (Dunn *et al.*, 1972). Estradiol has also been shown to increase insulin secretion and sensitivity, as well as promote glycogen synthesis in rat hearts (Gorski *et al.*,1976; Ahmed-Sorour and Bailey, 1981; Kendrick *et al.*, 1987; Nicklas *et al.*, 1989 and Colacurci *et al.*, 1998), an effect also antagonized by progesterone (Ahmed-Sorour and Bailey, 1981). Myocardial glycogen content has been shown to be 2-fold higher in female SHR rats compared to males (Wittnich & Wallen, 1997). Whether a 2-fold higher myocardial glycogen content exists in normotensive female rats is unknown.

The activity of the glycolytic hormones PFK, hexokinase in brain and pyruvate kinase in heart are increased with estradiol (Chainy & Kanungo, 1978; Kostanyan & Nazaryan, 1992; Baquer *et al.*, 1993 and Wallen *et al.*, 1999), whereas the activity of LDH has been shown to be

lower in female WKY rats compared to males (Wallen *et al.*, 1999), and inhibited by estrogen in human cervical mucus (Takehisa, 1980).

Recent data suggest the stage of the menstrual cycle, in particular the level of estradiol, affects the risk of an MI as well as injury such as infarct size (Smith *et al.*, 2000; Lloyd *et al.*, 2000 and Methot *et al.*, 2000). Acidosis has been related to infarct size after an MI, suggesting estradiol may affect the development of acidosis during ischemia.. Thus the development of acidosis may not only be gender-dependent, but also vary according to the stage of the ovarian cycle. Whether ovarian cycling affects the development of acidosis during ischemia and the ability to handle this acidosis is unknown and was an additional focus to this thesis.

HYPOTHESES
&
OBJECTIVES

Hypotheses

Based on the previous information, the following hypotheses have been formulated.

1. In the heart, compared to males, females will exhibit greater H^+ accumulation during global ischemia.
2. The higher H^+ accumulation during ischemia in females will be due to a lower BC and greater degree of anaerobic glycolysis.
3. Female responses will depend on the stage of the ovarian cycle.
4. Compared to males, females will display exaggerated LV-RV differences with respect to H^+ accumulation, BC and lactate levels at 30 minutes of global ischemia.

Objectives

The following objectives will assess the above hypotheses. In male and female normal hearts, to determine tissue:

1. A) *in vivo* hydrogen ion concentration ($[H^+]$).

   B) $[H^+]$ at thirty-minutes of global ischemia.

2. A) *in vivo* Buffering Capacity (BC)

   B) *in vivo* lactate levels

   C) lactate levels at thirty-minutes of global ischemia.

3. In hearts of females at each stage of their ovarian cycle, to determine:

   A) *in vivo* $[H^+]$, lactate levels and BC.

   B) $[H^+]$ and lactate levels at thirty minutes of global ischemia.

4. Between the left and right ventricles of males and females, to determine:

A) *in vivo* [H^+], lactate levels and BC.

B) [H^+] and lactate levels at thirty minutes of global ischemia.

MATERIALS and METHODS

Animal Model

Male and female Sprague-Dawley rats were used to conduct the following studies in normal hearts. The Charles River Breeding Laboratories supplied the rats as pure strain animals. Once in the facility, the rats were housed in plastic cages with free access to food and water. All animals were allowed to acclimatize for approximately two weeks under a 12-hour light-dark cycle. Surgery commenced at approximately 6 months of age, hence adult age. Adult age corresponds to a weight of approximately 450g and 320g for males and females, respectively. The animals were humanely treated in accordance with the Canadian Council on Animal Care guidelines.

Ovarian Cycling Model

In order to determine whether ovarian cycling affected female responses, vaginal smears were obtained in an additional sixteen females, which were then grouped into an ovarian stage based on cell types present (Freeman, 1994). Ultimately, 4 females were identified in each of the following stages of the ovarian cycle: estrus, metestrus, diestrus and proestrus.

Vaginal smears were conducted as follows:

1. Once the rat was anaesthetized, a 20μL Eppendorf tip containing 0.9% saline was used on the vaginal opening to flush the orifice and obtain vaginal fluid.
2. The vaginal fluid was smeared on a clean glass slide and viewed under a microscope (X 100 power). Based on the ratio of the following cell types: round epithelial, cornified squamous epithelial and leukocyctes., stages were classified and identified as: (1) estrus: predominance of cornified epithelium with few or no nuclei visible; (2) metestrus: high density of leukocytes; (3) diestrus: heterogeneous mixture of round epithelial cells and leukocytes; and (4) proestrus: predominance of round epithelial cells (Freeman, 1994).

Surgical Procedure and Sampling Protocol

Rats were anaesthetized using thiobutabarbital (INACTIN, Research Biochemicals, International, Natick, MD) intraperitoneally at a dose of 100 mg/kg. The abolition of all pain reflexes indicated the appropriate plane of anaesthesia. A tracheotomy was performed and a 16 gauge blunt needle inserted into the trachea and secured with suture material. Rats were ventilated with room air using a rodent ventilator (Kent model RSP 1002, Kent Scientific Corp., Litchfield, CT). The right carotid artery was exposed and cannulated with PE50 tubing, which was then sutured in place. Following cannulation, 100 IU heparin (Hepalean) was administered via the carotid artery to prevent coagulantion of the blood. Blood samples (0.5mL) were obtained via the carotid artery and analyzed for blood gases (PaO_2 and $PaCO_2$ mmHg) and acid-base status (pH, HCO_3^- mmol/L, base excess) using an ABL30 Acid-Base Ananlyzer (Radiometer, Copenhagen, Denmark). Ventilation parameters were adjusted as necessary to ensure normal blood gases and acid-base status. Once normal blood gases were obtained, a midline sternotomy was performed and the heart exposed. The pericardium was carefully opened, *in vivo* left ventricular (LV) and right ventricular (RV) biopsies were obtained via the freeze-clamp technique and placed in liquid nitrogen (Belanger *et al.*, 1992). The remaining LV and RV was then removed and placed in a beaker filled with glucose-free Kreb's Henseleit solution submerged in a 38°C water bath. At 30 minutes, a global, normothermic ischemic LV and RV biopsy was taken and placed in liquid nitrogen. All samples were placed in Eppendorf tubes and stored in a -90°C freezer until biochemical analyses.

Studies Conducted

In order to assess the hypotheses, the following intracellular parameters were measured in the left and right ventricle in non-pathological hearts:

***In vivo*:**

A)Hydrogen ion concentration ($[H^+]$)

B) Buffering Capacity (BC)

C) Lactate concentration

During 30 minutes of global, normothermic ischemia:

E) Hydrogen ion accumulation ($\Delta[H^+]$)

F) Lactate accumulation (Δ lactate)

In vivo Buffering Capacity (BC) and thirty-minute biopsies for hydrogen ion concentration ($[H^+]$) were determined using a pH meter. Lactate concentration was determined using the fluorometric method. See section Biochemical Analyses, page 71, for a description of the assays.

Biochemical Analyses

$[H^+]$ and Buffering Capacity Assay

The following assay is a modified version of the homogenate method taken from Van Slyke (1922) and Bates (1973). A pH meter (Metrohm 691) (Appendix 1) was used to determine initial and 30 minute $[H^+]$ and BC.

$[H^+]$:

Prior to commencing the assay, the pH meter was calibrated using standard pH buffer solutions, pH 4.0 and pH 10.0. An iodoacetic acid (IAA)/saline solution was prepared as follows: 0.0544g NaCl + 1.0810g KCl + 0.1040g IAA dissolved in 100 ml of water. Using 0.01M NaOH, the solution was brought to and maintained at a pH of 7.00 ± 0.01. Once the desired pH was achieved, the tissue biopsy, in the weight range of 0.09 g - 0.10 g, was removed from the freezer and submerged in liquid nitrogen until homogenization. Using a dilution factor of 19, the tissue was homogenized in the IAA/saline solution at 28,000 rpm for approximately 30 seconds using the Kinematic Polytron PT 3000 (Littau, Switzerland). The homogenizer is designed to disrupt only plasma membranes thus permitting the determination of mean intracellular pH readings.

The homogenate was then placed in a 37°C water bath for 10 minutes. After 10 minutes, 1500 µL of the homogenate was transferred to a new test-tube and lightly vortexed. The pH glass electrode was rinsed with distilled water, wiped and inserted into the test tube. A pH reading was obtained and the $[H^+]$ was calculated using the formula: $[H^+] = 1/10^{pH}$ expressed as mol/L.

Buffering Capacity Assay

In vivo biopsies were prepared as previously described. Once the initial pH was obtained,

the pH of the homogenate was lowered to 6.00 ± 0.05 using 0.02M HCl in order to eliminate HCO_3^- (Vasuvattakul *et al.*, 1992). The pH was then raised to 7.00 ± 0.05 using 0.01M NaOH, which was recorded as the initial pH (pH_i). The volume of 0.02M HCl required to lower the initial pH 1.00 ± 0.05 pH units was determined and the final pH was noted as pH final (pH_f). The BC of the tissue was then calculated as:

1. Tissue Weight = $\frac{\text{weight tissue (g) x 1500 µL}}{\text{volume of IAA/saline (µL)}}$

2. BC = $\frac{\text{0.02M HCl x volume to drop 1 pH unit (µL)}}{\frac{\text{tissue weight (g)}}{\Delta \text{ pH}}}$

Therefore BC = µmol/g wet wt/ pH unit

Lactate Assay

Fluorescence spectrophotometry (SpectroFluorometer RF-1501 Shimadzu, Japan) (Appendix 2) was used to measure lactate levels. Freeze-dry tissue is required for this assay, therefore all tissue biopsies were freeze-dried for 24 hours (Lyph-Lock 6 Lite Benchtop Model 77520, Labconco, Kansas City, MS). Connective and vascular tissue was carefully removed and 3-5 mg of myocardial tissue (LV or RV) was placed in an Eppendorf tube. The assay consists of two parts: 1) Tissue Extraction and 2) Lactate Analysis.

1) Tissue Extraction:

To each sample (Eppendorf tube), 600µL of 0.5M perchloric acid (PCA) was added. The tubes were placed in ethanol (EtOH) at 10°C for 10 minutes and were vortexed intermittently. To remove the protein precipitate, the tubes were then centrifuged at 14,000 rpm at 0°C for 10 minutes (Eppendorf model 5402, Brinkmann Instruments Inc., Rexdale Ont.). To a new Eppendorf tube, 540µL of supernatant was added and then placed in a -90°C freezer for

approximately 5 minutes. Adding 135µL of 2.3M $KHCO_3$ neutralized the frozen supernatant. The supernatant was allowed to thaw and was centrifuged a second time. The remaining supernatant was removed and stored at -90°C until step 2.

2) Lactate Analysis**:**

Acrylic cuvettes were prepared as follows: 3 blanks (20µL of distilled water), 3 standards (20µL lactate standard) and 2 cuvettes/sample (20µL sample supernatant). To each cuvette, 2 mL of a buffer solution containing 100mM hydrazine (Sigma-Aldrich, #H-7294), 100mM glycine (Sigma-Aldrich, #G-7403) and 0.5mM NAD^+ (Boehringer Mannheim, #127973) set at a pH of 10.00, was added. These cuvettes were slightly vortexed and read on the fluorometer at a wavelength range of 360 nm - 455 nm. These initial values (R1) were used to establish a baseline. Following the first reading, 250µL of lactate dehydrogenase (LDH, Boehringer Mannheim, #106984, 1250U/ml) was added to 1.0 mL of the above buffer solution. To each cuvette, 50µL of this diluted enzyme was added and incubated in the dark for 60 minutes. After one hour, the cuvettes were read a second time (R2) and the lactate concentration (µmol/ g dry weight) was determine based on the following chemical equation:

$$\text{Lactate} + NAD^+ \xrightarrow{LDH} \text{Pyruvate-hydrazone} + NADH$$

Figure 20: Determination of lactate via fluorometric analysis.

Finally, the actual concentration was calculated using the following mathematical formula:

$$\frac{\Delta\text{Sample} - \Delta\text{Blank}}{\Delta\text{Standard} - \Delta\text{Blank}} \times [\text{Standard}]/(\text{Tissue wt} \times \text{DF})$$

where: Δ = R2-R1; [standard] = 500 µM for *in vivo* samples and 1000 µM for ischemic samples; DF = dilution factor (1.333).

Statistical Analyses

All data were expressed as mean ± SD. The Statistical Analysis System (SAS) Program (Release 6.12, SAS Institute, Cary, NC) was used to perform all statistical analyses. The Student's t-test was used to assess differences between males and females. Within a gender, the left and right ventricles were compared using a paired t-test. To test the effect of one factor among the four female ovarian stages, a one-way analysis of variance (ANOVA) was used. To further examine differences among all possible pairs of means, the Tukey's *post hoc* test, which is the most widely accepted and most robust method, was performed with ANOVA (Zar, 1984). For all tests the level of significance (α) was $p<.05$ and trend at $.05 < p < 0.10$. Significance at .05 was chosen because at this level there is a small enough chance of committing a Type I error, that is rejecting the null hypothesis when in fact it is true. Accepting the null hypothesis when in fact false is a Type II error. Type II errors may occur when the sample size of the study is too small or quite variable. In such a case, one should compute the power of the test (Zar, 1984) (See Appendices for power values for NS (no significance) parameters).

