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AN EVALUATION OF MAGNESIUM STATUS AND INFLAMMATORY

RESPONSE DURING THE THIRD TRIMESTER OF NORMAL

PREGNANCY AND PREECLAMPSIA

By

Fauzia Asadullah Khan

A Dissertation
Submitted to the Faculty of
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in Nutrition
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Mississippi State, Mississippi

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Recent reports suggest that preeclampsia is the result of an excessive maternal
systemic inflammatory response. The role of magnesium and systemic inflammation in
normal pregnancy and preeclampsia needs to be defined.   The objectives of this study
were to compare (1) demographic and (2) anthropometric characteristics of normal and
preeclamptic pregnant women; and (3) to determine and compare serum iMg^{++} and tMg
concentration and the association between inflammatory makers such as TNFα, leptin,
adiponectin, IL10, ET-1, triacylglycerol, FFA, serum iMg^{++} and, tMg concentration of
normal and preeclamptic pregnant women during the third trimester of pregnancy. Thirty-
five healthy pregnant and thirty-five preeclamptic pregnant women in their third trimester
volunteered to participate in the study. Analyses were done by the use of ion-selective
electrode, atomic absorption spectrometry, spectrophotometry, and ELISA kits.

Pregnant normal and preeclamptic subjects were paired by age, parity, and week
of gestation. Gestational age, body weight, age, prepregnancy diastolic BP, intake of

birth control pills, supplements, miscarriage, still births, race, and employment status were statistically unchanged between the two groups. Current systolic and diastolic BP, family history of preeclampsia, urine albumin, minimum, maximum temperature and precipitation at the time of presentation were found to be significantly increased in the preeclamptic group. Number of children and pregnancies were significantly higher in the normal pregnant group.

In the preeclampsia group, TNFα, ET-1, leptin, FFA, and triacylglycerol blood concentrations increased while tMg, IL10, and adiponectin blood concentrations decreased. Significant positive correlation was recorded between iMg++ and tMg and between iMg++ and tMg with IL10 and adiponectin in the preeclamptic group. An inverse correlation of iMg++ and tMg with TNFα, ET-1, leptin, FFA, and triacylglycerol, was seen in the preeclamptic group.

Understanding the role of demographics and anthropometric characteristic during pregnancy and preeclampsia along with high correlation of iMg++ and tMg with each other and with inflammatory markers, increase in blood concentration of TNFα, ET-1, leptin, FFA, and triacylglycerol, and decrease in blood concentration of tMg, IL 10, and adiponectin could be an important step in understanding the pathophysiology of preeclampsia.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Preeclampsia is one of the most dreaded complications of pregnancy that puts the well being of the mother and fetus in jeopardy with increased risk of maternal and fetal death and with no satisfactory therapy readily available. The cause of this strictly pregnancy related disease is still unknown and is therefore a great challenge to all researchers in the field of pregnancy related pathophysiology. Preeclampsia is characterized by high blood pressure, proteinuria, and edema. Hypertension after 20 weeks of gestation is essential for diagnosis.

Preeclampsia is traditionally called the "disease of theories". To date the dominating theory of origin of preeclampsia is vascular endothelial cell activation and exaggeration of maternal inflammatory response. Recent reports suggest that the clinical features of preeclampsia are the result of an excessive maternal systemic inflammatory response. Several investigators claim that normal pregnancy is also a state of maternal systemic inflammation, which is not different from that of preeclampsia except that it is milder. Any factor that would increase the maternal systemic inflammatory response to pregnancy would predispose to preeclampsia. Thus, preeclampsia could be described as an exaggerated maternal systemic inflammatory response brought about by pregnancy in concert with some other predisposing factors.

Pregnancy has been stated as a condition of "physiological hypomagnesaemia" and a state of relative or mild insulin resistance and hyperinsulinemia. Magnesium deficiency is associated with inflammatory/pro-oxidant condition, hyperlipidemia, hypertension, insulin resistance, hyperinsulinemia, and endothelial cell dysfunction. Hyperlipemia resulting from accumulation of triacylglycerol rich lipoproteins, free fatty acids (FFAs), and inflammatory response with a marked leukocytosis and increase in plasma concentration of inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), interleukin-1 (IL-1), and interleukin-8 (IL-8) follows magnesium deficiency. Furthermore, magnesium deficiency is accompanied by activation of pro-inflammatory cells such as neutrophils, macrophages, and endothelial cells, which can contribute to the increased production of pro-inflammatory cytokines especially TNFα. Magnesium deficiency promotes endothelial cell dysfunction by promoting a proinflammatory, proatherogenic, and prothrombotic environment. Increased concentration of endothelin-1 (ET-1) in response to endothelial cell dysfunction further contributes to the vasoconstricted and inflammatory state. Concomitantly elevated plasma leptin concentration is associated with insulin resistance and also contributes to endothelial cell dysfunction by increasing FFA oxidation and inflammation.

Adiponectin is insulin sensitizing, anti-inflammatory, and anti-atherogenic, and a decreased plasma concentration of adiponectin leads to insulin resistance, inflammation, and endothelial cell dysfunction. Interleukin-10 (IL-10) inhibits the production of macrophage derived TNFα thus suppressing inflammation, and decreased plasma concentration of IL-10 promotes inflammation.

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There are several abnormalities that are common between magnesium deficiency and preeclampsia such as hypertension, dyslipedemia, endothelial cell dysfunction, insulin resistance, and systemic inflammation. Consequently, magnesium could be considered as the missing link or recurring factor between preeclampsia and inflammation, insulin resistance, hyperlipedimia, endothelial cell dysfunction, and hypertension.

The primary objectives of the proposed study were:

1. Demographic: To compare constitutional characteristics and medical history, age, race, parity, miscarriage, abortion, still births, number of children, and vitamin and mineral supplements of normal and preeclamptic pregnant women.

2. Anthropometric: To compare pre-pregnancy weight, current weight, and pre-pregnancy and current blood pressure of normal and preeclamptic pregnant women.

3. To determine and compare blood ionized magnesium (iMg++) and serum total magnesium (tMg) concentration and the association between inflammatory makers such as TNFα, leptin, adiponectin, ET-1, and IL-10, triacylglycerol, free fatty acid, iMg++, and serum tMg concentration of normal and preeclamptic pregnant women during the third trimester of pregnancy.

CHAPTER II

REVIEW OF LITERATURE

Magnesium

The usual daily intake of magnesium ranges from 300 to 500 mg. On average 40 % of ingested magnesium is absorbed. In a steady state, the same amount, 120 to 200 mg, is excreted in the urine. Magnesium can regulate its own concentration probably, at least in part, by interacting with calcium receptors, although the sensitivity of magnesium binding to calcium receptors is 2 to 3 fold less than that of calcium. Magnesium absorption takes place in the small intestine mainly by the jejunum and ileum and varies inversely with intake. Only ionized magnesium is available for absorption. Dietary factors such as phytate, fiber, and fatty acids may interfere with magnesium absorption, whereas vitamin D and lactose increase magnesium absorption. Excretion of magnesium is mainly through glomerular filtration and tubular reabsorption via the kidney and is dependent on plasma magnesium concentration. A decrease of circulating magnesium concentration leads to decreased magnesium excretion through the kidney and vice versa (Berne and Levy, 2004).

The average intake of magnesium by the United States (US) population is not adequate. A positive magnesium balance is maintained for humans by an intake of 6 to 8 mg/kg body weight, which is equivalent to 420 mg magnesium per day for males and 320 mg magnesium per day for females. During pregnancy and lactation, magnesium need

increases and approximately an additional 100 mg/day is required. Approximately 300

mg magnesium per day is available to an adult on a typical intake of food and drinking

water with consequential negative magnesium balance (Seelig, 1964 and Durlach, 1989).

The average serum concentration of magnesium has been reported to be < 0.8 mmol/L in

23% of US adults (ages 25 to 74 years) indicating hypomagnesaemia (Ford, 1999). The

median range of magnesium intake for 4,257 participants aged ≥20 years from the

National Health and Nutrition Examination Survey 1999 to 2000 was 326 mg/day in

Caucasian men and 237 mg/day in African American men. The intake of magnesium

was 237 mg/day in Caucasian women, and 177 mg/day in African American women, an

amount which is thought to be marginally adequate for maintenance of magnesium

concentration in adults (Ford and Mokdad, 2003). Magnesium is ubiquitous in foods.

Foods such as unpolished grains, whole grains, legumes, nuts, and green leafy vegetables

have high magnesium content, while meat, diary products, and most fruits have

intermediate amounts of magnesium. As a percentage of daily food intake, magnesium

content in food is: dairy products 20%; grain products 18%; vegetables 16%; poultry,

meat, and fish 15%; legumes, nuts, and soy products 13%; drinking water 25% (provided

it is hard and unsoftened). However, magnesium is less abundant in food now compared

to 50 years ago. Refining and processing of wheat to flour, rice to polished rice and corn

to starch reduces magnesium content by 82, 83, and 97%, respectively. In addition, there

are several factors that have led to a reduction of magnesium in the ecosystem such as

intensive farming of the soil and acid rain, which by lowering pH of the soil liberates

aluminum (aluminum is insoluble in neutral and alkaline pH water). Once free,

aluminum ion displaces magnesium from particles in the soil leading to deeper

penetration of magnesium in the soil and making it less available for plants and vegetables. This exchange between magnesium and aluminum leads to loss of magnesium in the soil. In addition, aluminum replaces magnesium from its binding sites on the roots of plants (Sanders et al. 1999).

There has been a progressive decline in the dietary intake of magnesium over the past 90 years. Refined foods have the least magnesium content, and it has been estimated that 75% of subjects surveyed in the US have intake of magnesium below the recommended levels because of consumption of refined foods. The advent of refining foods has lead to a decrease in magnesium intake from approximately 410 mg/day to less than 300 mg/day (Marier, 1986 and Marier, 1982). Magnesium in water is highly bioavailable, as waterborne minerals are in ionic form and are easily absorbed by the gastrointestinal tract. Thus drinking water is an important source of mineral intake. Waterborne magnesium is absorbed 30% faster than magnesium from food (Azoulay et al. 2001). The majority of drinking water in the US contains less than 10 mg/L of magnesium with the exception of certain areas in the Midwest where the water concentration of magnesium is above 10 mg/L. Hard water contains up to 30 mg/L of magnesium (Hopps and Feder, 1986). Mineral intake from tap water is generally low except when drinking from mineral-rich sources. Adult males may fulfill between 6 and 23% and adult females may fulfill between 8 and 31% of their magnesium requirement by drinking 2 liters of tap water per day. However, consumption of tap water has decreased and consumption of bottled water has increased in North America. Adults fulfill < 3% of their magnesium requirement when drinking most bottled spring water.

Bottled mineral water, however, can fulfill approximately 16 to 58% of magnesium

requirement depending on different brands of mineral water (Azoulay et al. 2001).

The divalent cation magnesium is the fourth most abundant cellular metal ion in

the body, and within the cell, it is the second most abundant cation after potassium.

Magnesium accumulates more in the intracellular than in the extracellular compartment.

Magnesium salts are highly soluble in water and thus are easily available to the cell. The

magnesium concentration in mammalian cells is between 14 and 20 mM. The normal

range of magnesium in plasma is 1.8 to 2.4 mg/dl (0.75 to 1 mmol/L). One third of

plasma magnesium is bound to protein. Intracellular free magnesium ion concentration is

0.5 to 0.7 mM, approximately 5% of the total body magnesium. Cellular free ionized

magnesium is in rapid equilibrium with bound magnesium. The body contains a total of

about 25 g of magnesium, of which 65% is present in bone, and almost all the rest is

present in the intercellular fluid. Approximately 32% of magnesium is complexed with

proteins and nucleic acids and the rest is present in plasma and other storage sites such as

nuclei, mitochondria, and endosarcoplasmic reticulum. In addition, 30% of the bone

magnesium and 25% of magnesium found in skeletal muscle is in the exchangeable form

(Wolf and Cittadini, 2003). Less than 1% of the total body magnesium is present in the

extracellular vascular compartment of serum and red blood cells. Approximately 2.5

mmol/L of magnesium is present in the red blood cells, with the older cells having the

least magnesium. Magnesium concentration of red blood cells is genetically controlled.

Serum magnesium comprises 0.3% of total body magnesium and is present in three

states, approximately 62% is ionized, 33% is protein bound mainly to albumin, and 5% is

complexed to anions such as phosphate and citrate (Cowan, 1995 and Elin, 1994). The

half life of magnesium in the human body is between 41 and 181 days, and equilibrium among most magnesium tissue pools is reached slowly. A close correlation between serum magnesium and bone magnesium of humans has been demonstrated (Elin, 1994). Magnesium sulfate is the drug of choice for the treatment, management and prophylaxis of eclampsia, and severe preeclampsia and has been used for therapy and prophylaxis of eclampsia for more than 70 years. Magnesium sulfate increases the production of the endothelial cell vasodilator prostacyclin. The clinical effects and toxicity of magnesium is linked to its concentration in the plasma. A concentration of 1.8 to 3.0 mmol/L of plasma is required for treatment while toxicity is seen at plasma concentrations starting from 3.5 to 5 mmol/L. At plasma concentration of 5 to 6.5 mmol/L, respiratory paralyses occurs, while a concentration above 7.5 mmol/L leads to alteration of cardiac conduction, and cardiac arrest occurs at a concentration of 12.5 mmol/L (Moran and Davison, 1999 and Lu and Nightingale, 2000). Normally, the magnesium content of various tissues ranges from 7 to 9 mmol/L (kg wet weight in liver and striated muscle) to 2 mM in erythrocytes, while plasma magnesium content is 0.89 ± 0.08 mM (Grubbs, 2002).

For almost three decades the view was held that cellular magnesium concentration was held constant and stable and no drastic changes of cellular magnesium were required or occurred for magnesium to carry out cellular functions, ion channels, metabolic pathways and cycles, signaling pathways, and enzymes. This view was held in lieu of the large quantity of both total and free magnesium within the cell in addition to the absence of changes of significant amplitude in free magnesium concentration and relatively slow turn over of cations across cell plasma membrane under dormant conditions. Recent data indicates that large fluxes of magnesium are transported across intracellular

compartments and cell membrane in either direction following metabolic and hormonal stimuli during a very short period of time resulting in major changes in total magnesium and to a lesser extent in free magnesium, and this change is in an opposite direction of circulating magnesium. Magnesium extrusion occurs via a cAMP-mediated process in addition to a variety of hormones capable of inducing fast fluxes of magnesium in both directions across the cell membrane. Na/Mg exchanger is considered the main extrusion mechanism in cells. Magnesium extrusion across biological membrane is through Na-dependent and Na-independent pathways. In addition, presence of magnesium entry mechanisms into the cell, majority of which operate as a channel, have been identified and cloned. Intracellular transport of magnesium is finely regulated by specific transport mechanisms. The transient receptor potential (TRP) super-family of ion channels has five subtypes, one of which is transient receptor potential cation channel super-family, melastatin subfamily (TRPM). Two of the TRPM channels designated TRPM6 and TRPM7 function as ion channels and also as protein kinase, a combination that is unique among proteins. Transient receptor potential melastatin 7 ion channel domain is controlled by intracellular concentration of magnesium or magnesium/nucleotide complexes. Transient receptor potential melastatin 7 is ubiquitous and is present in all tissues and cell lines and is responsible for control and adjustment of magnesium concentration at the cellular level. Transient receptor potential melastatin 6 is also regulated by intracellular magnesium concentration and is involved in mediating magnesium influx in intestinal and renal cells and is responsible for regulating magnesium at the level of the organ or organism (Romani and Maguire, 2002, Laires et al. 2004, Romani and Scarpa, 2000, Romani, 2007, Schmitz et al. 2007, Rasgado-Flores

and Gonzalez-Serratos, 2000, Gabriel and Gunzel, 2007 and Schmitz et al. 2004). Mutation of TRPM6 leads to hypomagnesemia with secondary hypocalcemia (Walder et al. 2002 and Schlingmann et al. 2002).