Sample Size Calculation

Sample sizes (n) were calculated using the following equation:

$$n = \frac{(\sigma^2_1 + \sigma^2_2)(Z_{1-\alpha/2} + Z_{1-\beta})^2}{(\mu_1 - \mu_2)^2}$$

where: $Z_{1-\alpha/2}$ and $Z_{1-\beta}$ are constants derived from the normal distribution, $\alpha = 0.05$ and $\beta = 0.20$.

Based on LV preliminary data for a few (n=2) males and females sample size requirements were 5 for each gender and thus the n numbers used in the study were no less than 5. Using preliminary ovarian cycling data with a few animals in each stage (n=2), the n number requirement was 2 and thus the n numbers per stage in the data were no less than 2.

RESULTS

Male vs. Female

Left Ventricle

Baseline $[H^+]$, Buffering Capacity and $\Delta[H^+]$:

At baseline, prior to ischemia, there was no statistical difference between male and female myocardial hydrogen ion concentration in the left ventricle (LV) (Table 2).

Table 2: Baseline $[H^+]$ in the LV of adult male vs. female Sprague-Dawley rats.

Gender	Baseline $[H^+] \times 10^{-8}$ mol/L
Male	6.60 ± 0.5
Female	6.69 ± 0.9
p - value	0.70

When buffering capacity (BC) was compared, females demonstrated a significantly ($p=.0001$) two-fold greater BC compared to males, that is 114.0 ± 14.0 (µmol/g wet wt/pH unit) vs. 62.8 ± 2.6 (µmol/g wet wt/pH unit), respectively (Figure 21A). The female group also demonstrated greater variability than males (Figure 21B) ranging in values from 93 µmol/g wet wt/pH unit to 130 µmol/g wet wt/pH unit.

Compared to males, by 30 minutes of global ischemia (Figure 22A), females had a trend to accumulate ($p = 0.07$) 2.5 times the amount of hydrogen ions. Once again, the female group exhibited a larger degree of variability, from 28 mol/L to 183 mol/L (Figure 22B).

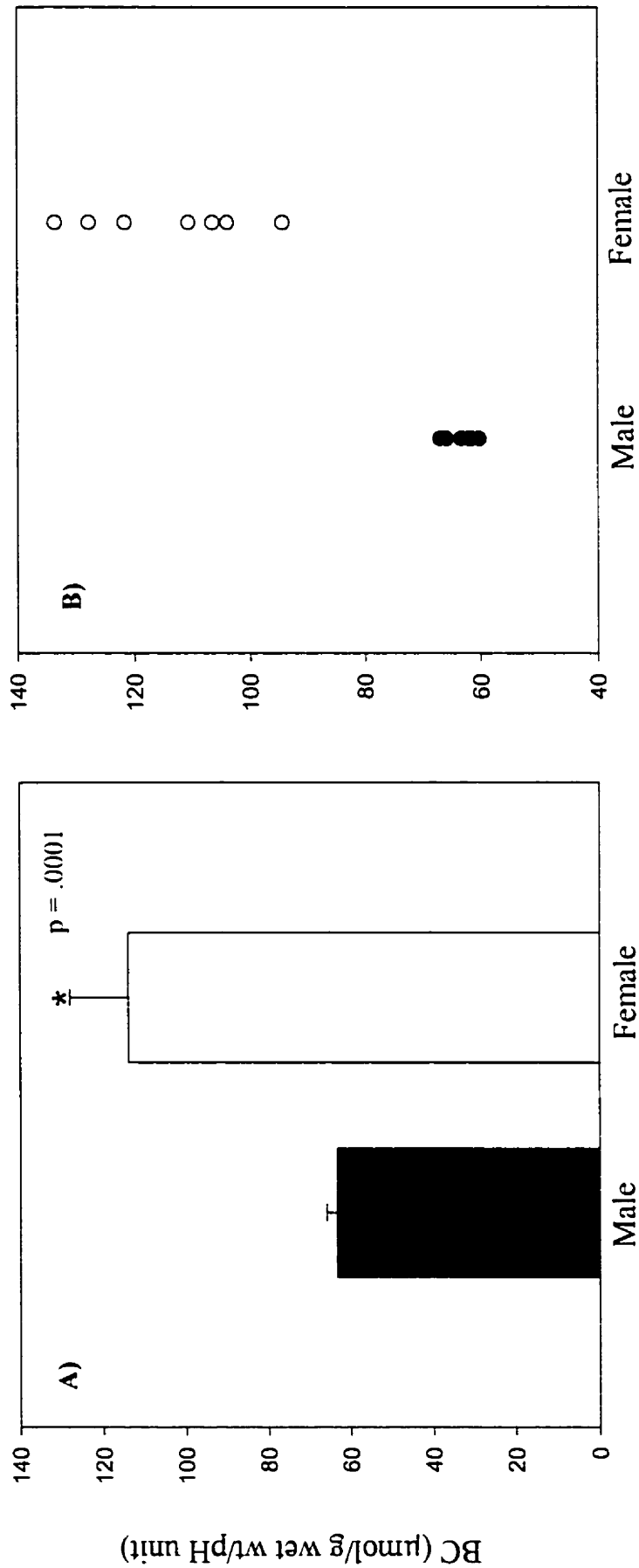

Figure 21: A) LV Buffering Capacity (BC) in adult male vs. female Sprague-Dawley rats. Values are mean ± SD. **B)** Scatter plot of the individual BC values. (Males = closed bar and circles; females = open bar and circles).

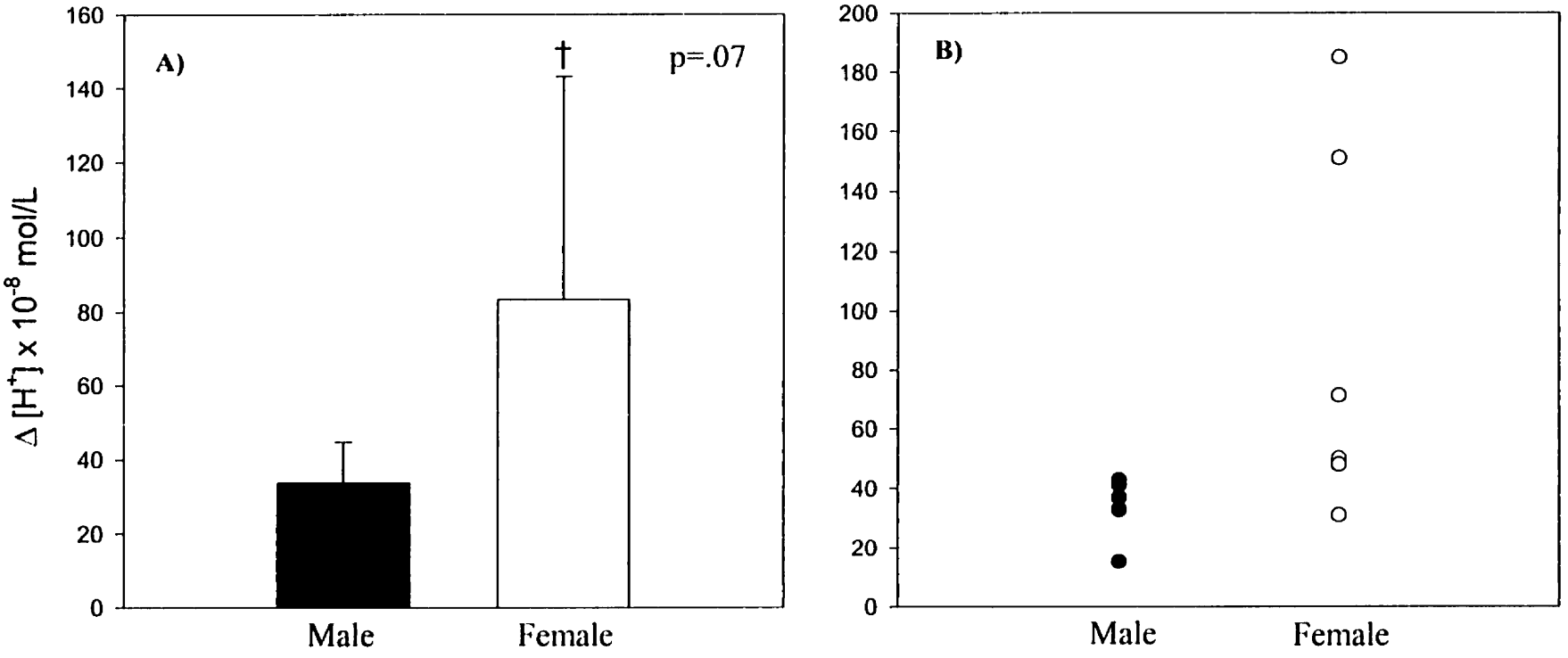

Figure 22: A) LV hydrogen ion accumulation ($\Delta[H^+]$) during 30 minutes of global ischemia in adult male vs. female Sprague-Dawley rats. Values are mean ± SD. **B)** Scatter plot of the individual $\Delta[H^+]$ values. (Males = closed bar and circles; females = open bar and circles).

Lactate:

There were no differences in initial lactate levels between males and females (Table 3).

Table 3: *In vivo* LV lactate levels (μmol/g dry wt) in adult male vs. female Sprague-Dawley rats.

Gender	*in vivo* lactate
Male	3.6 ± 1.2
Female	2.3 ± 1.2
p - value	0.10

In addition, at 30 minutes of global ischemia, females had accumulated approximately 11% less lactate (p=.03) compared to the males, 136.5 ± 25.6 μmol/g dry wt and 154.1 ± 17.0 μmol/g dry wt, respectively (Figure 23).

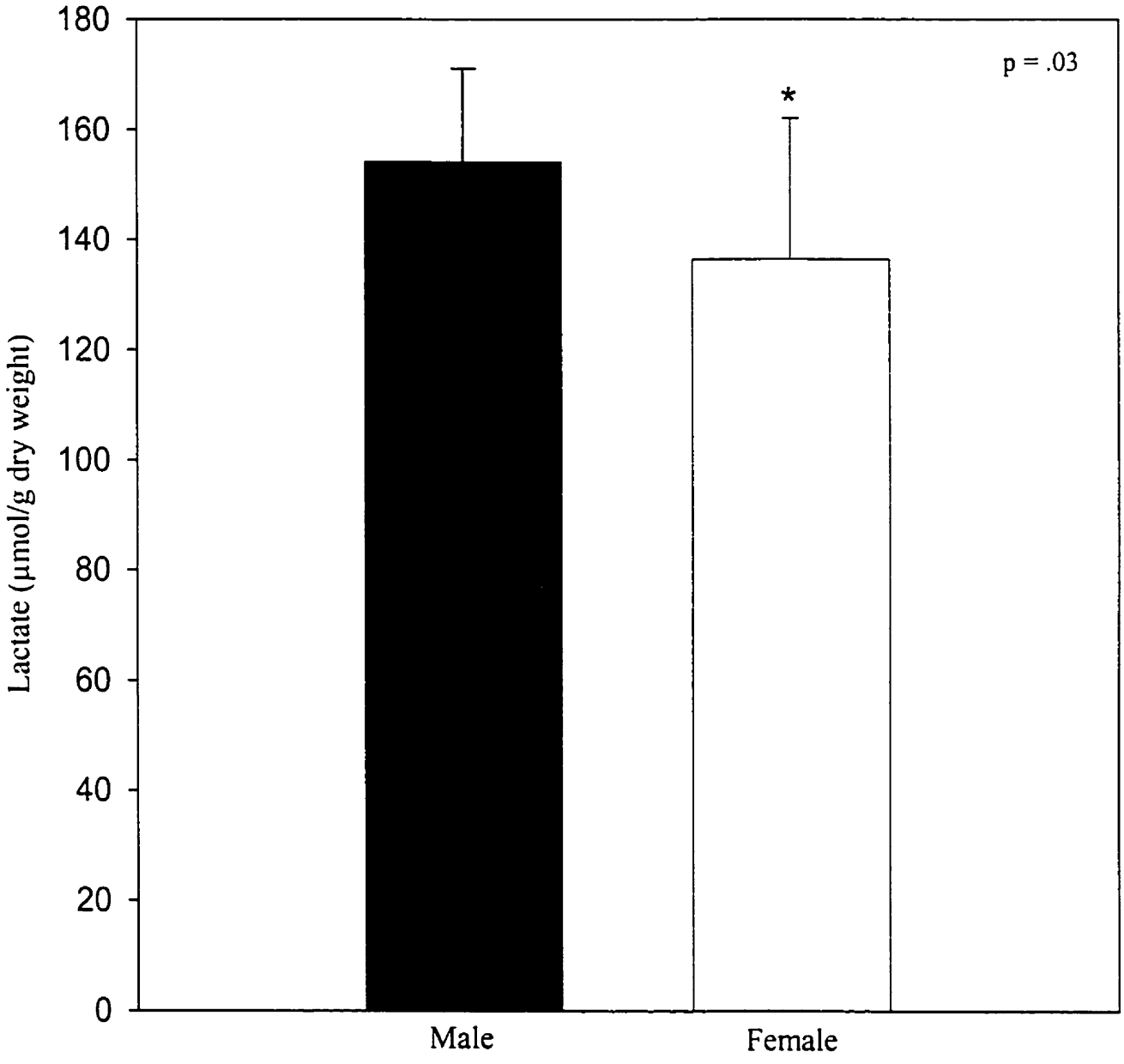

Figure 23: LV lactate levels at 30 minutes of global ischemia in adult male vs. female Sprague-Dawley rats. Values are mean ± SD. (Males = closed bar; females = open bar).

Right Ventricle

Baseline $[H^+]$, Buffering Capacity and $\Delta[H^+]$:

At baseline, there were no differences in the myocardial $[H^+]$ in the RV between males and females (Table 4). Unlike the LV, the RV of males and females exhibited similar BC values, 126.06 ± 30.94 (μmol/ g wet wet/pH unit) and 132.23 ± 28.55 (μmol/ g wet wet/pH unit), respectively (Table 4). As well, at 30 minutes of global ischemia, there were no differences in $\Delta[H^+]$ between males and females in the RV (Table 4).

Table 4: Baseline $[H^+]$, Buffering Capacity and $\Delta[H^+]$ in the right ventricle of adult male vs. female Sprague-Dawley rats.

Gender	Baseline $[H^+]$ x 10^{-8} mol/L	BC (μmol/g wet wt/pH unit)	$\Delta[H^+]$ x 10^{-8} mol/L
Male	6.3 ± 1.0	126.1 ± 31.0	17.0 ± 9.1
Female	5.6 ± 0.4	132.2 ± 28.6	7.8 ± 6.4
p-value	0.15	0.88	0.18

Lactate:

Although within physiological range, in contrast to the LV baseline RV lactate was significantly (p = .03) lower in females by 54% compared to males (Table 5). In addition, at 30 minutes of global ischemia, the RV of females accumulated approximately 65% (p=.002) less lactate compared to males (Figure 24).

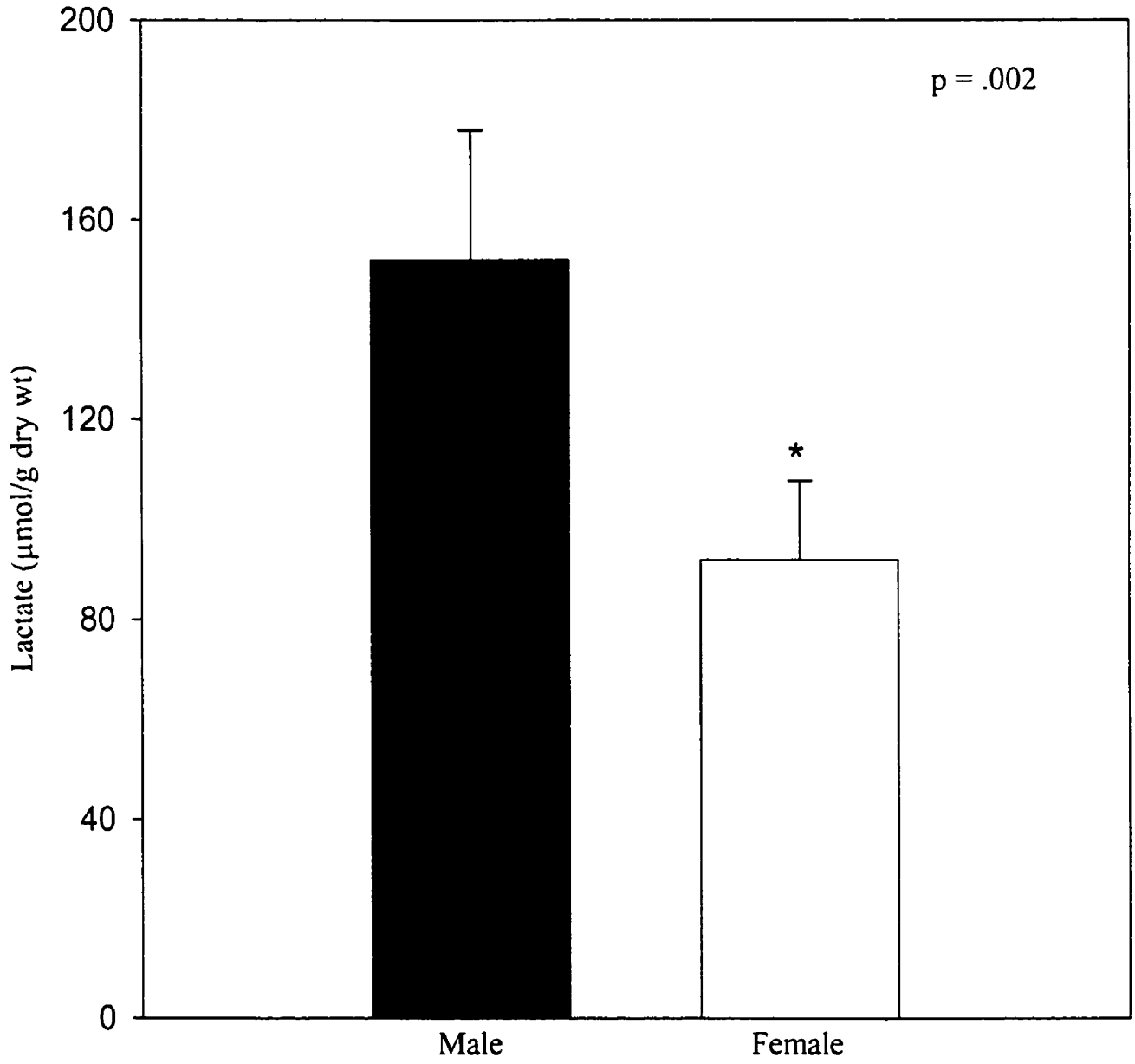

Figure 24: RV lactate levels at 30 minutes of global ischemia in adult male vs. female Sprague-Dawley rats. Values are mean ± SD. (Males = closed bar; females = open bar).

Table 5: *In vivo* RV lactate levels (μmol/g dry wt) in adult male vs. female Sprague-Dawley rats.

Gender	*in vivo* lactate
Male	3.7 ± 1.2
Female	2.4 ± 0.7
p - value	0.03

Females
Ovarian Cycling

Baseline $[H^+]$, Buffering Capacity and $\Delta[H^+]$:

There were no significant differences in baseline myocardial LV $[H^+]$ between the four stages of the ovarian cycle, (Table 6). When BC was compared, estrus and metestrus demonstrated 50% significantly ($p<.05$) lower values, 64.00 ± 5.2 (µmol/g wet wt/pH unit) and 60.65 ± 6.7 (µmol/g wet wt/pH unit), respectively, compared to proestrus, 125.05 ± 45.7 (µmol/g wet wt/pH unit) (Figure 25A). There were no differences in BC between diestrus and proestrus, 108.72 ± 30.7 (µmol/g wet wt/pH unit) and 125.05 ± 45.7 (µmol/g wet wt/pH unit), respectively (Figure 25A). Furthermore, proestrus values were the most variable (Figure 25B). When $\Delta[H^+]$ was compared, there were no statistical differences (ANOVA = .2634) between the four stages, however estrus appeared to accumulate the least H^+, 8.66 ± 1.9 (x 10^{-8} mol/L) versus metestrus, 19.96 ± 2.8 (x 10^{-8} mol/L), diestrus, 22.89 ± 8.1 (x 10^{-8} mol/L) and proestrus, 22.81 ± 18.0 (x 10^{-8} mol/L) (Figure 26A). Once again, proestrus exhibited the most variability (Figure 26B).

Table 6: Baseline LV $[H^+]$ (x 10^{-8} mol/L) in the four stages of the ovarian cycle; Estrus, Metestrus, Diestrus and Proestrus, from adult female Sprague-Dawley rats.

Stage	Baseline $[H^+]$ x 10^{-8} mol/L
Estrus	7.2 ± 0.3
Metestrus	6.3 ± 0.9
Diestrus	6.4 ± 1.3
Proestrus	6.4 ± 0.6
ANOVA	0.48

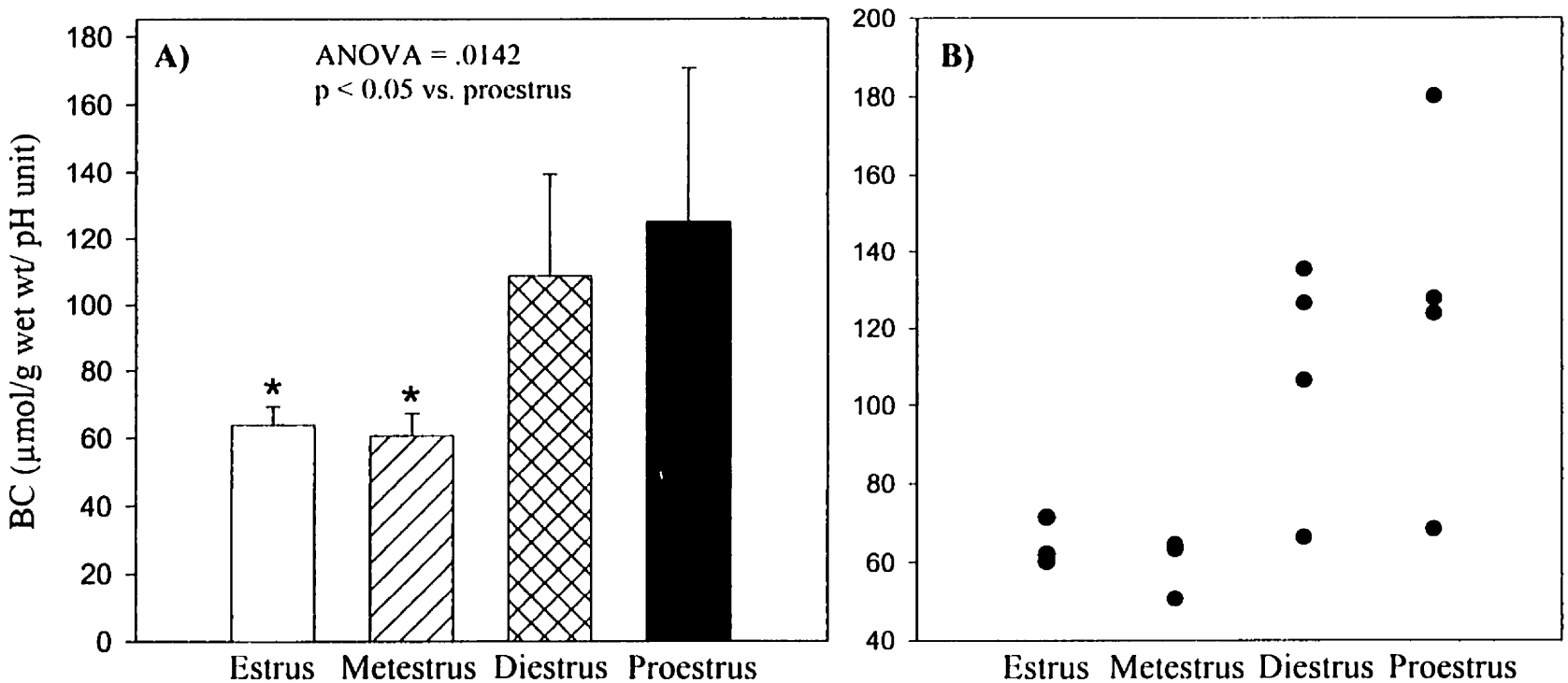

Figure 25: A) LV Buffering Capacity (BC) in adult female Sprague-Dawley rats during the four ovarian stages: Estrus (open bar), Metestrus (diagonal hatch), Diestrus (cross hatch) and Proestrus (closed bar). Values are mean ± SD.
B) Scatter plot of the individual BC values.