Magnesium and Preeclampsia

Kurzel (1991) investigated magnesium concentration in normal pregnancy and reported that normal pregnancy was a state of "physiological hypomagnesaemia." Magnesium blood concentration from 32 to 42 weeks of gestation was significantly lower than the concentration at 21 to 33 weeks of gestation. Serum magnesium concentration was significantly lower in preeclamptic women in comparison to normal pregnant women (Sukonpan and Phupong, 2005). Standley et al. (1997) investigated tMg and iMg++ serum magnesium concentration of normal and preeclamptic women during the first, second, and third trimesters. There was a decrease in serum tMg and iMg++ over the course of gestation of normal pregnant women. Serum tMg concentration decreased early during pregnancy in women who developed preeclampsia and was significantly lower by the second trimester when compared with normal pregnant women. In contrast, Sanders et al. (1999) reported increased serum iMg++ and serum tMg concentration of severe preeclamptic women in comparison to normal pregnant women. However, they did not detect any difference in serum tMg concentration between non-pregnant and mild preeclamptic women. In addition, intracellular iMg++ and tMg concentration in mononuclear blood cells and erythrocytes was similar in preeclamptic and normal pregnant women. In another study, no difference was detected in serum iMg++ concentration between preeclamptic and normal pregnant women, even though a

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significant difference was reported for serum iMg++ concentration between nonpregnant and normal pregnant women (Handwerker et al. 1995). In addition, plasma tMg concentration was not different between preeclamptic and normal pregnant women but was lower as compared to non-pregnant women. However, intraerythrocytic magnesium concentration was significantly lower in preeclamptic women in comparison to normal pregnant women (Kisters et al. 1990, Adam et al. 2001, Kisters et al. 2000 and Kisters et al. 1998). Magnesium deficiency could be considered as a predisposing factor for preecalmpsia.

Even after 200 years, preeclampsia remains an important syndrome both in the developing and developed world and is traditionally called the "disease of theories" (Higgins and Brennecke, 1998). It still remains one of the leading causes of maternal and infant mortality and extensive morbidity. Preeclampsia is characterized by high blood pressure, proteinuria, and edema.   The incidence of preeclampsia is between 5 and 7% of all pregnancies in the US (Podjarny et al. 1999). Preeclampsia is typically diagnosed by the fourth to the sixth month of pregnancy. The overall preeclampsia/eclampsia case fatality rate is 6.4 per 10,000 cases at delivery (from 1979 to 1992) and approximately half (51%) of preeclampsia- eclampsia deaths are associated with preeclampsia and the remainder with eclampsia (MacKay et al. 2001). According to Vital Statistics Mississippi 2001, the number of women suffering from eclampsia is 2,225 out of a total of 11,483, approximately 19.4% (Mississippi State Department of Health 2001). Very young and older women, as well as first-time mothers, are typically the ones who develop preeclampsia. In addition, women at high risk of developing the disease include those

who have multiple pregnancies, high blood pressure, diabetes mellitus, obesity, and a previous history of preeclampsia (Duckitt and Harrington, 2005).

Hypertension after 20 weeks of gestation is essential for the diagnosis of preeclampsia. The pathophysiological features of preeclampsia indicate that the disorder is early in gestation and predates its clinical presentation (Roberts, 2000). Mild preeclampsia is defined as systolic blood pressure of at least 140 mmHg and/or a diastolic blood pressure of at least 90 mmHg on at least two occasions at least 6 hours apart after 20 weeks of gestation in women known to be normotensive before pregnancy and before 20th week of gestation, plus proteinuria (300 mg or more per 24 hours urine collection). If 24 hour urine collection is not available, then proteinuria is defined as the concentration of at least 30 mg/dL (at least 1+ on a dip stick), of at least two random urine samples collected at least 6 hours apart. Severe preeclampsia is defined as sustained elevations of systolic blood pressure to at least 160 mmHg and/or in diastolic blood pressure to at least 110 mmHg for at least 6 hours in association with proteinuria, or if there is hypertension in association with severe proteinuria (at least 5 g per 24 hours period of urine collection). In addition, preecalmpsia is considered with multi organ involvement such as cerebral dysfunction manifesting as blurred vision, scotoma, headache, and cerebrovascular accidents; epigastric or right upper quadrant pain; renal failure or oliguria $\leq$ 500 ml of urine in 24 hours; pulmonary edema; impaired liver function (serum transaminase levels two times normal or greater); thrombocytopenia ($\leq$ 100,000 platelets/mm^3); coagulopathy; fetal growth restriction; eclampsia and generalized convulsions and HELLP "hemolysis, elevated liver enzymes and low platelets" (Tannirandorn, 2005). Subclassification of preeclampsia further categorizes

preeclampsia as early and late onset. Early onset preeclampsia is defined as onset earlier than 28 weeks and late onset preeclampsia after 28 weeks of gestation (Von Dadelszen et al. 2003).

In the Honolulu heart study, the relationship of various dietary variables to blood pressure was studied. Out of the entire nutrients studied, only magnesium had a strong relationship with blood pressure (Joffres et al. 1987). Further studies supported this relationship indicating increased intake of magnesium contributed to prevention of hypertension (Stuehlinger, 2001 and Mizushima et al. 1998). A significant negative correlation between intracellular magnesium concentration and blood pressure has been described under clinical conditions (Touyz et al. 1992), and magnesium deficiency promotes dyslipidemia, insulin resistance, vasospasm, and endothelial cell damage, a characteristic feature of small arteries in hypertension (Touyz and Milne, 1995 and Touyz, 2003). In vitro studies demonstrated hyperlipemia resulting from accumulation of triacylglycerol rich lipoproteins and inflammatory response with marked leukocytosis and increase in plasma concentration of inflammatory cytokines such as TNFα, IL-6, IL-1, and IL-8, following magnesium deficiency. Low serum concentrations of magnesium induced an increase in TNFα secretion and inflammation (Rodriguez-Moran and Guerrero-Romero, 2004).   Magnesium deficiency induced experimentally resulted in altered lipid profile and increased blood pressure (Blache et al. 2006, Laurant and Touyz, 2000 and Kh et al. 2000). In addition, in a rat experimental model of preeclampsia, magnesium infusion reduced high blood pressure and magnesium deficiency resulted in hypertension (Standley et al. 2006 and Saito et al. 1995). During preeclampsia and pregnancy induced hypertension, magnesium was reported to reduce high blood pressure,

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and Kisters et al. (1990) reported that decreased concentration of cellular magnesium of preeclamptic women contributed to development of hypertension in these subjects. An inverse relationship between magnesium content of water and cardiac disease has been documented. In areas where there was soft drinking water the incidence of cardiac disease was greater (Seelig, 1989). In addition, intake of large quantities of processed food leads to magnesium deficiency, as large quantities of magnesium are lost during processing, and it is reported that intake of processed food promotes increased incidence of cardiac disease (Maier, 2003).

In an investigation of the role of obesity during pregnancy induced hypertension, a longitudinal study was conducted and it was reported that the mean prepregnancy body mass index (BMI) was higher in women who had pregnancy induced hypertension compared with women who did not (Thadhani et al. 1999 and Odegard et al. 2000). Whereas prepregnancy BMI ($\geq$32.3 kg/m^2), a previous history of preeclampsia and nulliparity increased the risk of subsequent preeclampsia in future pregnancy (Stone et al. 1994, O' Brien et al. 2003 and Baeten et al. 2001). Obesity is a definite risk for preeclampsia and the risk increases with a greater BMI (overweight: BMI 25 to 29 kg/m^2 and obese: BMI $\geq$30 kg/m^2). With the world wide increase in obesity, the frequency of preeclampsia is likely to increase (Dekker and Sibai, 2001 and Hrazdilova et al. 2001). Primigravidae are at a higher risk than other pregnant women of developing preeclampsia as well as multigravidae having second or later pregnancies (Trupin et al. 1996). Preeclampsia is associated with maternal age, and there is a sharp rise in the incidence of preeclampsia in women above the age of 35, those with a family history of preecalampsia (Lyall and Greer, 1994) and those under the age of 20 (Sibai et al. 2005).

Magnesium, Inflammation, and Endothelial Dysfunction

In several animal studies, magnesium deficiency was reported to activate the inflammatory response or proinflammatory condition and hyperlipemia with an increase in serum concentration of triacylglycerol and FFAs in addition to other lipids (Maier, 2003). Inflammation rapidly increases serum triacylglycerol concentration by stimulating hepatic very low density lipoprotein production and by reducing triacylglycerol clearance. In addition, decreased concentration of lipoprotein lipase, hepatic lipase, and lecithin-cholesterol acyltransferase was also reported. The inflammatory response stimulated increased secretion of cytokines and increased blood circulating concentration of proinflammatory cytokine-activated macrophages and endothelial cells, resulting in endothelial cell dysfunction with increased cell permeability to lipids, further increase in cytokine secretion, and platelet activation (Maier, 2003).

Inflammation is the body's protective response against injury or insult caused to its cells and is not necessarily stimulated by antigen stimulation. The inflammatory response is fast, non specific, and is more primitive than the immune response. Inflammation is usually a healing response but sometimes acute local inflammation leads to a body wide response which can result in major organ failure and death (Tracey, 2002). Inflammatory response involves inflammatory leucocytes (granulocytes, macrophages and lymphocytes), cytokines, endothelium, and platelets, in addition to complement and clotting systems (Nathan, 2002).

 Normal pregnancy is a state of mild systemic inflammation that is not harmful to the mother or fetus. By the third trimester of pregnancy, inflammatory leucocytes and

endothelial cell activation is present along with a slight increase in the acute phase protein "C- reactive protein." Slightly raised C- reactive protein is normally present during the early part of the pregnancy. In preeclampsia, the maternal systemic inflammatory response during pregnancy intensifies and results in maternal symptoms (Redman et al. 1999 and Redman and Sargent, 2004). Consequently, an increase in inflammatory mediators and cytokines, inflammatory cell activation, and endothelial cell and organ dysfunction takes place (Dadelszen et al. 2000).

Faas et al. (1994) used rats as an experimental animal model for preeclampsia by infusion of endotoxin (1.0 μg/kg body weight, *Escherichia coli*) into pregnant rats. Endotoxin infusion produced potent activation of the inflammatory response with pathological signs in the pregnant rats such as proteinuria, increased blood pressure, and disseminated intravascular coagulation similar to those seen in preeclampsia. A comparable dose of endotoxin given to non-pregnant rats did not produce the same changes seen in pregnant rats. In a subsequent experiment, a persistent low dose infusion of endotoxin induced an inflammatory reaction in pregnant rats, and inflammatory activation was observed in the circulating inflammatory cells, granulocytes, and monocytes. Activation of granulocytes and monocytes was only observed in pregnant endotoxin treated rats but not in endotoxin treated non-pregnant rats. Leucocytes were activated but to a lesser extent in pregnant rats treated with saline (Faas et al. 2000). In a following experiment using the rat model for preeclampsia, the effect of endotoxin on monocyte cells was investigated. Activated monocytes produced cytokines, particularly TNFα, through which the monocytes activate the inflammatory response. Monocyte TNFα secretion was decreased while granulocyte and white blood cell counts were

increased in the endotoxin treated rats when compared with rats treated with saline. In

view of the decreased monocyte TNFα concentration in the endotoxin treated rats, it was

suggested that the monocytes were endotoxin-tolerant and were, therefore, activated.

These findings were similar to those observed concerning increased blood concentration

of granulocytes and TNFα of women with preeclampsia (Faas et al. 2004).

These results are consistent with findings in human preeclampsia and suggest that

experimental preeclampsia of pregnant rats result from a generalized inflammatory

response. Circulating inflammatory cells, leucocytes, are activated during preeclampsia

signifying an activated inflammatory response which includes both endothelial cells and

inflammatory cells in preeclampsia (Sacks et al. 1998).   Activated neutrophils can

mediate vascular damage in non-pregnant women, which is amplified in preeclampsia.

Activated neutrophils are confined to maternal circulation and contribute directly to

vascular cell damage (Harlan, 1987 and Greer et al. 1989).

Cell death by apoptosis (programmed cell death) has been identified in all cell

types of placenta in the second and third trimesters of pregnancy (Smith et al. 1997). The

proinflammatory cytokine TNFα is known to induce apoptosis in reproductive tissue and

endothelial cells (Winn and Harlan, 2005).   Preeclampsia is associated with abnormal

cytotrophoblast cell differentiation, shallow invasion, and decreased blood flow to the

placenta. DiFederico et al. (1999) investigated whether abnormal cytotrophoblast cell

differentiation and hypoxia lead to apoptosis and reported wide spread apoptosis of

cytotrophoblast in preeclampsia. In addition, several investigators examined the role of

apoptosis in preeclampsia and found an increase in trophoblast and cytotrophoblast

apoptosis in placenta (Allaire et al. 2000, Leung et al. 2001, Reister et al. 2001, Smith et

al. 2002, Kharfi et al. 2006 and Levy and Nelson, 2000). Enhanced apoptosis interferes

with the process of placentation, and causes placental ischemia and systemic endothelial

cell damage. Martin et al. (2003) reported apoptosis in primary culture of rat hepatocytes

in the presence of extracellular magnesium deficiency.

Normal pregnancy is associated with increased circulating lipid concentrations

and insulin resistance when compared with non-pregnant women (Potter and Nestel,

1979). While, during preeclampsia, an exaggerated degree of dyslipidemia and insulin

resistance is recorded. Insulin resistance promotes increased lipolysis and increases

circulating FFAs (Wolf et al. 2001).

Magnesium has antioxidant properties, and magnesium deficiency of cells make

them more susceptible to oxidative damage (Rayssiguier et al. 1993). A generalized

proinflammatory state and significant elevation of plasma concentration of macrophage

derived cytokines, IL-6, IL-1, and TNFα; endothelial cell derived cytokine, endothelin;

and substance P were observed after rodents were fed a magnesium deficient diet for

three weeks (Weglicki et al. 1992, Weglicki and Phillips, 1992 and Weglicki et al. 1994).

In a similar study on rats, inflammatory response was seen after rats were fed a severe

magnesium deficient diet for 4 or 8 days. Significant increase in plasma IL-6 as early as

day 4 was noted in addition to leucocytosis mainly due to an increased number of

polymorphonuclear leucocytes neutrophils, monocytes, and esinophils. In addition, an

increase in acute phase proteins and activated macrophages was also reported (Malpuech-

Brugere et al. 2000). Furthermore, increased blood pressure and plasma IL-6 blood

concentrations were noted in rats fed a magnesium deficient diet for 22 months.

Magnesium deficiency induced a chronic impairment of redox status associated with

inflammation, increased oxidized lipids, hypertension and endothelial cell dysfunction (Blache et al. 2006). Song et al. (2007) investigated the effect of a magnesium deficient diet fed to healthy women. Dietary magnesium intake was inversely associated with plasma concentration of C-reactive protein and E-selectin but not all markers of systemic inflammation and endothelial cell dysfunction.

A direct effect of low magnesium concentration on endothelial cell culture was demonstrated in promoting endothelial cell dysfunction by generating a proinflammatory, prothrombotic, and proatherogenic environment (Maier et al. 2004). In addition, high serum concentration of magnesium maintained endothelial cell function. Magnesium may have a protective effect in atherosclerosis and in promoting growth of collateral vessels in chronic ischemia (Maier et al. 2004)

Magnesium deficiency acts through the induction of proinflammatory cytokines and adhesion of monocyte cells to the endothelium. In addition, magnesium deficiency induces several features associated with cell senescence, and endothelial cell senescence contributing to atherosclerosis (Ferre et al. 2007, Altura and Altura, 2007). Upon evaluating the effect of magnesium on cultured endothelial cells, it was reported that high serum magnesium concentration stimulated endothelial cell proliferation, mitogenic response to angiogenic factors, attenuated the response to lipopolysaccharide and enhanced the synthesis of nitric oxide by up regulating endothelial cell nitric oxide synthase (Maier et al. 2004).

Several animal experiments have shown an inverse relationship between magnesium content of diet and atherosclerosis (Altura et al. 1990 and Ouchi et al. 1990). Magnesium deficiency produces a proinflammatory condition, a greater susceptibility of

lipoproteins to peroxidation, and an increased production of oxygen derived free radicals stimulating atherosclerosis (Weglicki et al. 1992 and Rayssiguier et al. 1993). In addition, several animal experiments have emphasized the role of magnesium in atherogenesis demonstrating the presence of spontaneous inflammation with marked leucocytosis, increased plasma concentration of inflammatory cytokines, hypertriacylglyceroldemia, and increased cholesterol blood concentration (Maier et al. 1998, Haenni et al. 1998, Sherer et al. 1999, Sherer et al. 2000 and Ravn et al. 2001). Hyperlipemia, increased plasma triacylglycerol, and increased plasma triacylglycerol rich lipoproteins were demonstrated after rats were fed a magnesium deficient diet for 8 days (Gueux et al. 1995). It can be suggested that magnesium has a role in the phenomenon of acute atherosis of pregnancy indicating vascular cell dysfunction. Acute atherosis in preeclampsia shares many common features with atherosclerosis, which is reported to be an inflammatory disease (Roberts, 1998 and Ross, 1999).

Preeclampsia is associated with maternal hyperlipidaemia in particular hypertriacylglyceroldemia (Sattar et al. 1997 and Hubel et al. 1996). During preecalmpsia, maternal spiral artery walls have lipid deposition and due to their resemblance with early stages of atherosclerotic lesions these lipid depositions are described as 'acute atherosis." Acute atherosis is characterized by an accumulation of lipid laden macrophage, perivascular lymphocytic infiltrate, and fibrinoid necrosis (Meekins et al. 1994 and Labarrere, 1988) and the early lesions are associated with endothelial cell damage. Acute atherosis and atherosclerosis are associated with inflammation (Rosenfeld et al. 1998 and Libby et al. 2002). The lipid laden macrophages

also contain the inflammatory cytokine TNFα, consequently linking the presence of acute atherosis with the inflammatory state of preeclampsia.