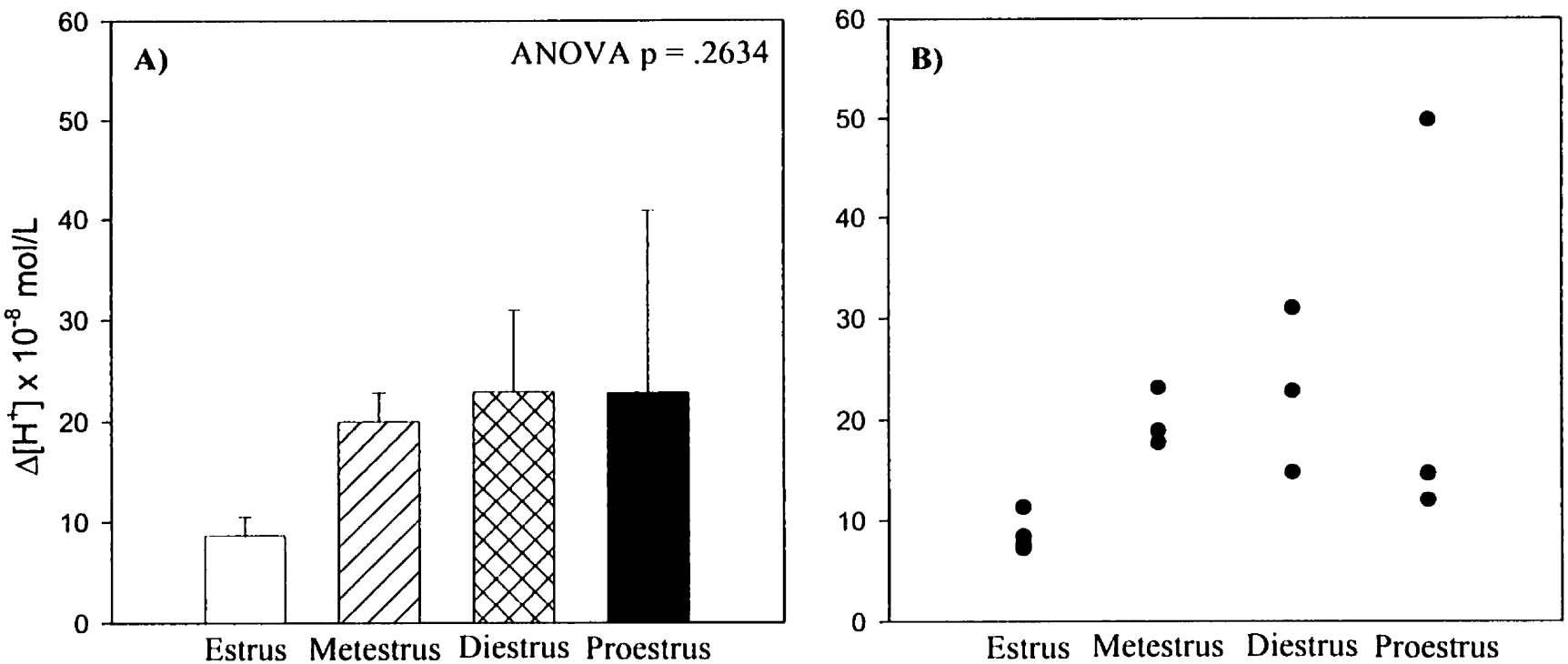

Figure 26: A) LV hydrogen ion accumulation ($\Delta[H^+]$) during 30 minutes of global ischemia in adult female Sprague-Dawley rats during the four ovarian stages: Estrus (open bar), Metestrus (diagonal hatch), Diestrus (cross hatch) and Proestrus (closed bar). Values are mean ± SD. **B)** Scatter plot of the individual $\Delta[H^+]$ values.

Lactate:

At thirty-minutes of global ischemia, there were no differences in lactate levels between the four stages of the ovarian cycle (Table 7) (Figure 27).

Table 7: Lactate levels (μmol/g dry wt) at 30 min. global ischemia in the four stages of the ovarian cycle; Estrus, Metestrus, Diestrus and Proestrus, from adult female Sprague-Dawley rats.

Stage	30 min.lactate
Estrus	130.50 ± 35.7
Metestrus	127.32 ± 14.8
Diestrus	126.05 ± 34.4
Proestrus	141.00 ± 17.5
ANOVA	0.83

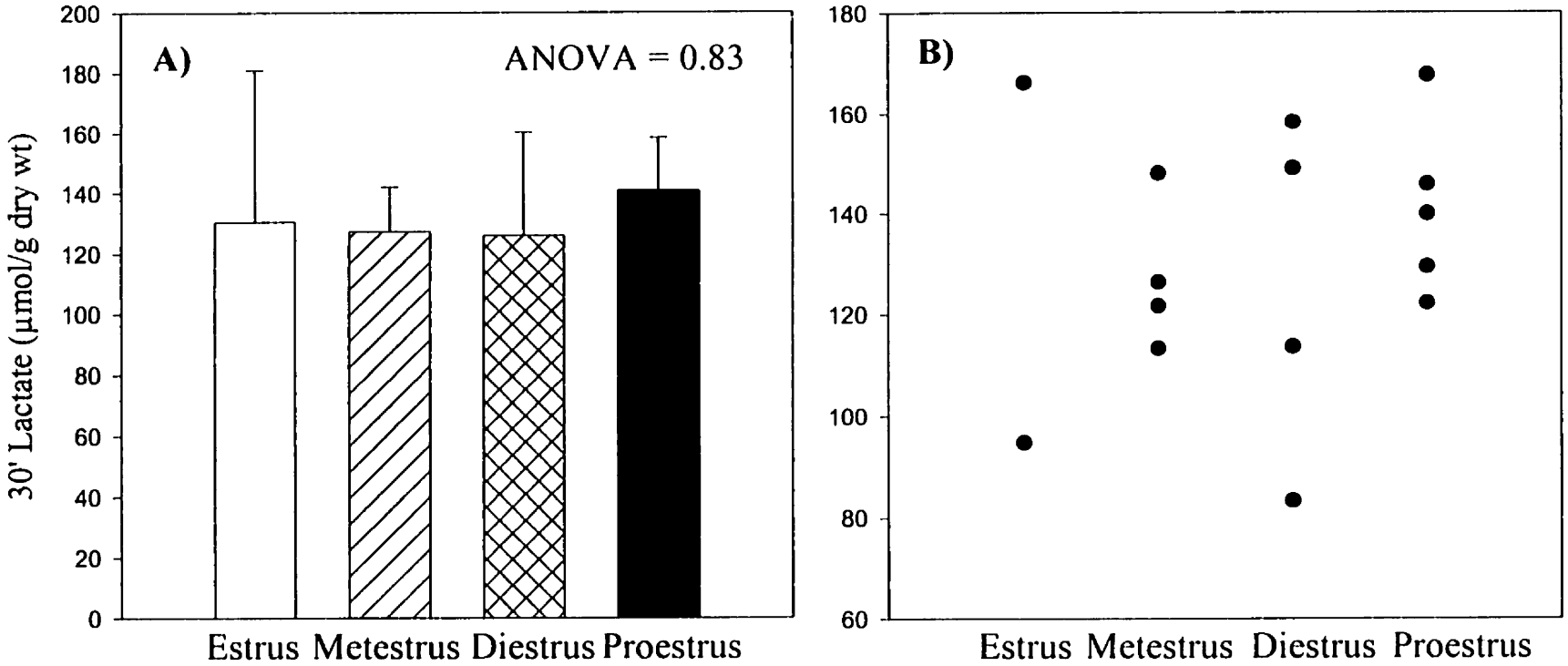

Figure 27: A) Lactate levels at 30 minutes of global ischemia in adult female Sprague-Dawley rats during the four ovarian stages: Estrus (open bar), Metestrus (diagonal hatch), Diestrus (cross hatch) and Proestrus (closed bar). Values are mean ± SD. **B)** Scatter plot of the individual lactate values.

Left Ventricle vs. Right Ventricle

Males and Females

Baseline $[H^+]$, Buffering Capacity and $\Delta[H^+]$:

There were no differences in baseline $[H^+]$ between the left and right ventricle in males and females (Table 8).

Table 8: Baseline $[H^+]$ (x 10^{-8} mol/L) in the left and right ventricle of adult male and female Sprague-Dawley rats.

Gender	LV	RV	p-value
Male	6.6 ± 0.4	6.4 ± 1.0	0.7
Female	5.9 ± 0.4	5.6 ± 0.4	0.3

When BC was compared in males, the RV exhibited a significantly ($p = .02$) two-fold higher BC than the LV, 126.1 ± 31.0 (µmol/g wet wt/pH unit) vs. 63.0 ± 1.8 (µmol/g wet wt/pH unit), respectively (Figure 28). In contrast, no differences ($p = .30$) in BC were found between the LV and RV in females, that is, 111.6 ± 37.3 (µmol/g wet wt/pH unit) vs. 132.2 ± 28.6 (µmol/g wet wt/pH unit), respectively (Figure 28).

In males, hydrogen ion accumulation ($\Delta[H^+]$) during 30 minutes of global ischemia was significantly ($p=.01$) less by 40% in the RV compared to the LV (Figure 29). Likewise, in females, $\Delta[H^+]$ was significantly ($p=.006$) 3.8 fold lower in the RV compared to the LV ($n=15$) (Figure 29).

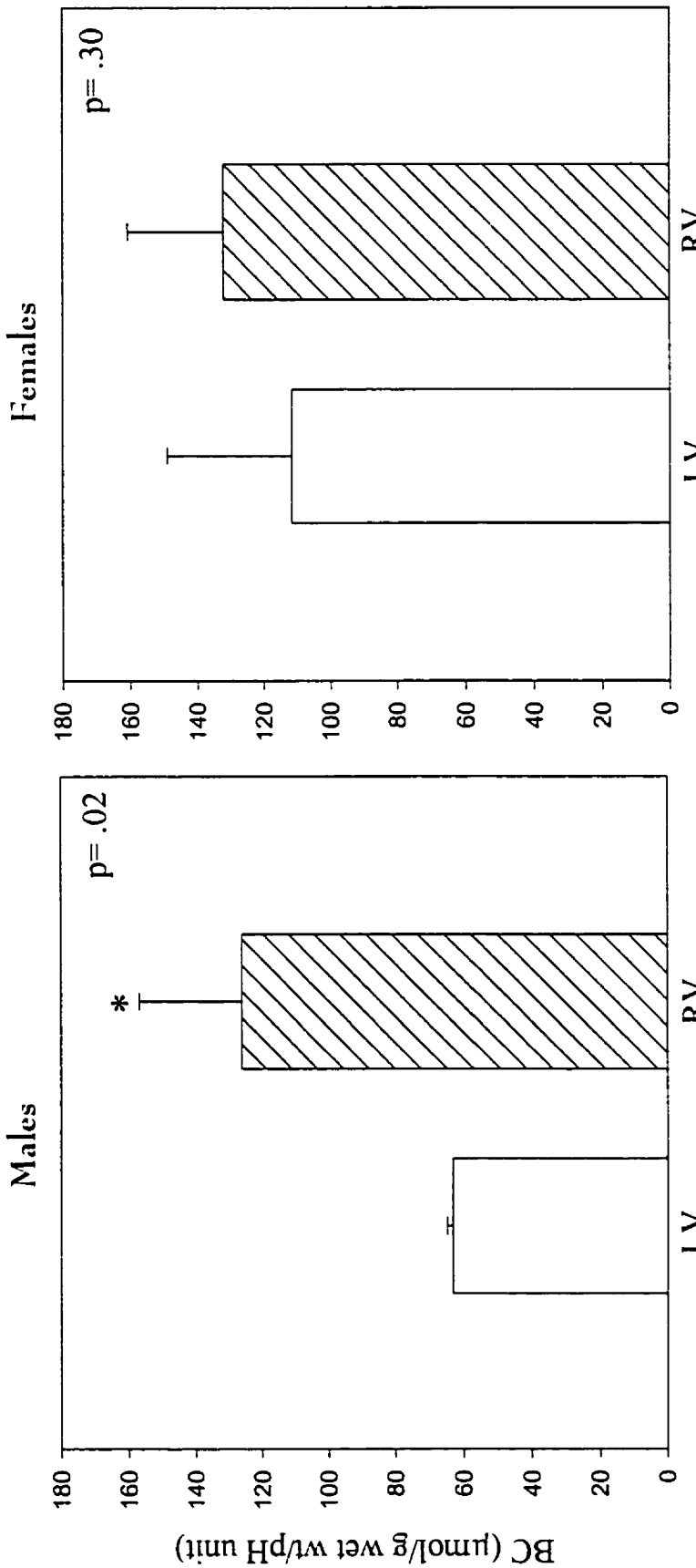

Figure 28: Buffering Capacity (BC) in the left (LV) (open bar) and right (RV) (diagonal hatch) ventricles of adult male and female adult Sprague-Dawley rats. Values are mean ± SD.