Acute atherosis is associated with placental infarcts and arterial thrombosis and represents a lesion that could reduce placental perfusion even in the absence of poor placentation (Pijnenborg et al. 1998 and Pijnenborg et al. 2006). In addition, activated endothelial cells are more resistant to trophoblast displacement than resting endothelial cells, which could contribute to shallow invasion of the spiral arteries by trophoblasts and reduced placental perfusion in preeclampsia (Chen et al. 2007).

Increased maternal fat deposits occur during the first half of normal pregnancy and are the source of physiological hyperlipidemia of late gestation. Hyperlipidemia results from increased adipose tissue lipolytic activity. In addition, adipose tissue lipoprotein lipase and hepatic lipase activity are decreased. All of these changes are secondary to insulin resistance and increased estrogen. This impairs the removal of triacylglycerol rich lipoproteins from the circulation. Increased insulin resistance during preeclampsia increases the mobilization of FFAs and triacylglycerol and results in their increased concentration in serum (Hubel, 1998). Accumulation of triacylglycerol in endothelial cells and increased serum concentration of FFAs can cause endothelial cell dysfunction (de Man et al. 2000, Stewart and Monge, 1993, Gianturco et al. 1980 and Speidel et al. 1990). Prepregnant obesity is associated with increased risk of preeclampsia and increasing body weight is likely to increase the occurrence of preeclampsia (O'Brien et al. 2003).

Obesity is associated with dyslipidemia, hyperinsulinemia, insulin resistance and impaired endothelial cell function (Freeman et al. 2004 and Ramsay et al. 2002)

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Circulating markers of inflammation are increased in obesity such as TNFα and C-reactive protein among others (Wolf et al. 2001, Esposito et al. 2002 and Esposito et al. 2003). Tumor necrosis factor alpha decreases lipoprotein lipase activity and increases adipose tissue lipolysis contributing to dyslipidemia and insulin resistance (Hotamisligil et al. 1995 and Kern et al. 1995).

Triacylglycerols are lipid constructs of fatty acids. They are composed of three fatty acids each in ester linkage with glycerol and are stored in adipocytes or fat cells. In early gestation, embryonic and fetal lipid requirements are derived from maternal free fatty acids crossing the placenta to provide energy to the fetus. However, in late gestation there is a gradual shift to de novo synthesis of fatty acids by the fetus for energy (Van Aerde et al. 1998). The various effects of pregnancy encompass a profound impact on lipid metabolism. However, dyslipidemia of preeclampsia is not a simple exaggeration of the physiological changes of lipid metabolism. Lorentzen et al. (1995) investigated 510 healthy primigravidae and reported that fasting serum concentration of FFAs and triacylglycerol were increased before 20 weeks of gestation in women who later developed preeclampsia. Similarly, in a longitudinal study increased serum concentrations of triacylglycerol and FFAs were demonstrated as early as 20 weeks of gestation (Chappell et al. 2002). Likewise, Gratacos et al. (1996) investigated pregnant women in first, second, and third trimesters and found a significant increase in serum concentration of triacylglycerol at 20 and 34 weeks of gestation in mild, severe, and superimposed preeclampsia. Also, an increase of triacylglycerol serum concentration was already present at 10 weeks gestation in mild and severe preeclampsia. Both studies

indicate that an increase in triacylglycerol concentration predates clinical onset of preeclampsia.

Dyslipidemia, especially triacylglyceroldemia, is not a result of preeclampsia as it is present before the onset of preeclampsia and tends to persist after pregnancy ends. An increase in triacylglycerol serum concentration was also noted in the third trimester of pregnant women suffering from mild or severe preeclampsia (Ouyang et al. 2007, Uzun et al. 2005, Bayhan et al. 2005 and Sattar et al. 1997). On the other hand, pregnant women with hyperlipidemia particularly with hypertriacylglyceroldemia are more prone to developing preeclampsia that tends to be severe (Ware-Jauregui et al. 1999). However, Clausen et al. (2001) reported that hypertriacyglyceroldemia before 20 weeks of gestation was associated with a risk of developing early but not late onset preeclampsia. Ray et al. (2006) reported that among 22 epidemiological studies, maternal plasma triacylglycerol was consistently elevated in women who developed preeclampsia. The highest triacylglycerol plasma concentration was associated with four fold higher risk of preecalmpsia compared to women whose triacylglycerol plasma concentration was normal. Bodnar et al. (2005) conducted a case control study of normal pregnant and preeclamptic pregnant women of ≤20 weeks of gestation and reported that triacylglycerol is an important mediator for preeclampsia second to inflammation.

Preeclamptic pregnant women had significant increases in fasting serum concentration of FFAs and the concentration and composition of circulating FFAs were already altered at 10 to 20 weeks gestation before clinical onset of preeclampsia (Lorentzen et al. 1995). In addition, sera from preeclamptic women demonstrated not only high availability of FFA, the sera also stimulated the synthesis of triacylglycerol by

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endothelial cells (Endresen et al. 1992). Pregnant women with severe preeclampsia have 2 to 3 fold higher serum concentration of FFAs than those with transient hypertension or normal pregnancy (Vigne et al. 1997). Likewise, pregnant preeclamptic women in their third trimester had higher serum FFA concentration in comparison to normal pregnant women (Murai et al. 1997 and Hubel et al. 1996).

Endothelial cell dysfunction is considered to underlie many pathological mechanisms of preeclampsia (Roberts et al. 1989, Belo et al. 2002 and Friedman et al. 1995). Endothelial cell dysfunction is part of the generalized maternal inflammatory reaction present during preeclampsia, and activated endothelial cells are an integral component of the inflammatory response (Redman et al. 1999 and Redman and Sargent, 2003). Garcia et al. (2007) conducted an investigation where they studied endothelial cell function with flow mediated vasodilatation. Flow mediated dilatation is a non-invasive method that uses high resolution ultrasonography to assess endothelial cell function. They reported decreased flow mediated vasodilatation and higher blood concentration of C-reactive protein and leucocytes of pregnant women (gestation age 22 weeks) who eventually developed preeclampsia. These alterations predate the clinical onset of preeclampsia and are consistent with the hypothesis that preeclampsia is caused by endothelial cell dysfunction and systemic inflammation during early pregnancy preceding the clinical onset of preeclampsia. On the other hand, an absence of maternal systemic inflammatory response at 18 and 20 weeks of gestation (as assessed by decreased C-reactive protein blood concentration and other markers of inflammation) of pregnant women who eventually developed preeclampsia was also reported (Djurovic et al. 2002 and Savvidou et al. 2002). Leucocyte, monocyte and granulocyte, particularly

neutrophil, activation was noted during preeclampsia as compared to normal pregnant women which is in agreement with maternal intravascular inflammation and endothelial cell activation (Belo et al. 2003, Gervasi et al. 2001 and Wang et al. 2001).

Activated endothelial cells are an integral component of the inflammatory response and activated endothelium or endothelial cell dysfunction is one aspect of a generalized systemic maternal inflammatory response of preeclampsia, which also involves circulating leukocytes, activated inflammatory cells such as neutrophils, monocytes, macrophages, and lymphocytes. Additionally, an increased release of pro-inflammatory cytokines such as TNFα, IL-6, IL-8, and IL-1 accompanies the maternal systemic inflammatory response during preeclampsia (Belo et al. 2002, Pleiner et al. 2002, Granger et al. 2001, Muller and Griesmacher, 2000, Clark et al. 1998, Baker et al. 1995, Taylor et al. 1998, Zammit et al. 1996 and Friedman et al. 1995). In addition, TNFα can induce insulin resistance and hypertriacylglyceroldemia by suppressing key enzymes such as lipoprotein lipase, acetyl CoA carboxylase, and fatty acid synthethase along with a marked stimulation of glycogenolysis and increased serum concentration of FFAs. Free fatty acids have been shown to activate endothelial cells (Carr et al. 2001, Heiskanen et al. 2002, Anim-Nyame et al. 2003, Serin et al. 2002, Rinehart et al. 1999, Williams et al. 1999, Conrad and Benyo, 1997, Kaaja et al. 1999 and Sigal and Ron, 1994).

C-reactive protein is an acute phase reactant of inflammation and is a marker for underlying systemic inflammation. High plasma concentration of C-reactive protein was demonstrated at 22 weeks of gestations in pregnant women, who eventually developed preeclampsia (Garcia et al. 2007). Upon activation or injury, during the process of

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inflammation, endothelial cells shed vesicles (endothelial microparticles) into blood circulation. These vesicles contain cytoplasmic components and negatively charged phospholipids bearing some of the cell surface protein. Elevation of endothelial microparticles is reported during preeclampsia and is an indication of endothelial cell dysfunction and activation during inflammation (Gonzalez-Quintero et al. 2003, VanWijik et al. 2002 and Preston et al. 2003).

Tumor necrosis factor alpha plasma concentration is increased during preeclampsia and mediates endothelial cell activation and dysfunction (Meekins et al. 1994, Cotran and Pober, 1990, Crews et al. 2000 and Pijnenborg et al. 1991) by activating neutrophils directly to adhere to the endothelial cells (Gamble et al. 1985). Activated leucocytes activate endothelial cells and activated endothelial cells activate leucocytes (Zimmerman et al. 1992 and Mantovani and Dejana, 1989).

In addition, endothelial cell dysfunction prior to the onset of preeclampsia can be demonstrated by the presence of markers of endothelial cell activation with secretion of cell surface proteins that mediate adherence of inflammatory cells. This induction is mediated by cytokines particularly TNFα produced by inflammatory cells and activated endothelial cells (Dekker and Sibai, 1998 and Garcia-Vallejo et al. 2006). Tumor necrosis factor alpha is one of the principal inflammatory cytokines that has been shown to induce structural and functional alterations in endothelial cells rendering them into a proinflammatory and prothrombotic phenotype known as "activated" by stimulating an increased expression of adhesion molecules. The cytokine endothelial cell interaction thus plays an important role during inflammation and atherosclerosis (Pober and Cotran, 1990 and Cotran and Pober, 1990). Tumor necrosis factor alpha through induction of

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insulin resistance and hypertriacylglyceroldemia promotes endothelium cell damage (Hotamisligil et al. 1994).

Women with preeclampsia have higher plasma concentration of ET-1 compared with normal pregnant women. Endothelin-1 contributes to a vasoconstricted and inflammatory state (Taylor et al. 1990 and August, 1995). Endothelin-1, by mediating release of cytokines, activates neutrophils, platelets, inflammation, and endothelial cell damage that are characteristic of preeclampsia (Clark et al. 1998). Tumor necrosis factor alpha is produced by neutrophils, macrophages, and adipocytes and can induce other cytokines such as IL-6, and IL-6 in turn regulates the expression of C - reactive protein. These mediators alone or in combination can damage endothelial cells and thus contribute to atherothrombosis (Schalkwijk and Stehouwer, 2005).

Interleukin-10 inhibits the secretion of macrophage derived TNFα and suppresses inflammatory response. Interleukin-10 is secreted by a variety of immune system cells including monocytes, macrophages (Pinchuk, 2002), and trophoblast cells (Roth et al. 1996). Adiponectin is an insulin sensitizing hormone (Tschritter et al. 2003) that is antiinflammatory (Mantzoros et al. 2005), antiatherogenic, and inhibits expression of adhesion molecules (Ouchi et al. 1999), reduces monocyte attachment to endothelial cells (Ouchi et al. 2001), suppresses TNFα secretion of macrophages (Yokota et al. 2000), stimulates nitric oxide synthesis (Chen et al. 2003), and suppresses endothelial cell activation (Ouchi et al. 2000).

Adiponectin sensitizes tissue to insulin by reducing serum FFAs and this then explains the decreased concentration of adiponectin with insulin resistance (Yamauchi et al. 2001 and Yamauchi et al. 2002). Adiponectin expression is regulated by TNFα and

IL-6 (Chen et al. 2006).   Leptin expression, on the other hand, is stimulated by TNFα

and IL-6, and a correlation of leptin with TNFα in pregnancy has been described

(Nuamah et al. 2004 and Bartha et al. 2001). Elevated plasma leptin concentration is

associated with insulin resistance (Segal et al. 1996). Leptin also contributes to

endothelial cell dysfunction by increasing FFA oxidation (Yamagishi et al. 2001). Leptin

receptors have been demonstrated in the endothelial cells and athererosclerosis plaques

indicating a role of leptin in angiogenesis and atherosclerotic changes (Bouloumie et al.

1998 and Bouloumie et al. 1999). High plasma concentration of leptin and low plasma

adiponectin concentration were demonstrated during preeclampsia (Shinohara et al.

2004).

There are reports of decreased serum concentration of ET-1 (Sagsoz and

Kucukozkan, 2003) and decreased ET-1 in placenta during preeclampsia (Holcberg et al.

2004) after magnesium treatment. Whereas, Mastrogiannis et al. (1992) reported a

significant reduction of plasma ET-1 concentration during preeclampsia after magnesium

treatment, which was not demonstrated in normal endothelial cells and pregnant women

without preeclampsia.   In a study conducted on rats, with initial infusion of ET-1

followed by magnesium, it was discovered that magnesium could reverse

vasoconstriction in a number of vascular beds (Kemp et al. 1999). However, Ariza et al.

(2005) reported that after magnesium treatment, serum concentration of ET-1

significantly increased in preeclamptic pregnant women and suggested that increased ET-

1 concentration may represent a compensatory mechanism for the vasodilatory effect of

calcitonin gene-related peptide. In a subsequent experiment, Ariza et al. (2007) reported

that during preeclampsia placental expression of ET-1$_A$ receptor (ET-1$_A$R) and ET-1 was

similar to that of normal pregnant women whereas placental expression of ET-1$_B$ receptor (ET-1$_B$R) was significantly lower in preeclampsia. After magnesium infusion (4 g loading dose and 1g maintenance dose) serum ET-1 decreased with an increase in ET-1$_B$R placental expression.

The endothelium has been viewed as an inert membrane responsible for the maintenance of vessel wall permeability and homeostasis. Overtime, this view of the endothelium has changed and the endothelium is now considered as an active heterogeneous tissue that performs critical synthetic, secretory, metabolic, and immunological functions and acts as a modulator of vasomotor tone, cell proliferation, hormone production, and inflammation. The endothelium secretes mediators to carry out different functions. These chemically different compounds are not stored in intracellular secretory granules and carry out their functions in an autocrine and paracrine manner rather than as a classical circulating hormone at multiple sites in the body. Their major biological effects are regulated by localization of specific receptors on the vascular wall, gene transcription, and rapid metabolism (Fishman, 1982 and Levin, 1995). The endothelium also has the ability to elicit both vasorelaxant and vasoconstrictor substances to modulate vascular smooth muscle tone.

The endothelin family consists of ET-1, ET-2, and ET-3 (21-amino acid peptides). The three isoforms of human endothelin are encoded by three separate genes and interact with different subtypes of endothelin receptors. Endothelins are synthesized and released by both vascular and non-vascular cells. Endothelin-1 is formed from the biologically inactive 203 amino acid precursor preproendothelin that is converted into a 39 amino acid prohormone "big endothelin." Big endothelin is transformed into the mature and active

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ET-1 by zinc metalloendopeptidases known as endothelin converting enzymes (Inque et al. 1989, Xu et al. 1994, Masaki, 2000 and Loffler, 2000). Endothelin-1 is one of the most potent endogenous vasoconstrictor and has antinatriuretic, mitogenic, and proinflammatory properties (Yanagisawa et al. 1988). Endothelin-1 is produced by many types of cells in addition to endothelial cells, including smooth muscle cells (Hahn et al. 1990), leucocytes (Sessa et al. 1991), monocytes, macrophages (Ehrenreich et al. 1990), mesangial cells (Bakris et al. 1991), and endometrial cells (Cameron et al. 1992 and Davenport et al. 1991). Neutrophils do not secrete ET-1, but they can convert exogenous big ET-1 to ET-1 (Sessa et al. 1991 and Kaw et al. 1992). Platelets up regulate messenger RNA expression and biosynthesis of ET-1 from endothelial cells (Ohlstein et al. 1991). There is an interaction between vascular endothelium and blood cells in modulating the production of endothelins in the circulation.