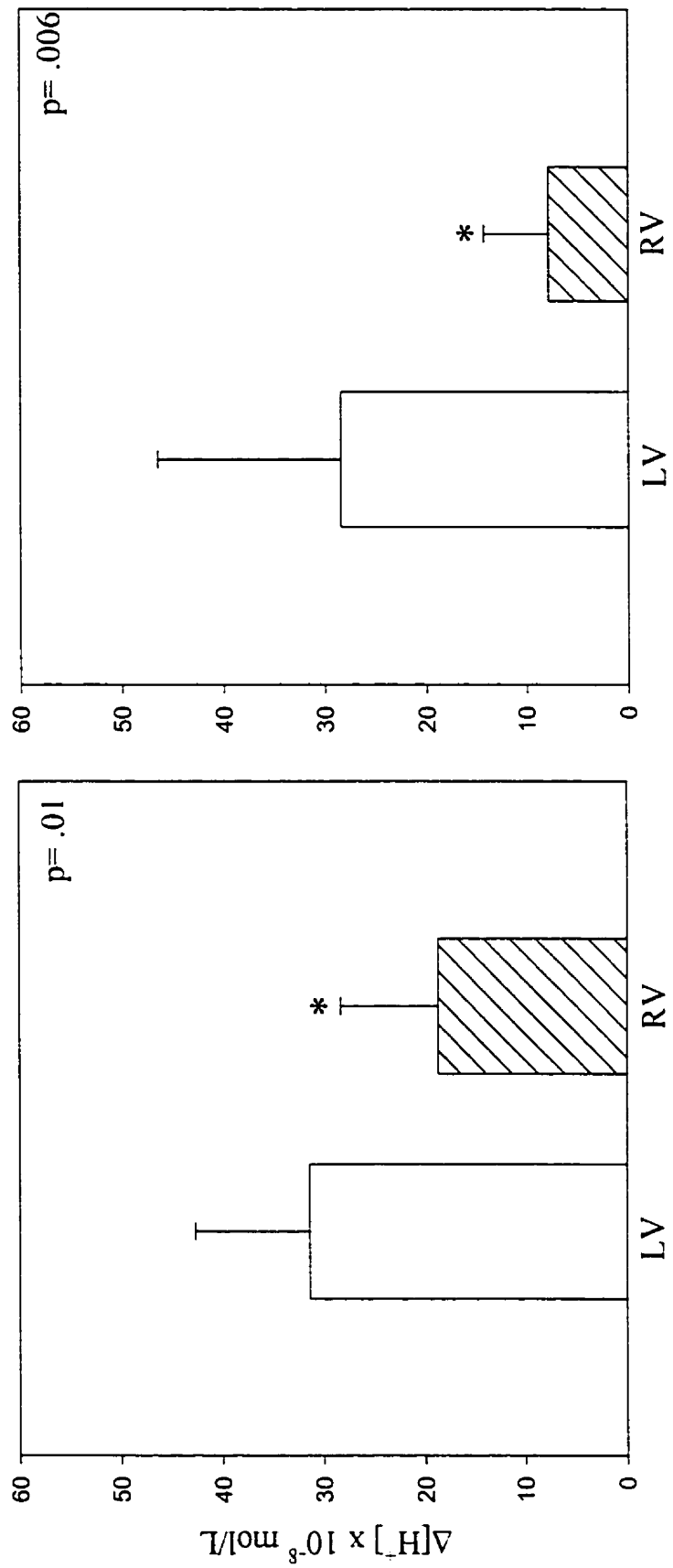

Figure 29: Hydrogen ion accumulation ($\Delta[H^+]$) during 30 minutes of global ischemia in the left (LV) (open bar) and right (RV) (diagonal hatch) ventricles of adult male and female adult Sprague-Dawley rats. Values are mean ± SD.

Lactate:

At baseline, there were no differences in lactate levels between the left and right ventricles in males and females (Table 9).

Table 9: *In vivo* lactate levels in the left and right ventricles of adult male and female Sprague-Dawley rats. (Units = μmol/g dry wt).

Gender	LV	RV	p-value
Males	3.9 ± 1.2	3.7 ± 1.2	0.84
Females	2.4 ± 1.3	2.4 ± 0.7	0.94

Although there were no differences in the amount of lactate accumulated after 30 minutes of global ischemia between ventricles in males (Table 10), the LV in females demonstrated significantly (p = .01) 34% more lactate compared to the RV (Table 10, Figure 30).

Table 10: Lactate accumulation (Δ lactate) over 30 minutes of global ischemia in males and lactate levels at 30 minutes of global ischemia in females. (Units = μmol/g dry wt)

Gender	LV	RV	p-value
Males	155.4 ± 20.8	144.3 ± 31.4	0.98
Females	136.5 ± 25.6	101.6 ± 12.3	.01

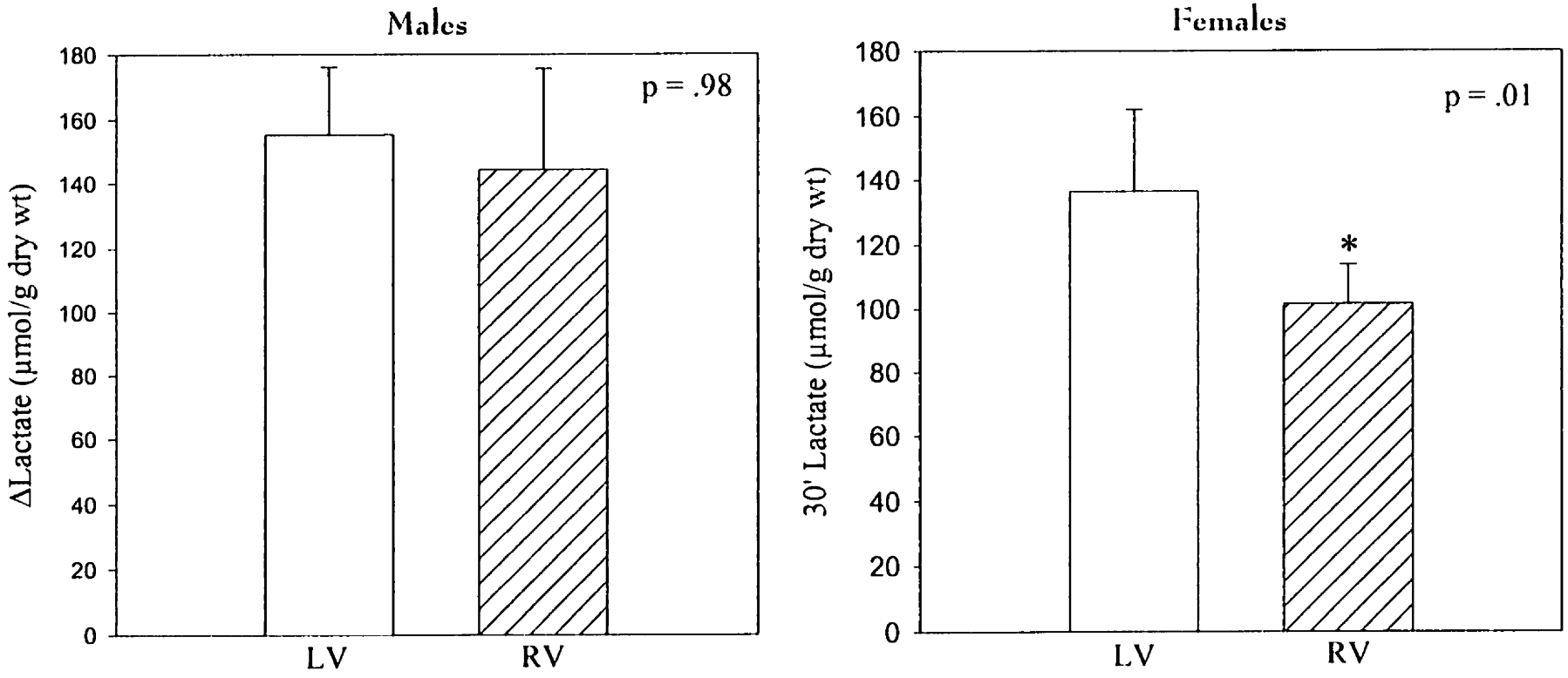

Figure 30: Lactate accumulation (Δlactate) during 30 min. of global ischemia in the left (LV) (open bar) and right (RV) (diagonal hatch) of adult male Sprague-Dawley rats and lactate levels at 30 min. of global ischemia in the LV and RV of adult female Sprague-Dawley rats. Values are mean ± SD.

DISCUSSION

Part 1:
Male vs Female

In vivo: $[H^+]$ and Lactate

In both males and females, LV and RV *in vivo* pH fell within the normal intracellular pH range (7.1 - 7.2) as obtained by studies using either the ^{31}PNMR method (Portner *et al.*, 1990 and Ng *et al.*, 1993) or a direct intracellular pH method (Ellis & Thomas, 1976). This confirms the accuracy in obtaining *in vivo* biopsies as well as the homogenate method as a reliable technique yielding comparable intracellular pH values (Horn *et al.*, 1998).

Compared to other studies using the same rat strain and enzymatic assay (Neely & Grotyohann, 1984; McNulty *et al.*, 1996; Higuchi *et al.*, 1997; Tanonaka *et al.*, 1996 and Geraldes *et al.*, 1997), *in vivo* LV and RV lactate levels were within physiological range (2 - 5 µmol/g dry wt). Again, this indicates the *in vivo* biopsies were not ischemic and metabolism in the heart was normal. Although within physiological range, baseline RV lactate levels were 54% lower in females compared to males. Physiologically, a concentration of any substance is a balance between production and utilization. Under normal conditions lactate may be utilized by the heart (Berne & Levy, 1997), and if the RV in females utilizes more lactate than the RV of males, this would result in lower baseline lactate levels. Lactate is produced from pyruvate by the enzyme lactate dehydrogenase (LDH). In the heart, LDH has a low affinity for pyruvate and thus under aerobic conditions pyruvate is converted to acetyl-CoA and enters the Kreb's Cycle (Katz, 1992). In WKY female rats, the activity of LDH was found to be 27% lower compared to males (Wallen *et al.*, 1999). This lower activity may further suppress any conversion of pyruvate to lactate, thus resulting in lower *in vivo* lactate levels compared to males.

Buffering Capacity (BC)

At the moment the cell experiences acidosis, bicarbonate is the first to buffer H^+. In the initial period of ischemia, CP hydrolysis, catalyzed by CK, attempts to sustain ATP and in the process consumes a H^+ (page 25) (Wolfe *et al.*, 1988; Do *et al.*, 1995 and Abelow, 1998). Several transporters, which are further activated by a decrease in pH_i, assist in minimizing acidosis by extruding H^+ into the extracellular space. However, in order for the transporters to function, H^+ must be removed from the extracellular space and thus maintain a gradient between the ICF and ECF (Lowenstein, 1993). During ischemia, H^+ ions pumped out accumulate in the extracellular space, which minimize the gradient, ultimately impairing transporter function. Once this occurs, the cell relies upon intracellular buffers, phosphates and histidine-rich proteins, in an attempt to minimize acidosis (Pierce & Philipson, 1985; Wolfe *et al.*, 1988 and Schussheim & Radda, 1993). As ischemia progresses, the last vital component responsible for buffering H^+ are histidine-rich proteins (Wolfe *et al.*, 1988). The protocol used to measure BC in this study incorporates buffering due to phosphates and histidine-rich proteins. An increase in either one or both of the latter buffer components would result in an increase in BC.

In the LV, females displayed a two-fold higher BC compared to males. Preliminary data in our lab indicate no gender differences with respect to the amount of CP in the heart. According to studies, the higher the histidine content, the higher the BC (Tait *et al.*, 1982 and Su *et al.*, 1994). Su et al. (1993) discovered a higher BC in the LV of adult pigs compared to the RV due to a significantly higher histidine content (μmol/g wet wt). In addition, the authors found a significant correlation between the amount of histidine and BC in the LV. Furthermore, studies have shown an increase in the BC of the heart as well as a reduction in acidosis when histidine was administered as a buffer prior to ischemia (Garlick *et al.*, 1979 and Tait *et al.*, 1982; Wilson

et al., 1990 and Takeuchi *et al.*, 1995). For example, Tait et al. (1982) infused a histidine buffer solution in adult dogs placed on cardiopulmonary bypass, followed by a 3 hour aortic cross clamping. The authors found the BC to be 60 times higher in the histidine buffered dogs compared to control. Although it is unknown whether the amount of histidine-rich proteins in the heart differs between males and females, one may speculate a higher histidine content in females is responsible for the higher BC. Oldershaw & Cameron (1989) observed a small but significant increase in mean protein concentration in male rats with LV hypertrophy compared to control, 164 ± 3 mg/g wet weight vs 154 ± 2 mg/g wet wt, respectively with a concomitant increase in the ability to regulate intracellular pH. The authors suggest this increase in the physiochemical buffering of the cell is secondary to the observed increase in protein content (Oldershaw & Cameron, 1989). Thus if no gender differences exist in the amount of CP, perhaps female hearts contain greater amounts of histidine-rich proteins, therefore resulting in a higher myocardial BC.

Not only were BC values in the RV similar in both males and females, but in contrast to the LV, BC values were as high as females. Thus perhaps in the RV, males and females exhibit equal quantities of phosphates and histidine-rich proteins, hence resulting in similar BC values. Or perhaps, males contain a higher amount of histidine-rich proteins in the RV, thus attaining similar BC values as females. In contrast to the RV, BC values in the LV of males did not vary, whereas the RV exhibited variability in the BC data. Females demonstrated variability in both ventricles and it is thought to be attributed to ovarian cycling. Although ovarian cycling does not occur in males, a diurnal cyclicity in plasma testosterone levels exist. In adult human males, testosterone levels increase during the early morning, reaching a peak (approximately 600 ng/dl) at noon and decreasing thereafter to 350 ng/dl (Montanini *et al.*, 1988). In adult male Wistar and Sprague-Dawley rats, plasma testosterone levels double from (1.0 ng/ml) early morning (8 am) to

noon (2.0 ng/ml), decreasing linearly afterwards to basal levels of 0.3 ng/ml by 2400 (Heywood, 1980 and Simpkins *et al.*, 1981). Our surgical protocol was conducted during daytime hours when testosterone levels are the most variable. Testosterone is known to influence protein synthesis in the heart via androgen receptors (Kinson *et al.*,1990; Marsh *et al.*, 1998 and Taylor & Al-Azzawi, 2000) which may occur within several hours (Degani *et al.*, 1984) . As previously mentioned, the slight increase in mean protein concentration observed by Oldershaw & Cameron (1989) resulted in a significant increase in the physiochemical buffering of the cell. Thus doubling of testosterone levels throughout the day may affect the BC outcome via affecting protein synthesis, thus possibly contributing to the variability observed in the RV. The lack of variability in the LV may be due to the differences in workload experienced. Perhaps the increased workload on the LV masks any testosterone affect on BC, whereas the RV may be more sensitive. On the other hand, perhaps the RV is more sensitive to testosterone compared to the LV.