The synthesis and release of ET-1 is in response to various stimuli, mediators, and vasoactive substances (Morganti et al. 2000) such as physical stimuli, hypoxia, shear stress, hormones angiotensin II (Toma et al. 2006) and vasopressin (Imai et al. 1992), insulin (Yang and Li, 2008, Oliver et al. 1991 and Hu et al. 1993), cytokines (Skopal et al. 1998, Corder et al. 1995, Lamas et al. 1992 and Marsden et al. 1992), and growth factor (Matsuura et al. 1998, Kurihara et al. 1989 and Yanagisawa et al.1989). Endothelin-1 is down regulated by nitric oxide, cyclic guanosine monophosphate, and artrial natruretic peptides (Morganti et al. 2000, Wada et al. 1996 and Stewart et al. 1994). Transcription of ET-1 messenger RNA is induced in response to various stimuli and the synthesis and release of ET-1 occurs within minutes. In order to achieve rapid mobilization of endothelin and endothelin converting enzymes the endothelial cells have

intracellular storage pools know as Weibel-Palade bodies in the cytoplasm (Fraser et al. 1998). The concentration of ET-1 in plasma of healthy subjects range from less than 0.3 pg/ml to around 3.0 pg/ml, on average. The plasma half life of ET-1 is 2 to 5 minutes (Neild, 1994) and the half life of ET-1 messenger RNA is 15 to 20 minutes (Anggard et al. 1989).

The endothelins (ET-1, ET-2, and ET-3) bind to two types of G-protein coupled receptors ET_A and ET_B (Sakamoto et al. 1991, Sakurai et al. 1990 and Arai et al. 1990). Endothelin$_A$ receptor and ET_BR are coupled to different G-protein receptors with regard to adenylate cyclase activity.   Endothelin$_A$ receptor activation leads to an increase in cyclic AMP via G-protein. While E_BR activation causes a decrease in cyclic AMP via G-protein (Eguchi et al. 1993). Endothelin receptors are widely expressed in all tissues including nonvascular organs. The ET_AR preferentially binds ET-1 resulting in vasoconstriction. Whereas the ET_BR is nondiscriminating, and binding of the receptor with endothelin results in vasodilatation when the receptor is located on the endothelial cells and vasoconstriction when the receptor is located on smooth muscle cells. The binding of ET-1 to its receptor is irreversible leading to long lasting vasoconstriction (Higgins et al. 1993 and Goraca, 2002).

Endothelin, upon binding with the endothelin receptor, activates a wide variety of intracellular mechanisms. Binding of ET-1 to ET_AR stimulates phospholipase C activation and generation of second messenger inositol triphosphate (IP_3) and diacylglycerol (DAG). Generation of IP3 and DAG stimulates release of intracellular calcium and eventually the entry of extracellular calcium and activation of protein kinase C. Another signaling pathway involves the activation of phospholipase D and A_2 and

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opening of ion channels (Miller et al. 1993, Muldoon et al. 1989 and Schiffrin, 2005).

Conversely, ET_BR activation stimulates nitric oxide and prostacyclin secretion and vasodilatation (D'Orleans-Juste et al. 2002).

Endothelial cell dysfunction and vasospasm is present during preeclampsia. Endothelin-1 is considered to play an important role in preeclampsia pathophysiology. Increased peripheral plasma concentration of ET-1 is seen in preeclampsia. The placenta has been postulated as a site of origin of ET-1. Rust et al. (1997) studied the site of origin of ET-1 and reported that peripheral plasma concentration of ET-1 was increased in severe preeclampsia but placenta was not the source of this increase. In preeclamptic women placenta, the expression of ET-1 immunoreactivity was uneven with a negative staining between 29 and 32 weeks of gestation in comparison with normal pregnancy placenta. This difference between normal and preeclamtic placenta does not support the suggestion that the increased plasma ET-1 concentration in preeclamptic women may have, at least partially, a trophoblastic origin (Barros et al. 2001). On the other hand, ET-1 concentrations were significantly higher in the placental tissues from women with preeclampsia (Singh et al. 2001 and Naiker et al. 2001). Similarly, an increased expression of ET-1 messenger RNA of trophoblast cells was seen in the placenta of preeclamptic pregnant women in comparison to the placenta of normal pregnant women (Vural, 2002). Ajne et al. (2003) in a longitudinal study discovered a significant increase in endothelin-converting enzyme activity and ET-1 plasma concentration of preeclamptic versus normal pregnant women. Several studies provide evidence that plasma ET-1 concentration is increased in preeclamptic pregnancy versus normal pregnancy (Slowinski et al. 2002, Khedun et al. 2002 and Kuwajima et al. 2001).

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Magnesium, Tumor Necrosis Factor Alpha, and Interleukin-10

Low concentration of serum magnesium induces an increase of TNFα secretion and inflammatory response (Rodriguez-Moran and Guerrando-Romero, 2004). Malpuech-Brugere et al. (1999) assessed the effect of different stages of magnesium deficiency on endotoxin response and TNFα secretion. Lipopolysacchride induced no lethal effects in control rats but resulted in 70% mortality in magnesium deficient rats within 3 hours. The rats had high plasma concentration of TNFα. Magnesium deficient rats that received magnesium supplement before the endotoxin challenge had significant increased survival rate. A significant increase of plasma TNFα concentration was observed in magnesium deficient rats compared to rats fed the control diet. Consequently, magnesium deficiency is associated with inflammatory response.

Cytokines are defined as soluble proteins synthesized by immune and non-immune cells. Cytokines are a vast array of relatively low molecular weight, pharmacologically active proteins that are secreted by one cell for the purpose of altering either its own functions (i.e. autocrine effect), or those of adjacent cell (paracrine effect), and distant cells (endocrine effect). Intercellular communication is carried out by transmitting information to target cells through receptor ligand interaction. In addition, cytokines function as stimulators or inhibitors of other cytokines and act as modulators of other regulatory systems. The secretion of cytokine is brief and self limited triggered by either specific antigen or nonspecific stimulus. In many instances, individual cytokine have multiple biological activities. Cytokines are pleiotropic i.e. one cytokine can perform more than one function acting on a variety of cells. Different cytokines can have

the same activity, resulting in biological redundancy within the inflammatory and immune systems. It is rare that loss or neutralization of one cytokine will markedly interfere with either the inflammatory or immune system. Besides, there is a hierarchy of cytokines, so that some of the "higher order" cytokines, such as TNFα orchestrate the synthesis, secretion and activity of other cytokines (McDermott, 2001 and Coppack, 2001).

Inflammatory cytokines act by modifying vasoregulatory responses, enhance vascular permeability, increase leucocyte adhesion to endothelium, and facilitate thrombus formation by inducing procoagulant activity (Schalkwijk and Stehouwer, 2005). Tumor necrosis factor alpha is a pleiotropropic cytokine, mainly produced by activated monocytes, macrophages, T cells, and neutrophils in addition to a wide variety of other cells such as lymphoid cells, mast cells, endothelial cells, adipose tissue, cardiac monocytes, fibroblasts, and neuronal tissue and is involved in systemic inflammation and endothelial cell activation. Tumor necrosis factor alpha also stimulates the release of other cytokines, and executes a variety of functions, and carries out a number of actions on various organ systems generally in concert with IL-1 and IL-6. Nearly all human cells are affected by TNFα and display receptors of TNFα on their cell surfaces. Tumor necrosis factor alpha receptors are of two types, tumor necrosis factor alpha –receptor 1 and tumor necrosis factor alpha –receptor 2. Tumor necrosis factor- receptor 1 is constitutively expressed on most tissues, while tumor necrosis factor alpha –receptor 2 is present on cells of the immune system (Goodsell, 2006 and Locksley et al. 2001).

Inflammatory response demonstrates pro and antiinflammatory reactions in which cytokines play a major role. The placenta is capable of secreting cytokines that

contribute to both local and systemic concentration of inflammatory molecules and cytokines. Inflammatory cytokines are important components of pregnancy complicated with preeclampsia. Maternal adipose tissue and placenta both contribute to the inflammatory condition by releasing common molecules (Hauguel-de-Mouzon and Guerre-Millo, 2006). Investigation in a longitudinal study of tumor necrosis factor alpha -receptor concentration of pregnant women with high risk of developing preeclampsia was conducted by Schipper et al. (2005) who reported no significant difference of plasma tumor necrosis factor alpha -receptor concentration between normotensive, hypertensive, and preeclamptic pregnant women in all three trimesters.

Cytokines may transfer from the fetal compartment to the maternal compartment and vice versa. No bidirectional transfer was noted for TNFα across the placenta (Aaltonen et al. 2005 and Zaretsky et al. 2004). Hayashi et al. (2005) and Benyo et al. (2001) also came to a similar conclusion and reported no significant increase of TNFα-total protein concentration of a placenta of preeclamptic group of women even in the presence of significantly elevated maternal serum TNFα concentration. Sources other than the placenta contributed to the increased serum concentration of TNFα in the circulation of preeclamptic women. Hamai et al. (1997) evaluated serum TNFα concentration in the first trimester of pregnancy and reported serum TNFα concentration increased during 11 to13 weeks of gestation in women who later developed preeclampsia suggesting an increase of TNFα serum concentration predates the onset of preeclampsia. Similarly, elevation of TNFα serum concentration was reported at 16 weeks of gestation of pregnant women who later developed preeclampsia (Williams et al. 1999). Serin et al. (2002) investigated longitudinal changes in maternal serum TNFα concentration during

pregnancy in the first, second, and third trimesters to determine if TNFα predates the clinical onset of preeclampsia. Out of ninety subjects, ten developed preeclampsia. Out of ten, four had severe and six had mild preeclampsia. The subjects were diagnosed with preeclampsia between 34 and 38 weeks of gestation. There was no significant difference of TNFα serum concentration during the first or second trimester between preeclamptic and normal pregnant subjects. However, TNFα serum concentration was higher in the third trimester of preeclamptic subjects compared to normal pregnant subjects. This was in agreement with the study done by Gulati (2005) who reported higher TNFα serum concentration in the third trimester of preeclamptic subjects compared to normal pregnant subjects. However, no difference was noted in TNFα and IL-10 serum concentrations between preeclamptic and normal pregnant women in the first trimester (Eneroth et al. 1998).

In another study, TNFα serum concentration was the highest in preeclamptic pregnant women in comparison to hypertensive and normotensive pregnant women during the third trimester of pregnancy. Furthermore, monocyte secretion of TNFα of the preeclamptic pregnant women was increased in comparison to the normotensive and hypertensive pregnant women (Carlos et al. 2007). Beckmann et al. (2004) reported increased capability of leucocytes for spontaneous TNFα secretion during preeclampsia suggesting activation of leucocyte by the disease process. In addition, several studies reported increased TNFα plasma concentration of preeclamptic pregnant women (Laskowska et al. 2006, Velzing-Aarts et al. 2002, Haeger et al. 1996, Kupferminc et al. 1994 and Vince et al. 1995) and also decreased TNFα plasma concentration of preeclamptic pregnant women (Schiff et al. 1994 and Greer et al. 1994).

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Cytokine IL-10 is a polypeptide that binds to type 2 cytokine receptors and activates the JAK-STAT signaling pathway. Interleukin-10 inhibits the production of macrophage derived TNFα and suppresses the inflammatory response. Interleukin-10 is secreted by a variety of the immune system cells including monocytes and macrophages (Pinchuk, 2002) as well as by trophoblast cells (Roth et al. 1996). In vitro studies of peripheral blood mononuclear cells (Orange et al. 2003, Darmochwal-Kolarz et al. 2002 and Jonsson et al. 2005) and placental trophoblast cells (Rein et al. 2003 and Makris et al. 2006) from preeclamptic and normal pregnant women demonstrated significantly less IL-10 secretion when preeclamptic pregnant women were compared to normal pregnant women. In addition, IL-10 from normal pregnancy placental trophoblast cells suppressed the mixed lymphocyte response in vitro (Roth et al. 1996). Furthermore, decreased IL-10 and increased TNFα immunolocalization was observed in the placenta of pregnant preeclamptic women at term along with deceased serum IL-10 concentration (Hennessy et al. 1999 and Azizieh et al. 2005). In contrast, Rinehart et al. (1999) reported increased expression of IL-10 and TNFα of third trimester placental tissue before the onset of labor in preeclamptic pregnant women (all pregnant subjects underwent cesarean section). Similarly, serum and term placenta IL-10 were significantly higher in preeclamptic women in comparison to normal pregnant women (Benian et al. 2002 and Madazli et al. 2003). In contrast, Borekci et al. (2007) specified that serum IL-10 concentration was different between eclamptic and preeclamptic subjects being significantly higher in eclamptic than either preeclamptic or control pregnant subjects. The lowest concentration was noted in preeclamptic subjects. Glucocorticoids are antiinflammatory and immunosuppressive and were shown in vitro to reduce production of

proinflammatory cytokine TNFα without altering or decreasing the antiinflammatory cytokine IL-10 in both normal and term placenta (Xu et al. 2005).

A study done on preeclamptic and normal pregnant women at 18 weeks of gestation showed no difference in IL-10 and TNFα serum concentration. In addition, there was no indication of intensified systemic inflammatory response at 18 weeks of gestation in women who later developed preeclampsia (Djurovic et al. 2002). Freeman et al. (2004) evaluated short and long term changes of plasma inflammatory markers associated with preeclampsia and normal pregnant women. No difference was detected in baseline changes in IL-10 and TNFα in normal pregnant and pregnant women who subsequently developed preeclampsia. Plasma concentration of IL-10 and TNFα were significantly increased between first and second trimesters in preeclamptic pregnant women when compared to pregnant control group of women. Interleukin-10 and TNFα plasma concentration were also assessed in women who had preeclampsia 20 years after the first preeclamptic pregnancy to determine whether short term inflammatory changes noted during first pregnancy are present later in life. There was a trend towards decreased IL-10 in women with a history of preeclampsia with a significantly higher IL-1β to IL-10 ratio.

Magnesium, Insulin, Adiponectin, and Leptin

Insulin is an important regulator of cellular magnesium, and low concentration of intracellular magnesium recorded in the presence of hyperinsulinemia is the effect of insulin on magnesium efflux via the Na^+/Mg^{2+} exchange activation (Ferreira et al. 2004). Barbagallo and Dominguez (2007) reported that insulin has specific ionic effect to

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stimulate the transport of magnesium from the extracellular to intracellular compartment and increase intracellular ionic magnesium concentration. This increase in ionic magnesium may depend on the activation of the tyrosine-kinase insulin receptor. Under physiological conditions insulin induces an increase in cellular magnesium followed by a decrease via the Na^+/Mg^{2+} exchange demonstrating a biphasic regulation of magnesium concentration by insulin. Tissue uptake of magnesium is regulated by insulin and impairment of the process could either cause or exacerbate intracellular magnesium deficiency (Ferreira et al. 2004 and Barbagallo and Dominguez, 2007).

On the other hand, magnesium is an important regulator of insulin and glucose metabolism. Decrease in ionic magnesium concentration can directly promote insulin resistance and altered vascular tone, magnesium being the cause of, rather than merely the result of, peripheral insulin resistance. The relationship between plasma concentration of insulin and glucose and their apposing effects on magnesium represent the two limbs of a biphasic regulatory system of magnesium in physiological and pathological conditions (Barbagallo and Dominguez, 2007). In addition, magnesium response to insulin is decreased during hypertension, and reduced magnesium in normal cells makes them insulin resistant (Barbagallo et al. 1997). Moreover, low magnesium concentration in cells in response to a magnesium deficient diet is associated with a decrease in insulin action (Nadler et al. 1993). This indicates that any alteration in magnesium concentration has a profound effect on insulin action. Consequently, altered cellular magnesium is an independent determinant of insulin action (Barbagallo et al. 1999). Insulin via its receptor also increases free magnesium level in normal human platelets (Hwang et al. 1993), red blood cells (Paolisso et al. 1990), and lymphocytes (Delva et al. 2006). There

are several metabolic abnormalities that are common between insulin resistance syndrome and preeclampsia such as systemic inflammation, hypertension, dyslipedemia, and obesity (Wolf et al. 2001). Consequently, magnesium could be a missing link between preeclampsia and inflammation, insulin resistance, hyperlipedimia, and hypertension.

In physiological and pathological conditions when the cellular action of insulin is impaired, insulin resistance develops. The pancreas, in order to compensate for the increasing resistance of the tissues to the metabolic action of insulin, augments its secretion of insulin resulting in hyperinsulinemia. Normal pregnancy is also a state of relative or mild insulin resistance and hyperinsulinemia in order to ensure greater fuel availability to the fetus. Insulin resistance is maximal during the third trimester and is mediated through hormonal changes during pregnancy. Plasma insulin returns to prepregnancy concentration after delivery (Barbieri, 1999 and Yen, 1973).