Global Ischemia: Hydrogen Ion and Lactate Accumulation

The primary glycolytic substrate during ischemia is glycogen (Wittnich *et al.*, 1987; Berne & Levy, 1997 and Weiss *et al.*, 1996). Glycogen enters the glycolytic pathway as glucose-6-phosphate (Lehninger *et al.*, 1997). Numerous studies have shown that an increase in glycogen content increases the development of acidosis and lactate levels during ischemia (Cross *et al.*, 1996). In a Langendorff-perfused male Wistar rat model, Cross et al. (1996) elevated the glycogen content by adding insulin to a glucose-containing buffer for 80 minutes prior to 32 minutes of low-flow ischemia (0.5 mL/min). At end ischemia, tissue lactate was significantly higher and pH was lower in the high-glycogen hearts compared to control. Cave and Garlick

(1997) also reported a correlation between pre-ischemic glycogen content and pH decrease during subsequent ischemic periods. Interestingly, adult hypertensive SHR female rats demonstrate a 2-fold higher myocardial glycogen content compared to males (Wallen *et al.*, 1999) and normotensive WKY females demonstrate a higher PFK activity compared to males (Wallen *et al.*, 1999). If this relationship exists in adult Sprague-Dawley rats, then according to the above studies, one would hypothesize, due to the higher myocardial glycogen content in females, a greater degree of acidosis and lactate levels during ischemia will occur in females compared to males. Interestingly, the LV of females exhibited a trend toward a 3-fold higher $\Delta[H^+]$ during ischemia compared to males, contrary to the expected finding of a low $\Delta[H^+]$ due to the 2-fold higher BC. The higher $\Delta[H^+]$ was not due to a greater degree of anaerobic glycolysis as indicated by the 11% lower lactate levels. Preliminary work in our lab has shown an 11% greater glycogen utilization in adult female rats at 10 minutes of global ischemia compared to males. The lower lactate levels in females may be due to an enhanced initial rate of anaerobic glycolysis, producing H^+ and lactate faster, which would in turn, inhibit PFK (King & Opie, 1998) and halt glycolysis sooner. This early halt in anaerobic glycolysis during ischemia in females would, with prolonged ischemia, result in lower lactate levels compared to males. In the RV, no gender differences in the accumulation of H^+ were observed. This may be explained by the fact that in the RV, males maintained roughly similar H^+ accumulation levels as the LV, whereas H^+ accumulation levels in the RV of females are much lower than levels observed in the LV and similar to the males. The latter may be attributed to the 65% lower lactate levels in the RV of females compared to the RV of males. The lower lactate levels in the RV of females may be due to either a lower amount of glycogen content compared to the RV of males, or an early halt in aerobic glycolysis as explained above.

Recall, an additional source of H^+ during ischemia is ATP hydrolysis (Gevers, 1977 and Dennis *et al.*, 1991). Therefore, if by end ischemia anaerobic glycolysis is significantly depressed in both the LV and RV of females, another source of H^+, namely ATP utilization, must be contributing the higher H^+ accumulation levels observed in the LV and the similar H^+ accumulation levels observed in the RV, compared to males.

ATP Degradation

Degani et al. (1984) noticed an increase in ATP utilization paralleled a decrease in CP concentration. Preliminary studies in our lab demonstrated a 50% less CP content after 10 minutes to global ischemia in adult females compared to males. Therefore perhaps ATP utilization rates are higher in females and thus a contributing source of H^+ during ischemia.

Murray et al. (1990) suggest a lower ATP utilization rate, due to lower metabolic requirements, results in fewer generation of protons via hydrolysis. A reduced rate of ATP utilization may, in turn, reduce the rate of anaerobic glycolysis and vice-versa (Murray *et al.*, 1990). Murray et al (1990) examined adult mongrel dogs of either sex and subjected them to preconditioning prior to 40 minutes of global ischemia. Preconditioning is a method in which the heart is exposed to several intermittent short ischemic bouts followed by reperfusion (Weis *et al.*, 1996 and McNulty *et al.*, 1996). Hearts of preconditioned dogs had lower lactate levels attributed to a decrease in glycolysis and glycogen breakdown. ATP degradation was also significantly slower in the preconditioned group. The authors believe a slower rate of ATP degradation, as indicated by a decrease in adenosine and inosine during the first 20 minutes, is responsible for driving anaerobic glycolysis at a slower rate. Using a pig model, Kida et al. (1991) also found a preservation of ATP as well as a significantly higher pH_i in the preconditioned group compared to control during 60 minutes of global ischemia. The authors

concluded the preservation of pH_i was due to a reduced ATP degradation. Schaefer et al. (1995) found results similar to Kida et al (1991) in isolated perfused hearts of adult male Sprague-Dawley rats subjected to 30 minutes of global ischemia. The authors observed a less rapid decline in pH_i in the preconditioned group as well as slower rates of ATP utilization, suggesting the two are linked. Therefore if ATP utilization is higher during ischemia in females compared to males, this would result in higher H^+ levels during global ischemia.

Reduced ATP degradation may be due to a decrease in reactions that consume ATP during ischemia. Such reactions may include mitochondrial ATPases, myosin ATPase, Na^+-K^+-ATPase or Ca^+-ATPase by the sarcoplasmic reticulum and phosphorylation of phosphorylase kinase. (Jennings & Reimer, 1981; Murray *et al.*, 1990; Schaeffer *et al.*, 1995 and Taegtmeyer *et al.*, 1998). Cross et al. (1996) found ATP consumption was due to the activation of myosin-ATPase in a Langendorff-perfused male Wistar rat model subject to 32 minutes of low-flow (0.5 mL/min) ischemia. Rouslin & Brogue (1993) also found myosin ATPase to be the most active ATPase reaction during 30 minutes of global ischemia in adult Sprague-Dawley rats of either sex using the Langendorff model. Whether gender differences exist in the activity of myosin ATPase under ischemia is unknown. There are conflicting results as to whether or not mitochondrial ATPase is a significant contributor to ATP depletion during ischemia (Rouslin *et al.*, 1990; Kobara *et al.*, 1996 and Green *et al.*, 1998). Under aerobic conditions, mitochondrial ATPase uses the driving force of the electrochemical proton gradient across the mitochondrial inner membrane to phosphorylate ADP. Under ischemic conditions mitochondrial ATPase is thought to reverse the reactions, thus hydrolyzing ATP in an attempt to maintain the electrochemical gradient (Kobara *et al.*, 1996 and Green *et al.*, 1998).

Thus, if gender differences exist in any one of the above mentioned ATP consuming

reactions, the female trend to accumulating more H^+ in the LV during global ischemia, despite lower degree or early inhibition of anaerobic glycolysis, may be due to enhanced ATP utilization compared to males.

In summary, baseline myocardial [H^+] in the LV and RV did not differ between males and females and all values fell within the normal physiological pH range. Physiological baseline lactate levels were obtained in both the LV and RV of males and females, however the RV of females had significantly lower lactate levels compared to the RV in males. This may be due to a greater utilization of lactate in the RV of females. In the LV, females demonstrated a significantly 2-fold higher BC compared to males. The latter may be due to higher amount of histidine-rich proteins in the LV of females. No differences in BC were found in the RV between males and females, possible due to equal concentrations of phosphates and histidine-rich proteins in the RV of males and females. Despite the 2-fold higher BC in females compared to males, $\Delta[H^+]$ during global ischemia in the LV of females appeared to be 2.5 times greater as well as variable. Anaerobic glycolysis may be inhibited earlier in the LV of females as indicated by the lower lactate levels at 30 minutes of global ischemia. Therefore perhaps ATP utilization is enhanced in females contributing to the higher H^+ levels. Lactate levels at thirty-minutes of global ischemia are also significantly lower in the RV of females compared to males with no differences in $\Delta[H^+]$. Again, perhaps ATP utilization rates are higher in the RV of females compared to males, resulting in no gender difference in $\Delta[H^+]$ in the RV. Furthermore, the variability in BC, $\Delta[H^+]$ particularly in the LV, may be due to ovarian cycling.

Part 2:
Females
Ovarian Cycling

Due to the large variability in female data compared to males, ovarian cycling is suspected to impact female responses, hence possibly explaining the variability observed. Therefore additional females were staged according to their vaginal smears and the study was repeated.

In vivo $[H^+]$

There were no differences in baseline myocardial $[H^+]$ between the four stages of the estrous cycle and all values fell within normal physiological range (Ellis & Thomas, 1976; Portner *et al.*, 1990 and Ng *et al.*, 1993). This indicates the following, 1) the heart was not experiencing any stress which may perturb values, 2) *in vivo* biopsies were accurately obtained and 3) the stage of the cycle does not affect *in vivo* $[H^+]$.

Buffering Capacity (BC)

Ovarian cycling did in fact affect the BC in females. Females in estrus and metestrus, when both estradiol and progesterone levels are basal, demonstrated a 50% lower BC than females in proestrus. In addition, BC values in estrus and metestrus were similar to the BC values in males. No differences in BC were observed between diestrus and proestrus. During diestrus estradiol levels begin to rise and the BC values in this stage were variable. However during proestrus, the BC values increased in variability, as do the estradiol levels, ranging from

extremely low to extremely high. Thus, as the hormone levels begin to fluctuate in diestrus and proestrus, BC values also began to fluctuate, suggesting the variability in proestrus and diestrus may be due to the variability in estradiol and/or progesterone hormone levels.

Estradiol has been shown to rapidly induce the activity of CK in the rat uterus (Philipson *et* al., 1985). Whether this occurs in the heart is unknown. If this does occur in the heart, this would translate into a rapid utilization of the CP pool, resulting in a greater reliance on histidine-rich proteins for buffering H^+. Conversely, at low estradiol levels, as in estrus and early metestrus, CK would not be as rapidly induced and thus utilization of the CP pool may occur at a slower rate. A slower utilization of CP in estrus and early metestrus may result in a slower reliance on histidine-rich proteins, possibly resulting in a low BC compared to that seen in late diestrus and early proestrus. Furthermore, perhaps the amount of histidine-rich proteins also varies according to the stage of the estrous cycle. Estrogen is known to stimulate protein synthesis in rat uteri (Degani *et al.*, 1984) and mRNA levels have been shown to increase with increasing estradiol levels within a few hours (Lephart *et al.*, 1992). If this occurs in the heart, then during the stages when estradiol levels are high, as in proestrus and late diestrus, protein synthesis will be increased resulting in a higher concentration of proteins in the cell, potentially enhancing the BC of the heart. This would explain the high BC values observed during diestrus and proestrus. However the BC values during diestrus and proestrus were also variable. The variability may be due to the effect of progesterone. Using mouse uterus, Paria et al. (1998) found progesterone to increase both the activity of histidine decarboxylase (HDC), the enzyme responsible for the breakdown of histidine to histamine, as well as the mRNA levels of the gene in a dose-dependent manner. If this occur in the hearts of Sprague-Dawley rats, free histidine levels would decrease due to the increase in breakdown, potentially limiting the amount of

histidine-rich proteins available for buffering. This would ultimately decrease the BC of the cell. Thus if females were obtained in early diestrus and late proestrus when progesterone levels are high, and if the latter applies to rat heart, this may not only potentially explain the low BC values obtained, but also the variability in the data.

Global Ischemia: Hydrogen Ion and Lactate Accumulation

It is interesting to note, despite a lower BC in estrus and metestrus, the LV of females in estrus appeared to accumulate the least H^+ followed closely by those in metestrus. The accumulation of H^+ appeared to be higher as well as variable in diestrus and proestrus. This potentially implies a production issue specific to the stages of the ovarian cycle, dependent on the profile of the hormones. That is to say, during estrus and metestrus, when estradiol and progesterone levels are basal, H^+ production appear to be consistently lower than diestrus and proestrus when estradiol and progesterone levels fluctuate. The variability in values observed in proestrus may be due to the extreme variations in the levels of sex hormones during this phase, possibly impacting glycogen stores and/or ATP consuming reactions and thus ultimately acidosis. Furthermore, no significant differences were noted with respect to the levels of lactate at thirty-minutes of global ischemia, implying similar rates of anaerobic glycolysis. However it is interesting that estrus and diestrus exhibited the greatest variability, potentially implying a modification in the rate of anaerobic glycolysis even within a stage of the ovarian cycle.

The effects of estradiol and progesterone on glucose metabolism are well established (Nolan & Proietto, 1995) and it is thought that the relative concentrations of estradiol and progesterone ultimately determines the extent of action of one hormone over the other (Kalkhoff, 1982 and Cano, 1999). Estrogen utilization has been shown to be related to the estrous cycle

(Dunn *et al.*, 1972 and Nehlig *et al.*, 1985). In adult female Sprague-Dawley rats, average glucose utilization in the brain was highest during proestrus and metestrus, whereas lower rates were observed during estrus and diestrus (Nehlig *et al.*, 1985). If this occurs during ischemia, this may explain the high lactate levels observed in proestrus and metestrus. Ovariectomized females displayed uniformly lower rates of glucose utilization, which matches the low lactate level in estrus. Nehlig et al. (1985) discovered a variability in glucose utilization rates when estrogen replacement was administered in low and high doses, clearly suggesting that hormones play a role in the regulation of cerebral energy metabolism. Thus if this also applies to the heart, the variability observed in proestrus may be due to the low and high estradiol levels which occurs within proestrus. Estrogen not only has been shown to increase glucose utilization in a variety of tissues (Bishop & Simpkins, 1995), but the addition of progesterone in combination with estradiol, has been shown to decrease glucose utilization (Porrino *et al.*, 1982). Progesterone has also been found to decrease glucose metabolism in a dose-dependent manner in rat adipose tissue in as little as twenty-minutes (Sutter-Dub, 1986 and Cordoba *et al.*, 1991). If this effect is also noted during ischemia, perhaps the high concentration of progesterone during early estrus and early diestrus, inhibits glucose utilization thus resulting in the lower lactate level observed in estrus and diestrus. Likewise, during the remainder of estrus and mid-late diestrus, progesterone levels are basal and thus perhaps the inhibition on glucose metabolism due to progesterone is released, resulting in an increase in anaerobic glycolysis and thus high lactate levels observed in late diestrus and estrus. In addition to glucose utilization, the substrate level of glycogen, which is the major endogenous source of glucose during ischemia, may impact the development of lactate levels.

In rats, estradiol is known to increase glycogen content in several tissue types, including

the heart (Kendrick *et al.*, 1987 and Nicklas *et al.*, 1989). Ovariectomy in adult female Sprague-Dawley rats reduced cardiac glycogen content and estradiol replacement restored the glycogen content (Kendrick *et al.*, 1987). When progesterone was administered in combination with estradiol, Ahmed-Sorour and Bailey (1981) found cardiac glycogen to increase although not to the same extent as when estradiol was administered alone. The indicates a potential antagonizing effect of progesterone on estradiol's effects. Therefore, glycogen content may potentially vary in diestrus, that is, low in early diestrus when progesterone levels are high and high in mid-late diestrus when progesterone levels are low. If this occurs, it may explain the observed variability in lactate levels in diestrus during ischemia. Furthermore, this study may also explain the outcome of lactate levels at thirty-minutes of global ischemia in metestrus compared to proestrus. Estradiol alone would mimick early to mid proestrus and the addition of progesterone would mimick late metestrus. The possible blunting effect of progesterone in metestrus may cause lactate levels to still be high, but not as high as in early proestrus when estradiol levels are high and progesterone levels are basal.

In addition to the above, steroid hormones have been shown to affect the activity of a number of glycolytic enzymes (Chainy and Kanungo, 1978; Takehisa, 1980; Kostanyan and Nazaryan, 1992 and Baquer *et al.*, 1993). Estradiol has been shown to induce the activity of pyruvate kinase in rat hearts (Chainy & Kanungo, 1978) as well as the activity of HK and PFK, with the highest activity observed in PFK (Kostanyan and Nazaryan, 1992) in brain. The authors speculate that variations in the physiological concentration of estradiol may regulate the glycolytic pathway. That is to say, in the presence of high estradiol levels, glucose entry into the glycolytic pathway would not only be increased due to HK but enhanced due to PFK. PK would stimulate glucose to completion, that is pyruvate. If this occurs during ischemia, an enhancement

in the glycolytic pathway may result in high lactate levels. As well, the activity of lactate dehydrogenase (LDH), the enzyme responsible for reversible conversion of pyruvate to lactate, has been shown to vary in cervical mucus according to the human menstrual cycle (Takehisa, 1980). The activity is highest during the proliferative or Follicular phase of the human menstrual cycle, lowest at ovulation followed by an increase afterwards. Estrogen was discovered to be responsible for the inhibition in activity whereas progesterone increased the activity (Takehisa, 1980). Perhaps the high concentration of progesterone during early estrus is acting to inhibit/reduce the activity of key glycolytic enzymes, thus depressing anaerobic glycolysis and therefore resulting in a low lactate level at thirty-minutes of global ischemia.

In conclusion, although no statistical difference existed between stages of the estrous cycle with respect to lactate levels at thirty-minutes of global ischemia, variability was observed. This variability may be due to alterations in the rates of anaerobic glycolysis in response to changes in estradiol and progesterone hormone levels.

ATP Degradation

Estrus appeared to accumulate the least $\Delta[H^+]$ during global ischemia, followed by metestrus with variability increasing in diestrus and proestrus. If ATP utilization rates vary across stages, this would affect $\Delta[H^+]$ outcome during global ischemia. Degani et al. (1984) demonstrated an increase in ATP utilization during the first four hours after estrogen administration as well as a subsequent decrease in the concentration of ATP. This increase in ATP utilization is thought to be due to the increase in DNA, RNA and protein synthesis (Degani *et al.*, 1984). Thus the increase in estradiol levels from estrus to proestrus, results in an increase in glucose utilization (Porino *et al.*,1982 and Nehlig *et al.*, 1985) as well as an increase in DNA,

RNA and protein synthesis (Degani *et al.*, 1984) and ultimately an increase in ATP utilization. Therefore if the cell is modified to a state in which ATP utilization is increased, then if this occurs during ischemia, the increase in ATP utilization would result in greater H^+ levels. An increase in ATP utilization during high estradiol levels, may explain the higher H^+ accumulation levels observed during global ischemia in diestrus and proestrus. Likewise, the lower H^+ levels observed during estrus and metestrus may be due to a decrease in ATP utilization. Furthermore, estrogen has been shown to modulate the activity of myosin ATPase, an enzyme which takes part in a major ATP consuming process (Dillmann, 1984). Schaible et al (1984) examined the activity myosin ATPase in adult female Wistar rat hearts (LV), intact and ovariectomized. The authors discovered a significant decrease in the activity of the enzyme in ovariectomized compared to intact rats. The alterations in the activity of myosin ATPase were associated with a reduction in the V1 myosin subunit and an increase in the V3 subunit. Furthermore, the changes in the isoenzyme patterns were restored upon estrogen replacement (Malhotra *et al.*, 1990 and Calovini *et al.*, 1995). If the effect of estradiol on myosin ATPase activity is dose-dependent, then ATP utilization would be enhanced during periods of high estradiol concentrations, that is late diestrus and early proestrus, thus producing more H^+ and ultimately resulting in a higher $\Delta[H^+]$ during global ischemia.

In summary, stages of the ovarian cycle affect the buffering capacity and possibly the degree of acidosis during global ischemia. This ovarian cycling effect may also explain the controversy in the literature relating to gender differences in the outcome after an MI, in that results will vary depending on a woman's phase of the ovarian cycle. Furthermore, the results of this thesis may explain the findings that infarct size after an MI in female Sprague-Dawley rats was greater in the ovariectomized females receiving17β-estradiol compared to those not

receiving the hormone. This is potentially true as studies have shown an increase in cell necrosis with an increase in acidosis (Poole-Wilson, 1989; Murray *et al.*, 1990 and Kida *et al.*, 1991). Therefore, the increase in H^+ levels observed during diestrus and proestrus could increase infarct size as this did lead to a trend toward increased mortality with the estradiol replaced group (Smith *et al.*, 2000). If this is the case, women experiencing an MI may be at varying risks of ischemic injury according to the stage of their menstrual cycle. According to the data presented in this thesis, women may be at a greater risk of injury when they are in the phase of their ovarian cycle when estradiol levels are high.

Part 3:
Right Ventricle vs. Left Ventricle
Males and Females

In vivo: $[H^+]$ and Lactate

In both males and females, there were no differences in myocardial baseline $[H^+]$ between the left and right ventricles and all values fell within the accepted physiological pH range (Ellis & Thomas, 1976; Portner *et al.*, 1990 and Ng *et al.*, 1993). Similarly, there were no differences in baseline lactate levels between ventricles in both males and females and all values fell within the normal lactate range (Neely & Grotyohann, 1984; Tanonaka *et al.*, 1996; Higuchi *et al.*,1997 and Geraldes *et al.*, 1997). This suggests the heart was not under any stress when *in vivo* biopsies were taken.

Buffering Capacity (BC)

In males, the BC of the RV was 2-fold greater compared to the LV. In addition, BC values in the RV were variable. As explained earlier, the variability may be due to the circadian rhythm of testosterone throughout the day. Once again, perhaps the lack of variability in the LV compared to the RV is due to an increased testosterone sensitivity in the RV. The 2-fold higher BC in the RV compared to the LV observed in males may be due to a greater concentration of the CP pool and/or histidine-rich proteins in the RV. Whether ventricle differences exist with respect to the amount of CP and histidine-rich proteins is unknown. Preedy et al. (1985) found protein synthesis and RNA/protein ratios to be 10% greater in the RV of rats compared to the LV and Oldershaw & Cameron (1989) observed an increase in the physiochemical buffering in

hypertrophied rat hearts which had a small but significant increase in mean protein concentration compared to control. Recall, previous work on adult pigs by Su et al. (1993) found a strong correlation between histidine content and BC. Thus perhaps the RV has a higher protein content compared to the LV and thus enhanced buffering as a result.

In contrast to the males, BC values were similar as well as equally variable in both the LV and RV in females. This implies the quantity of phosphates and histidine-rich proteins is similar in both ventricles. As previously discussed, the variability may be due to ovarian cycling.

Global Ischemia: Hydrogen Ion and Lactate Accumulation

In males, $\Delta[H^+]$ in the RV was 40% lower compared to the LV. Anaerobic glycolysis is similar between the LV and RV, as indicated by the similar lactate accumulation levels during global ischemia. Therefore, the lower accumulation of H^+ in the RV may be explained by the 2-fold higher BC. Also, perhaps ATP utilization is higher in the LV due to the greater workload experienced (Ganong, 1997) compared to the RV. As mentioned previously, myosin ATPase activity is involved in a major reaction which utilizes ATP. Male neutering has been shown to reduce the activity of this enzyme from the V_1 isoform to an energy conserving form V_3 (Dillmann, 1984; Schaible *et al.*, 1984; Calovini *et al.*, 1995). Testosterone replacement in males has been shown to restore the activity to the V_1 isoform (Schaible *et al.*,1984 and Calvolini *et al.*,1995). Due to the greater workload experienced by the LV, the variability in testosterone levels throughout the day may affect the level of myosin ATPase activity, which may affect ATP utilization during ischemia and thus ultimately H^+ accumulation. If this occurs, H^+ accumulation would be greater in the LV compared to the RV.

Despite similar BC values in the LV and RV of females, $\Delta[H^+]$ was 3.8 fold lower in the RV. The lower $\Delta[H^+]$ may be due to a lower rate of anaerobic glycolysis as observed by the 34% lower lactate levels at thirty minutes of global ischemia in the RV compared to the LV. Therefore, the higher $\Delta[H^+]$ in the LV may be due to a higher rate of anaerobic glycolysis in combination with a higher rate of ATP utilization due to the greater workload it experiences (Ganong, 1997). This increase in H^+ may overwhelm the BC system despite the high BC in the LV. Once again, the variability observed in both BC and $\Delta[H^+]$ in the LV may be related to ovarian cycling as previously discussed. Furthermore, LV-RV differences with respect to $\Delta[H^+]$ and lactate levels at thirty-minutes of global ischemia were exacerbated in females compared to males. This not only indicates LV-RV differences in response to ischemia, but also gender differences between ventricles.

In summary, although anaerobic glycolysis was similar in the LV and RV of males, $\Delta[H^+]$ was 40% lower in the RV than the LV. This lower accumulation in H^+ may be due to the two-fold higher BC in the RV compared to the LV. Furthermore, the ATP utilization may be higher in the LV thus resulting in a higher H^+ levels during ischemia. Despite similar BC values in the LV and RV of females, the RV accumulated 3.8 fold lower $\Delta[H^+]$ during global ischemia and 34% lower lactate levels at thirty-minutes of global ischemia compared to the LV. Thus the lower $\Delta[H^+]$ in the RV may be due to a lower rate of anaerobic glycolysis in addition to the high BC. The higher accumulation of H^+ during ischemia in the LV may be due to a higher degree of anaerobic glycolysis and/or ATP consuming reactions. This implies the LV may be facing a H^+ production issue.