Insulin resistance is also noted during conditions of inflammation particularly in association with proinflammatory cytokine hypersecretion such as TNFα (Festa et al. 2000 and Festa et al. 2003). Tumor necrosis factor alpha inhibits insulin signaling and insulin regulated glucose uptake and may be the mediator of insulin resistance in pregnancy (Kirwan et al. 2002). In addition, TNFα also inhibits the endothelium-dependent vasodilatation or insulin-stimulated endothelial cell function (Rask-Madsen et al. 2003). The effect of TNFα on insulin resistance is through the inhibition of insulin-dependent autophospohorylation of the insulin receptor and the phosphorylation of insulin receptor substrate-1 (Hotamisligil et al. 1994 and Kanety et al. 1995). Malek-Khosravi and Kaboudi (2004) conducted a longitudinal study of insulin concentration

changes in preeclamptic women during pregnancy and reported no change in serum glucose concentration in the second trimester between women who later developed preeclampsia in comparison to those who remained normotensive. Fasting blood glucose concentrations were similar in preeclamptic and normal pregnant women throughout the pregnancy. Fasting blood insulin concentration increased in both groups from the second to the third trimester. However, pregnant women who later developed preeclampsia had higher blood insulin concentration in the second trimester before the appearance of the clinical signs of preeclampsia. In another study, serial changes in fasting plasma concentration of TNFα were compared during the 16, 20, 24, 28, 32, and 36 weeks of gestation between normal pregnant women and pregnant women who eventually developed preeclampsia. The author reported a progressive increase in insulin resistance throughout normal pregnancy and preeclampsia but the increase was greater in women who later developed preeclampsia and predated the clinical onset of the disease. Similarly, blood TNFα concentration increased progressively throughout normal pregnancy and preeclampsia but the increase was greater in the second trimester in women who later developed preeclampsia. No correlation was found between increased insulin resistance and TNFα (Anim-Nyame et al. 2004). This is in contrast to the longitudinal study conducted during pregnancy where a significant correlation was reported between insulin resistance and TNFα during the third trimester of pregnancy irrespective of fat mass (Kirwan et al. 2002).

Several investigators have reported an increase in insulin resistance in preeclamptic pregnant women (Abundis et al. 1996, Kaaja et al. 1999 and Sowers et al. 1995). In contrast, it has been reported that insulin resistance in preeclamptic women

was the same as normal pregnant women (Roberts et al. 1998 and Salamalekis et al. 2005). Emery (2005) investigated 24 hour urine insulin excretion at 13 to 21 (base line 17 weeks) weeks of gestation and reported increased 24 hour urine insulin excretion in pregnant women. Among women who developed preeclampsia, adjusted urine insulin excretion was greater than the normal pregnant women but this increase was seen in pregnant women with mild preeclampsia and not severe preeclampsia. In addition, urine insulin excretion was not different between pregnant women who developed gestational hypertension and normal pregnant women.

Adiponectin is one of the bioactive peptides known as adipokines produced and secreted by adipose tissue and was discovered at the same time as leptin. The biological properties and effect of adiponectin are very different from other adipokines. In addition, contrary to other adipokines, adiponectin has high concentrations in blood approximately 10 μg/ml accounting for 0.01% of total serum protein. Human adiponectin is a 244 amino acid peptide hormone that belongs to the collagen superfamily and is homologous with TNFα, complement factor, and collagen. Adipose tissue is the principal site of adiponectin secretion. There are two types of adiponectin receptors, AdipoR1 (muscle cell) and AdipoR2 (liver cell). In addition, adiponectin receptors are found ubiquitously in the body. Globular adiponectin binds to both kinds of receptors resulting in glucose uptake and fatty acid oxidation through activation of AMP kinase and PPARλ, respectively. Adipo- receptor1 interacts with insulin receptor in muscle cells enhancing insulin signal transduction and resultant improvement of insulin resistance. In addition, activation of glucose transporter-4 further contributes to insulin sensitivity (Koerner et al. 2005 and Kiess et al. 2008). Furthermore, adiponectin has a protective role in the

pathogenesis of obesity and is inversely associated with obesity, insulin resistance, cardiovascular disease, and type 2 diabetes (Hug et al. 2005, Lawlor et al. 2005 and Hotta et al. 2000). Goldstein et al. (2004) described a negative correlation between plasma adiponectin and obesity, insulin resistance, dyslipidemia, and blood pressure. Likewise, high adiponectin plasma concentration increases insulin sensitivity, and the relationship is independent of low body mass and affects not only insulin stimulated glucose disposal but also lipoprotein metabolism and insulin mediated suppression of postprandial FFA release (Tschritter et al. 2003).

Adiponectin is a pleiotropic cytokine reported to have antiatherogenic, anti-inflammatory and antidiabetic properties (Trujillo et al. 2005 and Shetty et al. 2004). Adiponectin increases fatty acid oxidation in muscle and decreases plasma concentration of glucose, FFAs, and triacylglycerol (Fruebis et al. 2001). Furthermore, adiponectin acts as an endogenous regulator of endothelial cells in response to inflammatory stimuli via modulation of nuclear factor-κB signaling through a cyclic AMP/protein kinase A pathway attenuating the excessive inflammatory response in the vascular wall (Ouchi et al. 1999, Ouchi et al. 2000 and Ouchi and Walsh 2007). Consequently, adiponectin has an antiinflammatory and antiatherogenic effect on endothelial cells and macrophages (Kappes et al. 2000). Adiponectin reduces and suppresses the secretion of TNFα from monocytes, macrophages and in addition, lessens biological effects induced by TNFα (Ouchi et al. 2001). Conversely, TNFα suppresses adiponectin expression and secretion of adipocytes. In addition, C-reactive protein, an acute phase reactant and a major marker for systemic inflammation, was reported to have negative a correlation with plasma adiponectin concentration (Matsubara et al. 2003).

Adiponectin is considered to play an important role in mediating insulin sensitivity. Pregnancy is a state of relative insulin resistance with insulin concentration increasing throughout gestation with the highest concentration during the third trimester of pregnancy, which returns to normal after delivery (Yen, 1973 and Catalano et al. 1993). During preeclampsia, insulin resistance is exaggerated with the increase being more during the third trimester in comparison to normal pregnancy and predates the onset of clinical disease (Malek-Khosravi and Kaboudi, 2004). Naruse et al. (2005) investigated serum adiponectin concentration of pregnant women and reported decreased serum adiponectin concentration of pregnant women in comparison to nonpregnant women. However, this difference was attributed to haemodilatation when values were corrected for hematocrit. Reduced plasma concentration of adiponectin and increased concentration of leptin were reported for pregnant preeclamptic women indicating an altered ratio between leptin and adiponectin with further reduction of the ratio with disease severity during preeclampsia. In addition, a negative correlation was observed between adiponectin and leptin blood concentration (Ouyang et al. 2007 and Masuyama et al. 2007). This is in contrast to the finding reported by Takemura et al. (2007) where serum adioponectin concentration was increased in preeclampsia and the increase was speculated as a defensive reaction of the body. Similarly, Ramsay et al. (2003) reported a paradoxical elevation of plasma adiponectin concentration in preeclamptic pregnant women. In addition, adiponectin concentration was increased in normal weight pregnant preeclamptic women (BMI < 25 kg/m^2). Whereas, women with severe preeclampsia and BMI ≥ 25 kg/m^2 had decreased adiponectin and increased leptin concentration (Hendler et al. 2005).

In order to discover if adiponectin blood concentration increased before the clinical onset of preeclampsia, the total serum adiponectin concentration was estimated of serum samples stored in a serological biobank of pregnant women who later developed preeclampsia and those who did not. However, there was no difference for total serum adiponectin concentration between the two groups, and it was reported that total serum adiponectin concentration did not predate the clinical signs of preeclampsia onset (Odden et al. 2007). In another study, blood adiponectin concentration was examined in the first trimester of pregnancy and was discovered to be significantly lower in women who subsequently developed preeclampsia demonstrating decreased adiponectin blood concentration predated the clinical signs of preeclampsia onset (D'Anna et al. 2005). However, in a longitudinal study of serum adiponectin concentration during normal pregnancy, it was reported that serum adiponectin concentration increased in the first part of gestation, peaked in mid gestation, and the lowest concentration was noted in late gestation. In addition, an inverse relationship was observed with BMI of normal pregnant and non-pregnant women. The increase in adiponectin concentration in the first half of pregnancy is paradoxical. Adiponectin is produced mainly by adipocytes and its inverse relationship to the amount of adipose tissue is intriguing. In addition, an inverse association exists between insulin resistance and adiponectin concentration even after adjusting for BMI (Fuglsang et al. 2006). The main source of circulating blood adiponectin concentration is claimed to be from adipose tissue. However, Caminos et al. (2005) identified adiponectin messenger RNA and its receptor, Adipo-R2, in placental cytotrophoblast and syncytotrophoblast cells. The placental expression of adiponectin messenger RNA in the placenta was much weaker than it was in the adipose tissue. In

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addition, both adiponectin receptors, Adipo-R1 and R2, are abundantly expressed in the placenta (Yamauchi et al. 2003). In contrast, Hauguel-de Mouzon and Guerre-Millo (2006) did not detect adiponectin messenger RNA of placentas obtained from preeclampsia pregnant women. Ichida et al. (2007) identified a moderate immunointensity for adiponectin protein of endothelial cells of chorionic vessels exclusively of the term placenta of preeclamptic pregnant women. Very weak or no immunostaining for adiponectin protein in the endothelial cells of chorionic vessels was detected of term placenta of normotensive pregnant women. These finding are in contrast to the one reported by Corbetta et al. (2005) where adiponectin expression was identified in fetal tissue at mid and late gestation (14 to 36 weeks) but not for placenta. However, a strong immunostaining of adiponectin expression was present within the blood vessel lumina of chorionic villus in all placental samples but not of endothelial cells in samples of different gestational ages. Using an explant system adiponectin release was demonstrated in placenta and fetal membranes along with maternal subcutaneous adipose tissues and skeletal muscle obtained from normal pregnant women (Lappas et al. 2005).

For a long time adipose tissue was considered as only an energy storage tissue. Research revealed that white adipose tissue produces a wide variety of bioactive peptides known as adipokines or adipocytokines. Adipocytokines are defined as soluble mediators that are mainly but not exclusively produced by adipocytes and exert their biological effect in an autocrine, paracrine or systemic manner (Tilg and Moschen, 2008). Interestingly, many of these adipocytokines are not only involved in energy homeostasis, but they actively participate in development of inflammation, coagulation, and insulin sensitivity (Juge-Aubry et al. 2005). One of the adipocytokine is the protein leptin.

Leptin is a 16 kDa non-glycosylated peptide hormone named leptin from the Greek word for "thin." Leptin is encoded by the obesity gene "ob." The ob gene encodes a protein of molecular weight 18000 g/mol containing a signal sequence that is cleaved to produce the mature hormone of molecular weight 16000 g/mol (Zhang et al. 1994). Leptin was considered to be synthesized by white adipose tissue however with further studies it was recognized that leptin is expressed in several other sites such as placenta (Hassink et al. 1997), mammary gland (Aoki et al. 1999), ovaries, stomach, brain, brown fat, and muscle to name a few (Trayhurn et al. 1999 and 2001). Leptin receptor "OB-R" belongs to class 1 cytokine receptor family. There are four isoforms of the human leptin receptor and one or more isoforms are present in most tissues (Tartaglia et al. 1995 and Hoggard et al. 1997). Leptin receptors are present on cells in organs involved in energy storage, digestion, metabolism, reproduction, and immunity for example brown adipose tissue, placenta, ovaries, mammary gland, spleen, thymus, lymph nodes, haematopoietic cells, T cells, endothelium, and several other sites indicating that leptin acts as a endocrine, paracrine, and autocrine hormone. Leptin and its receptors have structures similar to pro-inflammatory cytokines and their receptors.

Leptin beside its main effects on the hypothalamus also acts as an inflammatory cytokine in the periphery. In other words, leptin is a pleiotropic hormone and cytokine. Ligand binding of the leptin OB-R_b receptor activates Janus kinases (JAK$_2$) and signal transducers and activators of transcription (STAT) proteins similar to class 1 cytokine receptor family and share some of the signaling pathways of class 1 cytokine family (Ghilardi et al. 1996 and Bjorbaek et al. 1997). In addition, signal transduction by

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cytokine 1 family is through mitogen-activated protein kinase (MAPK) and phosphotidyl inositol-3 kinase (PI-3) (Watowich et al. 1996).

Leptin is considered to play an important part in the regulation of appetite and energy expenditure via its effect on body fat mass. However, its role in pregnancy is not fully understood. Schubring et al. (1998) investigated longitudinal changes of maternal serum leptin concentration during pregnancy and determined that maternal serum leptin concentration continuously increased from 6 to 8 weeks up to 38 to 40 weeks of pregnancy. At 6 to 8 weeks of pregnancy maternal leptin serum concentration correlated significantly with body mass index and this correlation decreased with advancing gestation and at birth only poor correlation remained. Similar results were reported by Mukherjea et al. (1999), Tamas et al. (1998), and Tamura et al. (1998) who reported leptin concentration of plasma was increased early in pregnancy and remained elevated throughout pregnancy with no correlation with body weight or BMI during the third trimester of pregnancy. However, Sattar et al. (1998) investigated longitudinal changes in maternal leptin concentration during pregnancy and determined that maternal leptin concentration did not increase progressively throughout gestation but peaked at around 20 to 30 weeks before decreasing by term.

Leptin concentration during gestation is increased to a degree which cannot be explained by an increased body mass (Hardie et al. 1997). Leptin receptors were identified in the syncytiotrophoblasts of the placenta (Bodner et al. 1999). In addition to leptin, messenger RNA of leptin is also synthesized and secreted by the placenta cytotrophoblasts and syncytiotrophoblasts into the maternal circulation (Masuzaki et al. 1997, Schulz et al. 2000, Linnemann et al. 2000, Hoggard et al. 2001 and Lepercq et al.

2001). Leptin concentration in non-pregnant women is 3 to 4 times lower than in pregnant women (Schubring et al. 1998). During preeclampsia, leptin concentration is increased when compared with normal pregnant women and is independent of the maternal BMI. The normal relationship between maternal leptin concentration and adiposity may be altered in preeclampsia (McCarthy et al. 1999). William et al. (1999) noted that other factors in addition to the level of adiposity may influence serum leptin concentration in preeclamptic pregnant women.

Leptin concentration is similar in different grades of preeclampsia (mild to severe) and in normal pregnancy (Martinez-Abundis et al. 2000).   However, in a different study, leptin concentration was found to be elevated in normal pregnancy and is increased more in pregnant women with preeclampsia. This increase is due to an increase in the free fraction of leptin and indicates that the increase of leptin concentration in preeclampsia is biologically relevant (Teppa et al. 2000). In an investigation of longitudinal changes of blood leptin concentration during preeclampsia, it was reported that pregnant women that eventually developed preeclampsia had leptin concentrations consistently higher from 20 weeks of gestation, and in contrast to the normal pregnant control leptin concentration rose markedly from 32 weeks as preeclampsia developed. This study pointed out that leptin concentration increased during established preeclampsia and increased leptin concentration predates clinical manifestation of preeclampsia (Anim-Nyame et al. 2000).

On the other hand, no difference was noted for leptin concentration between normal pregnancy and preeclampsia pregnancy (Sattar et al. 1998). While lower blood leptin concentration was found during preeclampsia in comparison to normal pregnant

women (Lami et al. 2001 and Clausen et al. 2002). However, Vitoratos et al. (2001)

reported no difference for leptin concentration between normal pregnant women and

women with gestational hypertension but found leptin concentration was significantly

higher in preeclampsia in comparison to normal and hypertensive pregnant women. In

addition, Chappell et al. (2002) and Tommaselli et al. (2004) identified women who were at

risk for preeclampsia. Risk was assessed on the basis of an abnormal uterine artery Doppler flow

velocity waveform (FVW) results. A previous history of preeclampsia, and an early increase

in blood leptin concentration of high risk pregnant women who later developed

preeclampsia was reported. The difference of blood leptin concentration between normal

pregnant and preeclamptic women persisted even after correction for BMI. On the other

hand, blood leptin concentration was similar though out pregnancy between healthy

pregnant women and pregnant women who gave birth to small for gestation age infants.

During preeclampsia the normal relationship between leptin and adiposity is disturbed

and indicates that factors other than adiposity determine leptin concentration in

preeclampsia.

Tumor necrosis factor alpha has been reported to increase leptin concentration in

humans similar to that seen in hamsters and mice (Zumbach et al. 1997, Mantzoros et al.

1997, Grunfeld et al. 1996 and Sarraf et al. 1997). Longitudinal investigation of leptin

and TNFα concentration during pregnancy indicates a correlation between leptin and

TNFα in first and second trimesters but not in the third trimester of pregnancy.   Tumor

necrosis factor alpha concentration was low in early pregnancy and increased during the

second and third trimesters of pregnancy (Clapp et al. 2000), and significant correlation

was reported between TNFα and leptin in both normal pregnant women and pregnant

women with preeclampsia during the third trimester of pregnancy. In addition, higher blood concentration of TNFα of pregnant women with preeclampsia was associated with higher blood concentration of maternal leptin concentration. This points to a main role of TNFα in regulating leptin concentration in preeclampsia since the relationship between maternal leptin concentration and BMI is lost in preeclampsia (Bartha et al. 2001). On the other hand, Soh et al. (2000) reported no significant change in leptin concentration when WISH, JEG3 cell, and amnion, choriodecidua explant were treated with TNFα.

CHAPTER III

AN EVALUATION OF MAGNESIUM STATUS AND INFLAMMATORY

RESPONSE DURING THE THIRD TRIMESTER OF NORMAL

PREGNANCY AND PREECLAMPSIA-PART I

Abstract

Recent reports suggest that preeclampsia is the result of an excessive maternal systemic inflammatory response. Normal pregnancy has been stated as a condition of "physiological hypomagnesesaemia" and mild systemic inflammation. The role of magnesium and systemic inflammation in normal pregnancy and preeclampsia needs to be defined. The aim of this study was to investigate whether the demographic and anthropometric characteristics are of relevance in the development of preeclampsia. Thirty five healthy pregnant women (third trimester) and thirty five preeclamptic (third trimester) women volunteered to participate. Pregnant and preeclamptic subjects were paired by age, parity, and gestation. Selected information was collected from the subject's medical chart and questionnaire after they had given their written informed consent. Current blood pressure, body weight, and urine albumin were also recorded. Maternal gestational age, body weight, age, prepregnancy diastolic blood pressure, intake of birth control pills, supplements, and occurrence of miscarriage, still births, race, and employment status were statistically unchanged between the two groups. Significant difference was noted in prepregnancy systolic blood pressure, which was higher in the

preeclamptic group but was within normal range. Current systolic and diastolic blood pressure, family history of preeclampsia, urine albumin, minimum and maximum temperature and precipitation at the time of presentation were increased in the preeclamptic group. Number of children and pregnancies were significantly more in the normal pregnant group. Temperature and precipitation, nulliparity, second pregnancy, hypertension, proteinuria, and family history of preecalmpsia appear to be risk factors for preeclampsia. Multiparity was statistically significant in normal pregnant women.

Introduction

Preeclampsia and eclampsia remain one of the leading causes of maternal and infant morbidity and mortality. The incidence of preeclampsia is between 5 and 7% of all pregnancies in the USA (Podjarny et al. 1999). Preeclampsia is typically diagnosed by the fourth to the sixth month of pregnancy. The overall preeclampsia/eclampsia case fatality rate is 6.4 per 10,000 cases at delivery (from 1979 to 1992) and approximately one half (51%) of preeclampsia- eclampsia deaths are associated with preeclampsia and the remainder with eclampsia (MacKay et al. 2001). According to Vital Statistics Mississippi 2001, the number of women suffering from eclampsia is 2,225 out of a total of 11,483, approximately 19.4% (Mississippi State Department of Health 2001). Very young and older women, as well as first-time mothers, are typically the ones who develop preeclampsia. In addition, women at high risk of developing the disease include those who have multiple pregnancies, high blood pressure, diabetes mellitus, obesity, and a previous history of preeclampsia (Duckitt and Harrington 2005). Hypertension after 20 weeks of gestation is essential for diagnosis of preeclampsia.

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The pathophysiological features of preeclampsia indicate that the disorder is early in gestation and predates its clinical presentation (Roberts, 2000). Mild preeclampsia is defined as systolic blood pressure of at least 140 mmHg and/or a diastolic blood pressure of at least 90 mmHg on at least two occasions at least 6 hours apart after 20 weeks of gestation of women known to be normotensive before pregnancy and before 20 weeks of gestation, plus proteinuria (300 mg or more per 24 hours period of urine collection). If 24 hour urine collection is not available, then proteinuria is defined as concentration of at least 30 mg/dL (at least 1+ on dip stick) in at least two random urine samples collected at least 6 hours apart. Severe preeclampsia is defined as sustained elevations in systolic blood pressure to at least 160 mmHg and/or diastolic blood pressure of at least 110 mmHg for at least 6 hours in association with proteinuria, or if there is hypertension in association with severe proteinuria (at least 5 g per 24 hours period of urine collection). In addition, preecalmpsia is considered with multiorgan involvement such as cerebral dysfunction manifesting as blurred vision, scotoma, headache, and cerebrovascular accidents; epigastric or right upper quadrant pain; renal failure or oliguria ≤ 500 ml of urine in 24 hours; pulmonary edema.; impaired liver function (serum transaminase levels two times normal or greater); thrombocytopenia ($\leq 100,000$ platelets/mm^3); coagulopathy; fetal growth restriction; eclampsia and generalized convulsions and HELLP "hemolysis, elevated liver enzymes and low platelets" (Tannirandorn, 2005). Subclassification of preeclampsia further categorizes preeclampsia as early and late onset. Early onset preeclampsia is defined as onset earlier than 28 weeks and late onset preeclampsia after 28 weeks of gestation (Von Dadelszen et al. 2003).

A longitudinal study investigating the role of obesity during pregnancy induced hypertension was conducted, and it was reported that the mean prepregnancy body mass index was higher in women who had pregnancy induced hypertension compared with women who did not (Thadhani et al. 1999 and Odegard et al. 2000). Whereas, prepregnancy BMI $\geq$32.3 kg/m^2 and a previous history of preeclampsia and nulliparity increased the risk of subsequent preeclampsia in future pregnancy (Stone et al. 1994, O' Brien et al. 2003 and Baeten et al. 2001). Obesity is a definite risk for preeclampsia and the risk increases with a greater BMI (overweight: BMI 25 - 29 kg/m^2 and obese: BMI $\geq$30 kg/m^2). With the worldwide increase in obesity, the frequency of preeclampsia is likely to increase (Dekker and Sibai 2001 and Hrazdilova et al. 2001).

Primigravidae are at a higher risk than other pregnant women of developing preeclampsia as well as multigravidae having second or later pregnancies (Trupin et al. 1996). Preeclampsia is also associated with maternal age, and there is a sharp rise in the incidence of preeclampsia in women above the age of 35, those with a family history of preecalampsia (Lyall and Greer, 1994), and those below the age of 20 (Sibai et al. 2005).

The incidence of preeclampsia has also been associated with seasonal variation. This may help in identifying exposures to potential etiological risk factors such as physical inactivity, cold temperature, and infectious agents. An increased incidence of preeclampsia of primigravida in Washington peaked among those women who had conceived during spring and summer months (Rudra and Williams 2005). Phillips et al. (2004) reported seasonal variation for preeclampsia that was related to timing of conception than delivery with increased incidence in summer. Wacker et al. (1998) in their study in Zimbabwe noted increased prevalence of preeclampsia in dry hot seasons

and in the first month of the rainy season. They explained the increased incidence of preeclampsia during these months as due to humidity and temperature effects on blood vessels or production of vasoactive substances, since dry rainy seasons influence agriculture yields which impacts nutritional intake. A similar study, done in Mississippi, reported a lack of seasonality in Mississippi on the incidence of preeclampsia (Magann et al. 1995).

Magnesium sulfate is the drug of choice for treatment and management of preeclampsia and eclampsia and has been used for therapy and prophylaxis for more than 70 years. The Honolulu heart study investigated various dietary variables and their relationship to high blood pressure. Out of all of the nutrients studied, only magnesium had a strong relationship with blood pressure (Joffres et al.1987). Further studies supported this relationship, showing that an increased intake of magnesium contributed to the prevention of hypertension (Stuehlinger 2001 and Mizushima et al. 1998). A significant negative correlation between blood magnesium concentration and blood pressure has been described under clinical conditions (Touyz et al. 1992), and magnesium deficiency has been shown to promote dyslipidemia, insulin resistance, vasospasm, and endothelial cell damage, a characteristic feature of small arteries in hypertension (Touyz and Milne 1995 and Touyz, 2003). Magnesium deficiency induced experimentally resulted in an altered lipid profile and increased blood pressure (Blache et al. 2006, Laurant and Touyz, 2000 and Kh et al. 2000). In addition, using experimental models of preeclampsia, magnesium infusion reduced high blood pressure and magnesium deficiency resulted in hypertension (Standley et al. 2006 and Saito et al. 1995). During preeclampsia and pregnancy induced hypertension, magnesium was reported to reduce

high blood pressure. In addition, Kisters et al. (1990) reported that decreased concentration of cellular magnesium of preeclamptic women contributed to the development of hypertension in these subjects.

Magnesium deficiency is common due to a variety of reasons but often remains misdiagnosed and undetected (Liebscher et al. 2004). A seasonal and geographical pattern of magnesium deficiency has been described for Mississippi (Franz and Bailey, 2004). An inverse relationship between magnesium content of water and cardiac disease has been documented. In areas where there was soft drinking water the incidence of cardiac diseases was greater (Seelig, 1989). In addition, intake of large quantities of processed food results in magnesium deficiency as a large amount of magnesium is lost during processing, and it has been reported that this leads to increased incidence of cardiac disease (Maier, 2003). Pregnancy has been stated as a condition of "physiological hypomagnemia" (Laires et al. 2004) and a state of maternal systemic inflammation. Any factor that would increase the maternal systemic inflammatory response to pregnancy (Redman and Sargent, 2005) and magnesium deficiency could predispose to preeclampsia (Vormann et al. 1992).

Objectives

The primary objectives of this study were: 1. Demographic: To compare constitutional characteristics and medical history, age, race, parity, miscarriage, abortion, still births, number of children, and vitamin and mineral supplements of normal and preeclamptic pregnant women.

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2. Anthropometric: To compare prepregnancy weight, current weight, and prepregnancy and current blood pressure of normal and preeclamptic pregnant women.

Methods

Selection of Subjects and Research Design

Normal pregnant women (third trimester) and preeclamptic pregnant women (third trimester) were matched according to the eligibility criteria. Thirty five healthy pregnant women (third trimester) and thirty five preeclamptic women (third trimester) that were patients of Oktibbeha County Hospital, (OCH) Starkville, Mississippi volunteered to participate. The study was approved by Mississippi State University (MSU) Institutional Review Board (IRB) IRB Docket #04-209. The eligibility criteria were: age eighteen to forty years; no medication; no previous pathology; no intake of contraceptives; no intake of alcohol; no use of tobacco; and no medical history of diabetes or chronic hypertension. Selected information was collected from the subject's medical chart after they had given their written informed consent. Information was also collected through a self-administered questionnaire pertaining to the subject's medical history and constitutional characteristics.

Preeclampsia was defined by a blood pressure elevation of $\geq$140/90 mm Hg after 20 weeks gestation in association with proteinurea, either 1+ or more by dipstick or 300 mg/24 hour urine collection or more. Women with preeclampsia had no significant past medical history such as diabetes or chronic hypertension. Women with preeclampsia were matched for gestational age, parity, and age with women with normal pregnancy.

Data were analyzed by analysis of variance (ANOVA), Tukey's test, and Chi-Square. Differences in continuous variables between groups were tested by ANOVA and Tukey's test and reported as mean ± SEM. Chi square test was used for categorical data and reported as percentages. Means were separated by Fisher's protected least significant difference method (at $\alpha = 0.05$). The Statistical Analysis Software (SAS) was used to analyze data (SAS, version 9.1.3, 2006).

Results

Data were collected during the months of August, 2005 through April, 2007. No statistical difference was noted between normal and preeclamptic pregnant women gestational age. In addition, there was no significant difference between the two group's age and body weight (Table 3.1). There was no significant difference in the pre-pregnancy diastolic blood pressure. The prepregnancy systolic blood pressure was noted to be significantly higher in the preeclamptic group than the controls $P \leq 0.01$. At the time of data collection (August, 2005 through April, 2007), the diastolic and systolic blood pressure was significantly higher in the preeclampsia group $P \leq 0.001$ (Table 3.2). There was no significant difference between the two groups' number of miscarriages, abortions or stillbirths. However, a significant difference was noted in the number of pregnancies and children between the two groups, with increased number of pregnancies and children of the normal pregnant women (Table 3.3). The month, maximum and minimum temperatures, and precipitation at the time of diagnosis were significantly different between normal and preeclamptic pregnant women (Table 3.4). A history of preeclampsia reported for the preeclamptic women's mothers was significantly higher in

the preeclampsia group than the normal pregnant women P ≤ 0.02. At the time of data

collection, the urine albumin was significantly higher in the preeclampsia group P ≤

0.001. Intake of vitamins and minerals, birth control pills and employment status was

similar between the two groups (Table 3.5).

Discussion

Preeclamptic pregnant women were matched with the normal pregnant women by

week of gestation in order to prevent the influence of gestational age on the study (Laires

et al. 2004). As expected no statistical difference was noted between normal and

preeclamptic pregnant women gestational age. In addition, there was no significant

difference between the two groups age and body weight. It is reported that preeclampsia

is associated with maternal age and there is a sharp rise in the incidence of preeclampsia

above the age of 35 (Lyall and Greer, 1994) and age younger than 20 years (Sibai et al.

2005). However, in this study the average age of the preeclamptic and normal pregnant

women was 26 and 25 years, respectively. Obesity is also considered a definite risk

factor for preeclampsia and the risk increases with a higher BMI (overweight: BMI 25 -

29 kg/m^2 and obese: BMI ≥ 30 kg/m^2). In addition, prepregnancy BMI (≥ 32.3 kg/m^2), a

previous history of preeclampsia, and nulliparity increases the risk of subsequent

preeclampsia in future pregnancy (Stone et al. 1994, O' Brien et al. 2003 and Baeten et

al. 2001).

One of the eligibility criteria for selection of subjects was that both groups be

normotensive before their pregnancy. There was no significant difference in the pre-

pregnancy diastolic blood pressure. The prepregnancy systolic blood pressure was found

to be significantly higher in the preeclamptic group than the control. These data may be of no clinical significance as the values were within the expected range. At the time of data collection, the diastolic and systolic blood pressure were significantly higher in the preeclampsia group, validating the diagnosis of preeclampsia. In addition, women at a higher risk of developing preeclampsia include those who have multiple pregnancies, high blood pressure, diabetes mellitus, obesity and a previous history of preeclampsia (Duckitt and Harrington, 2005). Kisters et al. (1990) reported that decreased concentration of cellular magnesium in preeclamptic women contributed to the development of hypertension in these subjects. Whereas, during preeclampsia and pregnancy induced hypertension, magnesium was reported to reduce high blood pressure.

The occurrence of miscarriage was similar between the two groups which may be explained by the low parity of the preeclampsia group. It was either their first or second pregnancy. The normal pregnant group had more children and pregnancies and this was the reason for the significant difference noted (Mostello et al. 2002). Primigravidae are at a higher risk than other pregnant women of developing preeclampsia as well as multigravidae having second or later pregnancies (Trupin et al. 1996). Trupin's report is in agreement with this study; the preeclamptic women were either nulliparous or it was their second pregnancy, however, normal pregnant women were mostly multiparous, which is in contradiction of the above statement.

In the current study, the majority of preeclamptic subjects presented in the winter and spring season, during the months of December, February, March, and April which is in agreement with the results reported by Magnus and Eskild (2001). However, a number of preeclamptic subjects presented in summer in the current study, a finding which was

similar to the one reported by Franz and Bailey (2004) and as these preeclamptics subjects in the current study were on hypertension medication they were excluded from the study. Mississippi spring and fall seasons have a mean maximum temperature of 22.8^0 C (73^0 F) and 18.3^0 C (65^0 F) with relative humidity of 85 and 84%, respectively. The summer season has a mean temperature of 32.7^0 C (90.8^0 F) and relative humidity of 89.8% (Magann et al. 1995).

Climatic conditions that increase sweating may result in excessive loss of magnesium that is not compensated by diet or water intake. In addition, areas of high precipitation usually have low water hardness which could further lead to magnesium deficiency (Franz and Bailey, 2004), and dietary deficiencies during spring would predispose to more frequent occurrence of preeclampsia in the late autumn and winter months (Magnus and Eskild, 2001). Dry and rainy seasons influence agricultural yields and may result in magnesium deficient crops such as grains, vegetables, and fruits further compounding magnesium deficiency. Preeclampsia is associated with a family history of preecalampsia and there is a sharp rise in the incidence of preeclampsia when it is present in ones family history (Lyall and Greer 1994 and Duckitt and Harrington, 2005). In this study, a history of preeclampsia reported for the preeclamptic women's mothers was higher and statistically significant in the preeclampsia group. It may be possible that the families of the preeclamptic women were from the same area (Mississippi) and similar seasonal factors were operating on the mother leading to nutrient deficiencies and subsequent preeclampsia.

Another eligibility criterion for selection of subjects was that the preeclamptic women should have albumin present in their urine. At the time of data collection, urine

albumin was significantly higher in the preeclampsia group validating the diagnosis of preeclampsia. The intake of vitamins, minerals, and birth control pills and employment status were similar between the two groups and the influence of these risk factors was analogous between the two groups.