Conclusions

In conclusion, in the LV, females exhibited a trend in the accumulation of H^+ during asystolic global ischemia compared to males, whereas in the RV no differences in $\Delta[H^+]$ were observed. This trend in the LV was not due to an increase in anaerobic glycolysis as indicated by the significantly 11% lower lactate levels at thirty-minutes of global ischemia. Lactate levels at thirty-minutes of global ischemia were also 65% lower in the RV of females compared to the RV of males. The lower lactate levels in females may be due to the higher PFK activity which may increase anaerobic glycolysis rapidly during early ischemia, resulting in an early inhibition in anaerobic glycolysis and thus lower lactate levels compared to males. Due to a depressed anaerobic glycolysis in the LV and RV of females, ATP utilization must be greater in both ventricles of females in order to achieve the trend to a higher H^+ accumulation or similar $\Delta[H^+]$ levels in the LV and RV, respectively, compared to males. A low BC would explain a higher H^+ accumulation during ischemia, however females exhibited a two-fold higher BC in the LV compared to males. Estradiol has been shown to increase protein synthesis and therefore, the higher BC may be due to a greater content of histidine-rich protein in females compared to males. In the RV, BC was similar between males and females as well as variable. Testosterone is also known to increase protein synthesis which may affect BC via affecting the content of histidine-rich proteins. A circadian rhythm of plasma testosterone exists in males, and perhaps the RV is sensitive to this rhythm thus contributing to the variability in BC values in the RV compared to the LV.

Buffering capacity in the LV was affected by the stage of the estrus cycle in females.

Estrus and metestrus exhibited a 50% significantly lower BC compared to proestrus. Females in estrus and metestrus, when estradiol and progesterone levels are basal, had BC values similar to males in the LV. Thus perhaps when estradiol levels are low, protein content is low, thus contributing to a low BC in estrus and metestrus. Significance is lost as the BC values begin to vary in diestrus and proestrus, an effect probably due to the large variation in estradiol and progesterone levels during diestrus and proestrus. Although not significant, $\Delta[H^+]$ appeared to be the lowest in estrus followed by metestrus. Once again, the variability in diestrus and proestrus may be due to the large variability in hormone levels during the latter two stages. Lactate levels at thirty-minutes of global ischemia were not significant across stages, however variability existed within each stage. This variability may be due to a variability in anaerobic glycolysis caused in turn by the effects of estradiol and progesterone on substrate levels and/or enzyme activity. Overall, the ovarian cycling affect noticed in this study may explain the controversy in the literature relating gender differences in the outcome after an MI.

Females demonstrated exaggerated LV-RV differences with respect to $\Delta[H^+]$ during global ischemia and lactate levels at thirty-minutes of global ischemia. The 50% greater BC in the RV of males compared their LV may be due to an increase in protein content in the RV. The lower $\Delta[H^+]$ in the RV compared to the LV in males was not due to a lower rate of anaerobic glycolysis, and therefore probably due to the higher BC in the RV. The 3.8 fold lower $\Delta[H^+]$ during global ischemia in the RV of females compared to their LV may be due to a lower degree of anaerobic glycolysis and/or decrease in ATP utilization.

Implications

This data potentially implies the following,

1. Despite the higher BC in females compared to males, hydrogen ion accumulation appeared to be higher and therefore females may be at a greater risk of ischemic injury after an MI compared to males.

2. A woman may be at varying risks depending on the stage of her menstrual cycle when experiencing a myocardial infarction.

3. In both males and females, the LV may be at a greater risk of ischemic injury compared to the right. Furthermore, this difference between ventricles is exaggerated in females, thus the RV in females may recover better after a myocardial infarction.

Limitations

There are several limitations to this thesis.

1. A single method for quantifying myocardial BC is not universally accepted and many methods exist ranging from acid loading to ^{31}PNMR (Ng *et al.*, 1993). The pH of the homogenate in this protocol is dropped to 6.00 ± 0.5 thus removing bicarbonate as a contributing factor to the final BC measurement. This protocol is an accepted and reliable method (Pörtner *et al.*, 1990 and Corbett *et al.*, 1992) and includes the contribution of phosphate compounds and histidine-rich proteins. A limitation to this protocol is it does not allow for a quantification of each component. In order to determine how much phosphate and histidine-rich proteins contributed to the buffering of the cell, one would determine, both before and after; 1.the amount of creatine phosphate, 2. phosphates, and 3. histidine-rich proteins.

2. Tissue homogenate consists of intracellular (66.7%) and extracellular fluid (33.3%) (Ganong, 1997). Thus a pH reading obtained from the homogenate consists mainly of the intracellular environment. The pH of intracellular organelles is generally lower than the cytosol and could contribute to false low intracellular pH readings if included. This is avoided using a homogenizer designed to disrupt only plasma membranes (Brinkmann, 1992), thus permitting the determination of mean intracellular pH readings. Although the pH values in this study were comparable to other methods, one way to avoid any errors would be to directly measure intracellular pH *in vivo* with a pH probe.

3. This study also did not identify the source of H^+. In order to do so, one would measure the amount of ATP, before and after, as well as the intermediates, ADP, AMP and adenosine. In addition to lactate (measured in this study), intermediates such as glucose-6-phosphate and

fructose 1,6-bisphosphate in the glycolytic pathway would provide an overall picture of anaerobic glycolysis. Measurement of glucose-1-phosphate, before and after, would allow for an interpretation of glycogen breakdown.

4. The rate of anaerobic glycolysis with respect to gender was an issue in this study. A measurement of glycolytic intermediates as well as the activity of glycolytic enzymes during the entire time course of ischemia would allow for the rate to be determined. Furthermore, enzyme activity analysis of phosphorylase *a* during ischemia would assess the rate in glycogen breakdown.

5. Determination of plasma hormone concentration would clearly identify the effects of hormones on BC and H^+ accumulation. Furthermore, incorporating neutered and hormone (estradiol or progesterone)- replaced females would tease out which hormone is responsible for the cycling affect.

6. The activity of transmembrane transporters would be of interest in order to understand the exact mechanisms of H^+ handling during early ischemia.

7. This study does not mimic a typical MI seen clinically and thus caution should be taken when interpreting these results and extrapolating them to an MI setting. The present model does however identify gender differences in the response to a maximal ischemic stress in a non-beating heart. In order to mimic an MI, the left anterior descending coronary artery (LAD) would be ligated and the study repeated.

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Appendices

Appendix 1

pH Meter

A pH meter (Figure 31) measures the pH of a solution with the immersion of two electrodes into the solution and reading the pH value on a screen. The electrodes measure pH based on a membrane potential across the electrode. A pH dependent electrode governs the system and a pH independent electrode acts as the reference point. The voltage between the two electrodes is proportional to pH and read on the pH meter. The glass electrode is the most commonly used pH dependent electrode which is permeable to H^+ and impermeable to other cations and anions (Freidfelder, 1982).

Although the P.N.M.R (phosphorous (^{31}P) nuclear magnetic resonance) method is rapid and noninvasive, in measuring pH, use of a pH meter, for the purpose of this study, has obtained pH values similar to those studies using NMR spectroscopy (Pörtner *et al.*, 1990) and thus is a reliable technique. Furthermore, homogenate derived intracellular pH values (pH_i) represent effective mean pH_i values (Kienlin *et al.*, 1998).

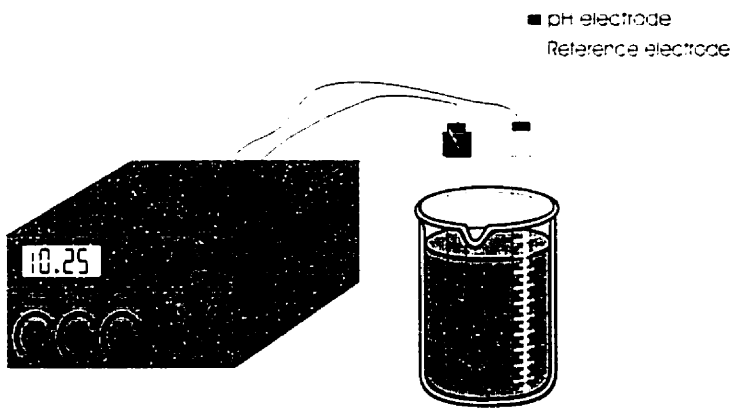

Figure 31: A pH meter. Modified from Freifelder 1982.

Appendix 2

Fluorescence Spectroscopy

Fluorescence spectroscopy is based on the theory that molecules possess energy. If a molecule is unexcited it is said to be in a resting or ground (G) state. Light energy can be absorbed from a molecule once it is excited to an excited state (S). When the molecule returns to its G state, it emits light (fluorescence) with a given wavelength.

The spectrofluorometer follows a series of steps in measuring fluorescence of a sample. Refer to Figure 32 below. Initially, a high intensity light beam passes through a monochromator causing an excitation wavelength. This excited light then passes through the sample port containing the sample to be analyzed. The fluorescence emitted by the sample then passes through a monochromator for wavelength analysis and finally a photosensitive detector.

Other spectrophotometric methods exist which measure lactate, however the fluorometric method is currently the most valuable and acceptable method for measuring lactate. It is very sensitive due to the employments of two wavelengths and thus provides a variety of valuable information requiring only small amounts of the material (Freifelder, 1982).

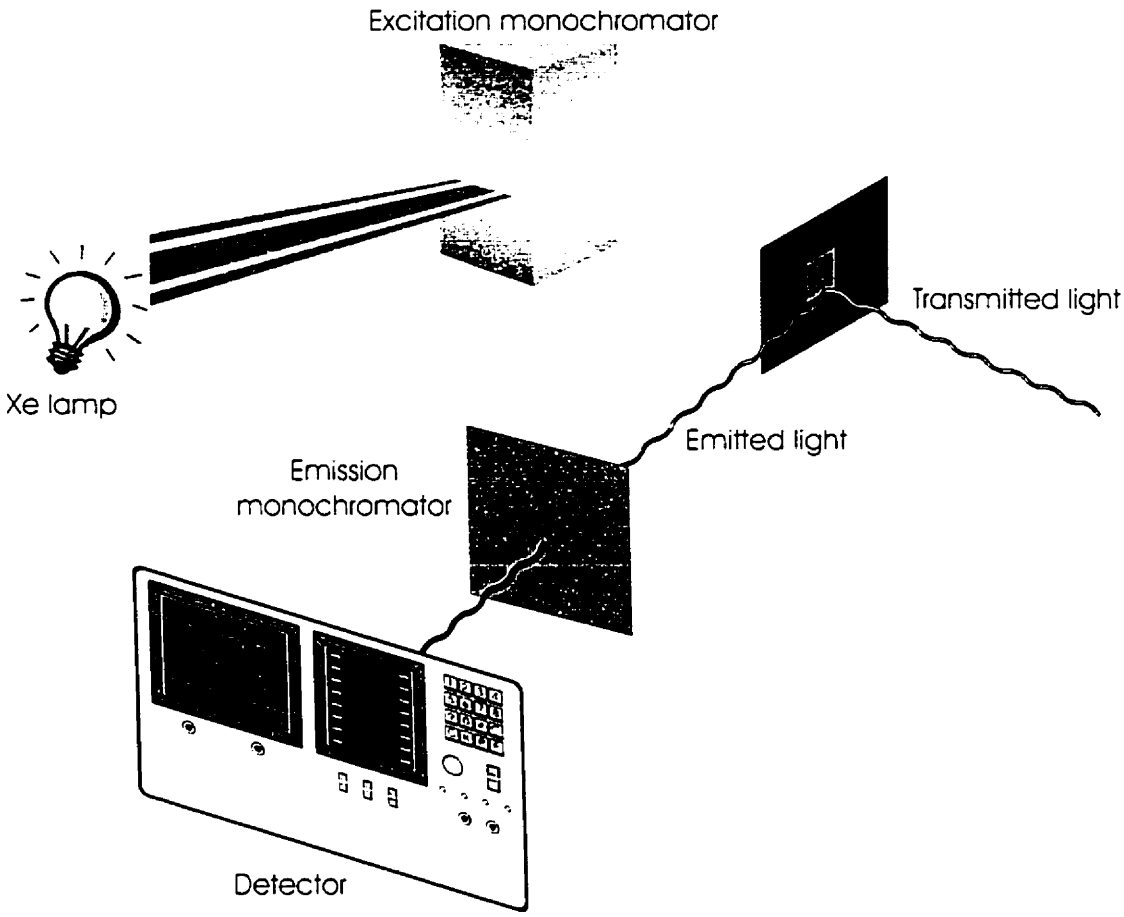

Figure 32: Theory of a fluorescence spectrophotometer. (Xe = Xenon)
Modified from Freifelder 1982.

Appendix 3

Power calculations were conducted for results which demonstrated no statistical significance.

Table 11: Power calculations for non statistical significant results.

Data	Power (%)
RV-BC (males vs females)	87
RV-$\Delta[H^+]$ (males vs females)	57
BC-LV vs RV (females)	45
Δlactate LV vs RV (males)	98

Therefore, a power of 87% states that 87 percent of the time there is no difference in BC between males and females in the RV. The low power of 57% states that 57 percent of the time there is no difference with respect to hydrogen ion accumulation in the RV between males and females. However, according to the preliminary sample size calculations on page 57 and the power n number calculation, the numbers used in this analysis should have sufficed. Interestingly, when the n number was recalculated using the formula on page 57 with the final data, an n number of 11 is required to ensure significance. A low power resulted for BC-LV vs RV in females, stating 45 percent of the time there is no difference. Similarly, when preliminary data was used in the formula on page 57 and the power n number calculation, the number used in the study once again should have sufficed. Again when using the final data was used in the n number calculation on page 57, 40 animals would be required to ensure significance. For Δlactate in males the power was 98% and clearly no difference between ventricles with respect to lactate accumulation.