Conclusion

This study demonstrated that the significant risk factors associated with preeclampsia were ambient temperature, precipitation, nulliparity, second pregnancy, hypertension, proteinuria, and family history of preeclampsia, which are in agreement with reported risk factors. However, additional risk factors like multiparity were only noted in the normal pregnant group. Hypertension and proteinuria are prerequisite for diagnosis of preeclampsia and are secondary to an initiating factor inducing preeclampsia. As a majority of normal pregnant women were multiparous, parity as such did not seem to play a key role. Ambient temperature and precipitation however were associated with preeclampsia and seem to be an important factor. Family history of preeclampsia was also relevant. Understanding the role of demographics and clinical characteristics during pregnancy and preeclampsia are important steps in understanding the pathophysiology of preeclampsia and may provide a better prenatal identification of pregnant women at risk of developing preeclampsia.

Table 3.1   Descriptive and Clinical Characteristics of Normal and Preeclamptic Pregnant Women-age, body weight before pregnancy, current body weight, and weeks of gestation*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance
Age (year)	25 ± 0.90	26 ± 0.80	N.S.¶
Body Weight Before Pregnancy (lbs)	147 ± 60	160 ± 7.0	N.S.¶
Current Body Weight[a] (lbs)	177 ± 60	193± 7.0	N.S.¶
Weeks of Gestation (wks)	38 ± 0.30	37 ±0.30	N.S.¶

[a] Bodyweight recorded at consent to participate in the study *Values are means ±SEM Significant differences (P≤ 0.05) ¶No significance at P ≥0.05

Table 3.2   Descriptive and Clinical Characteristics of Normal and Preeclamptic Pregnant Women-current diastolic and systolic blood pressure and before pregnancy diastolic, and systolic blood pressure*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance
Current Diastolic BP (mmHg)	75 ± 1.5	97 ± 1.04	$P \leq 0.001$
Current Systolic BP (mmHg)	124 ± 1.7	152 ± 2.2	$P \leq 0.001$
Before Pregnancy Diastolic BP (mmHg)	65 ± 1.5	69 ± 1.5	N.S.¶
Before Pregnancy Systolic BP (mmHg)	112 ± 1.6	118 ± 1.7	$P \leq 0.010$

*Values are means± SEM   Significant differences (P≤ 0.05) ¶No significance at P ≥0.05

Table 3.3   Descriptive and Clinical Characteristics of Normal and Preeclamptic
             Pregnant Women-number of miscarriages, children, pregnancies, and still
             births*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance
Number of Miscarriages	0.40 ± 0.14	0.28 ± 0.07	N.S.¶
Number of Children	0.08 ± 0.90	0.60 ± 0.13	$P \leq 0.02$
Number of Pregnancies	2.50 ± 0.25	$2.00 \pm .15$	$P \leq 0.01$
Number of Stillbirths	0.29 ± 0.03	0.00 ± 0.00	N.S.¶

*Values are means$\pm$ SEM   Significant differences (P$\leq$ 0.05) ¶No significance at P $\geq$0.05

Table 3.4   Descriptive and Clinical Characteristics of Normal and Preeclamptic
             Pregnant Women-month of presentation, minimum and maximum
             temperature of day arrived in hospital, and precipitation*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance
Month of Presentation	2.3 ± 0.10	6.0 ± 0.60	$P \leq 0.001$
Minimum Temperature Day Arrived in Hospital (F)	41 ± 1.9	49 ± 2.7	$P \leq 0.020$
Maximum Temperature Day Arrived in Hospital (F)	60 ± 2.1	74 ± 2.7	$P \leq 0.001$
Precipitation (Rain Yes)	35 †	6 †	$P \leq 0.001$

*Values are means$\pm$ SEM   †Values are % Significant differences (P$\leq$ 0.05)

Table 3.5   Descriptive and Clinical Characteristics of Normal and Preeclamptic Pregnant Women-race, intake vitamin and mineral before and during pregnancy, family history of preeclampsia, employment status, took birth control pills, and albumin in urine*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance
Race (white %)	59	47	N.S.¶
Intake Vitamin & Min. Before Pregnancy (Yes %)	29	32	N.S.¶
Intake Vitamin & Min. During Pregnancy (Yes %)	82	82	N.S.¶
Family History of Preeclampsia (Yes %)	3	21	$P \leq 0.020$
Employment Status (Yes %)	47	32	N.S.¶
Took Birth Control Pill (Yes%)	62	74	N.S.¶
Albumin In Urine (dipstick) (%)	0	100	$P \leq 0.001$

*Significant differences ($P \leq 0.05$) ¶No significance at $P \geq 0.05$

CHAPTER IV

AN EVALUATION OF MAGNESIUM STATUS AND INFLAMMATORY

RESPONSE DURING THE THIRD TRIMESTER OF NORMAL

PREGNANCY AND PREECLAMPSIA-PART II

Abstract

Recent reports suggest that preeclampsia is the result of an excessive maternal systemic inflammatory response. The role of magnesium and systemic inflammation in normal pregnancy and preeclampsia needs to be defined. The objectives of this study were to determine and compare serum ionized magnesium (iMg++) and plasma total magnesium (tMg) concentrations in normal pregnant and preeclamptic pregnant subjects during the third trimester of pregnancy and to determine the association between inflammatory makers and magnesium status. The demographic and anthropometric characteristics of the subjects were previously described in Part 1 of the study. In the current study, iMg++ and tMg concentrations and associations with inflammatory markers: tumor necrosis factor alpha (TNFα), endothelin-1 (ET-1), leptin, adiponectin, Interleukin-10 (IL-10), triacylglycerol, and free fatty acids (FFA) were examined. Analyses were done by the use of ion-selective electrode, atomic absorption spectrometry, spectrophotometer, and ELISA kits. In the preeclampsia group, blood concentration of TNFα, ET-1, leptin, FFA, and triacylglycerol increased while tMg,

IL-10, and adiponectin blood concentration decreased, and a strong significant (P ≤ 0.001) difference was noted between normal and preeclamptic pregnant women. Significant (P ≤ 0.001, P ≤ 0.04, P ≤ 0.001, P ≤ 0.001, and P ≤ 0.02) inverse correlation was noted between iMg++ and tMg with TNFα, ET-1, leptin, FFA, and triacylglycerol Furthermore, a significant (P ≤ 0.001) positive correlation was demonstrated between iMg++ and tMg and of iMg++ and tMg with IL-10 and adiponectin between normal and preeclamptic pregnant women. This study is the first human study to evaluate the role of magnesium and its relationship with inflammatory response in normal and preeclamptic pregnancy. Decreased plasma tMg concentration of the preeclampsia group in concert with high correlation of iMg++ and tMg with each other and inflammatory markers suggests a pathophysiological role of magnesium in the development of the inflammatory model of preeclampsia.

Introduction

Preeclampsia and eclampsia remain one of the leading causes of maternal and infant morbidity and mortality. According to The World Health Organization, hypertensive disorders of pregnancy are among the top three specific causes of maternal death all over the world representing 9 to 26% maternal death in developing countries and 16% in the developed world (Khan et al. 2006). According to Vital Statistics Mississippi 2001, the number of women suffering from eclampsia is 2,225 out of a total of 11,483, approximately 19.4% (Mississippi State Department of Health, 2001).

Magnesium deficiency is common due to a variety of reasons but often remains misdiagnosed and undetected (Liebscher et al. 2004). Preeclampsia is traditionally called

the "disease of theories" (Higgins and Brennecke, 1998). To date, the dominating theory of origin of preeclampsia is vascular endothelial cell activation and exaggeration of maternal inflammatory response (Von Dadelszen et al. 2000). Recent reports suggest that the clinical features of preeclampsia are the result of an excessive maternal systemic inflammatory response. Several investigators claim that normal pregnancy is also a state of maternal systemic inflammation, which is not different from that of preeclampsia except that it is milder. Any factor that would increase the maternal systemic inflammatory response to pregnancy would predispose the mother to preeclampsia. Consequently, preeclampsia could be described as an exaggerated maternal systemic inflammatory response brought about by pregnancy in concert with some other predisposing factors (Redman et al. 1999, Sacks et al. 1998, Schuiling et al. 1997, Faas and Schuiling, 2001, Dietl, 2000 and Redman and Sargent, 2000).

Pregnancy has been stated as a condition of "physiological hypomagnesaemia" (Kurzel, 1991, and Semczuk and Semczuk-Sikora, 2001) and a state of relative or mild insulin resistance and hyperinsulimia. Magnesium deficiency is associated with inflammatory/prooxidant condition (Vormann et al. 1992), abnormalities of lipid metabolism, hyperlipidemia, oxidation of lipoproteins, hypertension (Rayssiguier, 1986 and Cubizolles et al. 1993), vasospasm, ischemia, increased vascular reactivity, and lipid infiltration in the endothelium, and endothelial cell damage and dysfunction (Yukoyama et al. 1994). Low plasma concentrations of magnesium induced an increase in TNFα secretion and inflammation (Rodriguez-Moran and Guerrero-Romero, 2004). Magnesium deficiency is also accompanied with induction and activation of proinflammatory cells such as neutrophils, macrophages, and endothelial cells and

contributes to increased secretion of proinflammatory cytokines in particular TNFα (Malpuech-Brugere et al. 2000, Maier et al. 1998, Weglicki et al. 1992 and Weglicki and Phillips, 1992). In addition, insulin resistance and reduced antioxidant capacity is reported in the presence of magnesium deficiency (Sebekova et al. 2002 and Barbagallo et al. 1999). Furthermore, insulin stimulates magnesium uptake in platelets, lymphocytes, and erythrocytes (Nadler et al. 1993, Barbagallo et al. 1999, Sebekova et al. 2002, Sanders et al. 1999, Rosolova et al. 1997, Paolisso et al. 1990 and Hwang et al. 1993).

A direct effect of low extracellular magnesium concentration on endothelial cell culture resulted in endothelial cell dysfunction by generating a proinflammatory, prothrombotic, and proatherogenic environment (Maier et al. 2004). In addition, high extracellular concentration of magnesium maintained endothelial cell function. Endothelin-1 is expressed in the presence of activated endothelial cells. Concomitantly, elevated plasma leptin concentrations are associated with insulin resistance (Segal et al. 1996) and contribute to endothelial cell dysfunction by increasing FFA oxidation (Yamagishi et al. 2001). Inflammation is the causal pathway through which obesity predisposes to preeclampsia (Wolf et al. 2001). Additionally, increased serum concentration of FFAs and triacylglycerol amplifies insulin resistance and contributes to endothelial cell dysfunction and inflammation during preeclampsia (Kaaja, 1998, Moschos et al. 2002, Sattar et al. 2000, 1999 and 1996, Murai et al. 1997, Hubel et al. 1996 and Tripathy et al. 2003). Insulin resistance is also associated with elevated serum leptin and TNFα concentrations (Laivuori et al. 2000, Wolf et al. 2002, Martinez et al. 1996, Kirwan et al. 2002, Agata et al. 1997 and Fruhbeck et al. 2001).

Leptin hormone is expressed by adipose tissue and placenta (Masuzaki et al. 1997 and Mise et al. 1998) and blood leptin concentration is reported to increase during preeclampsia mostly in situations associated with release of cytokines and is an indication of proinflammatory activity for the most part with TNFα (Bartha et al. 2001, Anim-Nyame et al. 2000, McCarthy et al. 1999, Teppa et al. 2000 and Van Dielen et al. 2001). Adiponectin is an insulin-sensitizing hormone and its blood concentration is markedly decreased during preeclampsia indicating insulin resistance (Ramsay et al. 2003, Gale et al. 2004, Yamauchi et al. 2001, Bettowski, 2003 and Diez and Iglesias, 2003). Adiponectin is antiinflammatory and antiatherogenic, and decreased blood concentration of adiponectin leads to inflammation and endothelial cell dysfunction (Trujillo et al. 2005 and Shetty et al. 2004) further enhancing the inflammatory state of preeclampsia. Interleukin-10 inhibits the secretion of macrophage derived TNFα, thus suppressing inflammation and decreased blood concentration of IL-10 promotes inflammation. Interleukin-10 is secreted by a variety of immune system cells including monocytes, macrophages (Pinchuk, 2002), and trophoblast cells (Roth et al. 1996).

Examining the magnesium status of normal and preeclamptic pregnant women can help determine if magnesium deficiency is a risk factor in the inflammatory model of preeclampsia. The goal of this research was to determine if magnesium deficiency is an integral part of and plays a role in the pathophysiology of the inflammatory process during pregnancy.

Objectives

The primary objectives of the proposed study were: 1. To determine and compare serum ionized magnesium (iMg++) and plasma total magnesium (tMg) concentration and the association between inflammatory makers tumor necrosis alpha (TNF α), endothelin-1 (ET-1), leptin, and interleukin-10 (IL-10), adiponectin, free fatty acids (FFAs), triacylglycerol, serum iMg++ , and plasma tMg concentration of normal and preeclamptic pregnant women during the third trimester of pregnancy

Methods

Selection of Subjects and Research Design (Appendix G)

Briefly, the study was approved by Mississippi State University Institutional Review Board (IRB), IRB Docket #04-209, and Institutional Biological Safety Program (IBC), IBC Protocol #009-05. Pregnant and preeclamptic subjects were paired by age, parity and gestation.

The eligibility criteria for selection of subjects were: Age eighteen to forty years; no medication; no previous pathology; no intake of contraceptives; no intake of alcohol; no use of tobacco; and no medical history of diabetes or chronic hypertension. Preeclampsia was defined by a blood pressure elevation of $\geq 140/90$ mm Hg after 20 weeks gestation in association with proteinurea, either 1+ or more by dipstick or 300 mg/24 h of urine collection or more. Women with preeclampsia had no significant past medical history such as diabetes or chronic hypertension. Relevant information to the

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study was collected from the subject's medical chart and a questionnaire pertaining to the subject's medical history and constitutional characteristics after they had given their written informed consent.

Thirty five healthy pregnant women (third trimester) and thirty five preeclamptic women (third trimester), patients of Oktibbeha County Hospital Starkville, Mississippi volunteered to participate. Blood analyses were done by the use of ion-selective electrode, atomic absorption spectrometry, spectrophotometer and TNF α, IL-10, ET-1, adiponectin, and leptin ELISA kits. Ionized magnesium assay was performed by ion select electrode (AVL Scientific Cooperation 988-4, Roche, Indianapolis, Indiana USA), Department of Laboratory Medicine, National institute of Health (NIH), Bethesda, Maryland USA. Total magnesium assay was performed by atomic absorption spectrometry (Perkin Elmer Analyst 800, Norwalk, Connecticut, USA), according to manufacturer instructions, (Cox and Campbell 1991). Procedure for ionized and total magnesium assay is described in Appendix H. ELISA was read by use of ELISA Microplate Reader (Molecular Devices Thermomax, Sunnydale, California USA). Tumor necrosis factor alpha assay was performed by Quantikine Human ELISA kit (R&D Systems DTA00C, Minneapolis, Minnesota, USA), according to manufacturer instructions, (Cesari et al. 2003). The intra and inter-assay coefficient of variation was 4.6 and 5.8 %, respectively. The minimum detection limit of tumor necrosis factor alpha was 0.5 to 5.5 pg/ml; the mean detection limit was 1.6 pg/ml. The procedure for sample preparation, reagents, and analysis as explained by R&D systems manual is described in Appendix I. Interleukin-10 assay was performed by Quantikine Human ELISA kit (R&D Systems D1000B, Minneapolis, Minnesota, USA), according to manufacturer

instructions, (Freeman et al. 2004). The intra and inter-assay coefficient of variation was

3.6 and 6.9 %, respectively. The minimum detection limit of interleukin 10 was less than

3.9 pg/ml. The procedure for sample preparation, reagents, and analysis as explained by

R&D systems manual is described in Appendix J. Endothelin-1 assay was performed by

QuantiGlo Human ELISA kit (R&D Systems QET00B, Minneapolis, Minnesota, USA),

according to manufacturer instructions, (LaMarca et al. 2005). Endothelin-1 ELISA was

read at Life Sciences and Biotechnology Institute, Mississippi State University by use of

L-max microplate Luminometer, Molecular Devices, ID # 35333-27, Part number 0112-

0073, (Berthold Technologies, 75323 Bad Wildbad, Germany) according to manufacturer

instructions. The intra and inter-assay coefficient of variation was 3.1 and 6.7 %,

respectively. The procedure for sample preparation, reagents, and analysis as explained

by R&D systems manual is described in Appendix K. Adiponectin assay was performed

by Quantikine Human ELISA kit (R&D Systems DRP300, Minneapolis, Minnesota,

USA), according to manufacturer instructions, (Donghong et al. 2006). The intra and

inter-assay coefficient of variation was 3.5 and 6.5 %, respectively. The minimum

detection limit of adiponectin was 0.079 to 0.891 ng/ml; the mean detection limit was

0.246 ng/ml. The procedure for sample preparation, reagents, and analysis as explained

by R&D systems manual is described in Appendix L. Lepin assay was performed by

Quantikine ELISA kit (R&D Systems DLP00, Minneapolis, Minnesota, USA), according

to manufacturer instructions, (Rizos et al. 2002). The intra and inter-assay coefficient of

variation was 3.1 and 4.4 %, respectively. The minimum detection limit of leptin was 7.8

pg/ml; the mean detection limit was 0.064 pg/ml. The procedure for sample preparation,

reagents, and analysis as explained by R&D systems manual is described in Appendix M.

Free fatty acid assay was performed by NEFA-HR (2) kit (Wako Pure Chemicals, Richmond, Virginia USA) (Hubel et al. 1996) according to manufacturer instructions and samples read using a Perkin Elmer 552A UV/VIS Spectrophotometer (Norwalk, Connecticut USA). The within run precision was 0.68 % and total precision was 0.029 %. The minimum detection limit was 0.0014 mEq/L. The procedure for sample preparation, reagents, and analysis as explained by Wako Pure Chemicals manual is described in Appendix M.

Triacylglycerol assay was performed by Triacylglycerol (GPO) Pointe Scientific, Inc. kit (Canton, Michigan USA) (Bodnar et al. 2005) according to manufacturer instructions and samples read using Perkin Elmer 552A UV/VIS Spectrophotometer (Norwalk, Connecticut USA). The precision was 1.6 %. Sensitivity was calculated as absorbance, and 1 mg/dl of triacylglycerol gave absorbance of 0.001. The procedure for sample preparation, reagents, and analysis as explained by Pointe Scientific, Inc. manual are described in Appendix N.

Data were analyzed by analysis of variance (ANOVA), Tukey's test and Pearson Correlation analysis. Differences in continuous variables between groups were tested by ANOVA and Tukey's test and reported as mean ± SEM. Relationship in continuous variables between groups was tested by Pearson Correlation and reported as R and ± P values. Means were separated by Fisher's protected least significant difference method (at $\alpha = 0.05$). The Statistical Analysis Software (SAS) was used to analyze data (SAS, version 9.1.3, 2006).

Results

Preeclamptic pregnant women were matched with the normal pregnant women by week of gestation, body weight, and age. As expected, no significant difference was found between normal and preeclamptic pregnant women's gestational age, in addition to body weight and age (Table 4.1). There was no significant difference between the concentrations of serum iMg++ of normal pregnant and preeclamptic pregnant women. However, plasma tMg was significantly lower in the preeclamptic groups ($P \leq 0.001$) (Table 4.2). There was a significant difference between plasma TNFα (inflammatory cytokine) ($P \leq 0.001$) and plasma IL-10 (anti inflammatory cytokine) ($P \leq 0.001$) concentration between the two groups (Table 4.3).

In addition, there was a significant increase in serum ET-1 concentration in the preeclamptic group ($P \leq 0.001$). While plasma adiponectin was significantly lower in the preeclamptic group (normal range 6 to 10 μg/ml) ($P \leq 0.001$). Serum ET-1 and plasma leptin concentrations were significantly greater in preeclamptic women than normal pregnant women ($P \leq 0.001$) (leptin normal range 6.01 to 27.61 ng/ml) (Table 4.4). There was a significant increase in serum FFA and serum triacylglycerol concentration of the preeclamptic group ($P \leq 0.001$) (triacylglycerol normal range 44 to148 mg/dl) (Table 4.5).

Serum iMg++ and plasma tMg correlated positively with each other (R 0.68, $P \leq 0.001$). In addition, there was a negative correlation of iMg++ and tMg with plasma TNFα (R -0.45, $P \leq 0.001$); serum ET-1 (R -0.41, $P \leq 0.001$); plasma Leptin (R -0.33, $P \leq 0.001$); serum FFA (R -0.38, $P \leq 0.001$) and serum triacylglycerol (R -0.39, $P \leq 0.001$); all were increased in preeclampsia group. In addition, there was positive correlation of

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serum iMg++ and plasma tMg with plasma IL-10 (R 0.52, P ≤ 0.001) and plasma

adiponectin (R 0.54, P ≤ 0.001).   Interleukin-10 and adiponectin concentration was

decreased in the preeclampsia group (Table 4.6).

Discussion

The purpose of this study was to investigate the concentration of serum iMg++

and plasma tMg and their relationship to inflammatory and antiinflammatory mediators

in the third trimester of pregnancy between normal and preeclamptic pregnant women.

Preeclamptic pregnant women were matched with the normal pregnant women by week

of gestation in order to prevent the influence of gestational age on the study (Laires et al.

2004). The preeclamtic group had lower values of serum iMg++ and plasma tMg relative

to normal values. Although there was no significant difference between the

concentrations of iMg++ of normal pregnant and preeclamptic pregnant women, plasma

tMg was significantly lower in the preeclamptic group. The normal expected range of

iMg++ is 1.2 to 1.7 mg/dl (0.5 to 0.7 mmol/L). Serum iMg++ concentration in the

present study was 1.13 mg/dl (0.46 mmol/L) and tended to decrease and at the lower end

of the spectrum in comparison to normal values. The normal expected range of tMg is 1.8

to 2.4 mg/dl (0.74 to 1 mmol/L). Values of tMg in the range of 0.75 to 0.85 mmol/L are

considered to be deficient. The range of tMg is expected to be ≥ 0.85 mmol/L for health,

and concentrations ≤ 0.85 mmol/L are proposed to be deficient (Ford, 1999 and Elin,

2007). A strong correlation between plasma tMg and serum iMg++ concentration has

been reported in healthy and disease subjects (Saha et al. 1996 and Markell et al. 1993),

which is in agreement with the present study. Recent data indicates the occurrence of

major fluxes of magnesium across the cell membrane in either direction, resulting in major changes in total and plasma tMg concentrations. These major fluxes of magnesium result in limited variation of iMg++ concentration and larger variations in total and plasma tMg concentrations (Romani, 2007). Standley et al. (1997) investigated serum tMg and iMg++ concentration in normal and preeclamptic women during the first, second and third trimester of pregnancy. These workers observed a decrease in serum tMg and iMg++ over the course of gestation in normal pregnant women. Serum tMg concentration decreased early during pregnancy in women who developed preeclampsia and was significantly lower by the second trimester when compared with normal pregnant women. In another study, no difference was detected in serum iMg++ concentration between preeclamptic women and normal pregnant women, even though a significant difference was reported in serum iMg++ concentration between non-pregnant and normal pregnant women (Handwerker et al. 1995).

In the current study, there was an increase in proinflammatory mediators, and a decrease in antiinflammatory mediators, along with a decrease in plasma tMg and a strong correlation of serum iMg++ and plasma tMg with the inflammatory and antiinflammatory mediators. The results of this study agree with experimental results suggesting that hypomagnesemia promotes circulating proinflammatory environment (Mazur et al. 2007). Compelling evidence now demonstrates that magnesium deficiency modulates cellular events particularly involving inflammation with activation of endothelium, macrophages, and release of inflammatory cytokines (Mazur et al. 2007). Magnesium has antioxidant properties and magnesium deficiency of cells makes them more susceptible to oxidative damage (Rayssiguier et al. 1993). It is reported that low

serum concentrations of magnesium induce an increase in plasma concentration of TNFα and inflammatory response (Rodriguez-Moran and Guerrero-Romero, 2004). Tumor necrosis factor alpha is secreted in the early phase of inflammation and functions as a mediator of endothelial cell dysfunction during magnesium deficiency (Malpuech-Brugere et al. 1999). Consequently, magnesium deficiency is associated with the inflammatory response. Furthermore, cytokines may transfer from the fetal compartment to the maternal compartment and vice versa. No bidirectional transfer was noted for TNFα across the placenta (Aaltonen et al. 2005 and Zaretsky et al. 2004). In addition, no significant increase was seen in the TNFα/total protein concentration of the placenta of preeclamptic women even in the presence of significantly elevated maternal serum TNFα concentration. These results indicated sources other than placenta contributing to the increased concentration of TNFα in the blood circulation of preeclamptic women (Hayashi et al. 2005 and Benyo et al. 2001). Increased plasma concentration of TNFα has been observed in the first trimester of pregnancy of women who later developed preeclampsia (Hamai et al. 1997, Williams et al. 1999). Higher plasma TNFα concentration was also reported in the third trimester of preeclamptic subjects compared to normal pregnant subjects (Serin et al. 2002 and Gulati, 2005), which is in agreement with the results of the current study. Tumor necrosis factor alpha can induce other cytokines such as IL-6, and IL-6 in turn regulates the expression of C-reactive protein. These mediators alone or in combination can damage the endothelial cell (Schalkwijk and Stehouwer, 2005). A high inverse relationship of serum iMg++ and plasma tMg with TNFα was demonstrated in the current study and supports the hypothesis that there is a connection between hypomagnesaemia and the inflammatory response.

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Interleukin-10 is an important antiinflammatory cytokine in pregnancy and may contribute to the clinical condition of preeclampsia. Interleukin-10 inhibits the production of macrophage derived TNFα and suppresses inflammation (Pinchuk, 2002). Borekci et al. (2007) specified that serum IL-10 concentration was different between eclamptic and preeclamptic subjects being significantly higher in eclamptic than either preeclamptic or control subjects. The lowest serum concentration of IL-10 was reported in preeclamptic subjects, which is similar to the present study.

Endothelin-1 is a potent vasoconstrictor, and recent data indicates that magnesium decreases ET-1 concentration (Ariza et al. 2007). Endothelin-1 is secreted in response to vascular injury and indicates endothelial cell activation. Magnesium deficiency elevates and supplementation decreases plasma ET-1 concentration (Touyz, 2003). Endothelin-1, by mediating release of cytokines, activates inflammatory cells, which result in inflammation and endothelial cell damage that are characteristic of preeclampsia (Clark et al. 1998). Women with preeclampsia have higher plasma concentration of ET-1, which contributes to the vasoconstricted and inflammatory state of preeclampsia (Taylor et al. 1990 and August and Lindheimer, 1995). Additionally, several investigators have reported decreased serum concentration of ET-1 during preeclampsia after magnesium treatment (Sagsoz and Kucukozkan, 2003) and decreased ET-1 in placenta (Holcberg et al. 2004). Gammill and Roberts (2007) reported a significant increase of serum ET-1 concentration of preeclamptic women in comparison to normal pregnant women, which is in agreement with the results of the current study. In a study conducted on rats with initial infusion of ET-1 followed by magnesium, it was discovered that magnesium could reverse vasoconstriction in a number of vascular beds (Kemp et al. 1999). A direct effect

of low serum magnesium concentration on endothelial cell culture was demonstrated in promoting endothelial cell dysfunction by generating a proinflammatory, prothrombotic, and proatherogenic environment (Maier et al. 2004) and high serum concentration of magnesium maintained endothelial cell function. Magnesium may have a protective effect during inflammation, atherosclerosis, and in promoting growth of collateral vessels in chronic ischemia (angiogenesis) (Maier et al. 2004). In the current study, serum ET-1 concentration increased and a high negative correlation of serum ET-1 with serum iMg++ and plasma tMg was noted, which further gives support to the role of magnesium in the process of endothelial cell activation and the inflammatory response.

Reduced plasma concentration of adiponectin and increased plasma concentration of leptin was reported in pregnant preeclamptic women implying an altered ratio between leptin and adiponectin, and this ratio decreased with disease severity during preeclampsia (Ouyang et al. 2007 and Masuyama et al. 2007). The findings of the current study are similar to the above study. Adiponectin plasma concentration was significantly lower in the preeclamptic pregnant women (normal range 6 to 10 μg/ml). In addition, a significant positive correlation of serum adiponectin was demonstrated with serum iMg++ and plasma TMg. Adiponectin is considered to play an important role in mediating insulin sensitivity and a negative correlation is observed between adiponectin and leptin (Ouyang et al. 2007 and Masuyama et al. 2007).   In addition, adiponectin is an insulin sensitizing hormone (Tschritter et al. 2003), antiinflammatory (Mantzoros et al. 2005), and antiatherogenic with inhibition of expression of adhesion molecules (Ouchi et al. 1999, and Kappes et al. 2000). Adiponectin also reduces monocyte attachment to endothelial cells (Ouchi et al. 2001), suppresses TNFα secretion by monocytes and macrophages and

decreases the biological effects induced by TNFα (Yokota et al. 2000, and Ouchi et al. 2001), stimulates nitric oxide synthesis (Chen et al. 2003), and suppresses endothelial cell activation (Ouchi et al. 2000). In addition, adiponectin sensitizes tissue to insulin by reducing serum FFAs and this explains adiponectin low serum concentration in the presence of insulin resistance (Yamauchi et al. 2001 and Yamauchi et al. 2002).

Adponectin expression is regulated by TNFα and IL-6 (Chen et al. 2006). The occurrence of decreased plasma adiponectin concentration along with a positive correlation with serum iMg++ and plasma tMg in the current study suggests a role of magnesium in the inflammatory process and endothelial cell activation.

Leptin concentration during gestation is increased to a degree that cannot be explained by an increased body mass (Hardie et al. 1997). Leptin beside its main effects on the hypothalamus also acts as an inflammatory cytokine in the periphery (Ghilardi et al. 1996 and Bjorbaek et al. 1997). Leptin expression is stimulated by TNFα and IL-6, and a correlation of leptin with TNFα during pregnancy has been described (Nuamah et al. 2004 and Bartha et al. 2001). Elevated serum leptin concentration is associated with insulin resistance (Segal et al. 1996). Leptin also contributes to endothelial cell dysfunction by increasing FFA oxidation (Yamagishi et al. 2001), and leptin receptors have been demonstrated in the endothelial cells and athererosclerosis plaques indicating leptin's role in angiogenesis and atherosclerotic changes (Bouloumie et al. 1999 and Bouloumie et al. 1998). Leptin plasma concentration was found to be elevated during normal pregnancy and is increased more in women with preeclampsia. This increase is due to an increase in the free fraction of leptin and indicates that the increase of serum leptin concentration in preeclampsia is biologically relevant (Teppa et al. 2000). Plasma

leptin concentration increases in preeclampsia mostly in situations associated with release of cytokines and is an indication of proinflammatory activity for the most part with TNFα (Bartha et al. 2001, Anim-Nyame et al. 2000, McCarthy et al. 1999, Teppa et al. 2000, Van Dielen et al. 2001, Ramsay et al. 2003, Gale et al. 2004, Yamauchi et al. 2001, Bettowski, 2003 and Diez and Iglesias, 2003). A significant correlation was reported between TNFα and leptin in normal pregnant and pregnant women with preeclampsia in third trimester of pregnancy. In addition, higher plasma concentration of TNFα in pregnant women with preeclampsia was associated with higher plasma concentration of maternal leptin (Bartha et al. 2001).   Increased plasma concentration of leptin and significant inverse relationship with serum iMg++ and plasma tMg in the present study suggests that leptin may have been elevated in response to magnesium deficiency and the presence of proinflammatory environment induced by low blood concentration of magnesium.

In several animal studies, magnesium deficiency was reported to activate the inflammatory response or proinflammatory condition and hyperlipemia with increased serum concentration of triacylglycerol and serum FFAs, in addition to other lipids together with increased cell permeability to lipids (Maier, 2003). The inflammatory responses also stimulated increased secretion of cytokines. The association of magnesium deficiency with increased blood concentration of FFAs and triacylglycerol is well documented. Furthermore, recent research indicates that in the presence of magnesium deficiency, triacylglycerol and FFA concentrations are increased in humans (Randell et al. 2006).   In the present study, inverse correlation of serum iMg++ and plasma tMg with serum triacylglycerol and FFA was demonstrated and suggests a role of magnesium

deficiency in promoting inflammation and endothelial cell dysfunction. Preeclampsia is

associated with maternal hyperlipidemia in particular hypertriacylglyceroldemia (Sattar et

al. 1997 and Hubel et al. 1996). Increased insulin resistance noted in preeclampsia

increases the mobilization of FFAs and triacylglycerol and promotes increased

concentration in serum (Hubel, 1998). Accumulation of triacylglycerol in the endothelial

cells and increased concentration of FFAs can cause endothelial cell dysfunction (de Man

et al. 2000, Stewart and Monge, 1993, Gianturco et al. 1980 and Speidel et al. 1990).

Several animal experiments demonstrated the presence of spontaneous inflammation with

marked hyper- triacylglyceroldemia and cholesterol increase in the presence of low

serum magnesium (Maier et al. 1998, Haenni et al. 1998, Sherer et al. 1999, Sherer et al.

2000 and Ravn et al. 2001) and indicate a role of magnesium deficiency in the process of

inflammation and acute atherosis.   In addition, Morrill et al. (1997) reported fatty streaks

and lesions in the intima of the vascular smooth muscle similar to atherogenic lesions in

the presence of magnesium deficiency and demonstrated the role of magnesium

deficiency in the development of acute atherosis. Incomplete invasion of the maternal

blood vessels by trophoblast cells results in defective transformation of the spiral arteries

in the decidua and superficial myometrium. The decidual vessels along with chorion may

be affected by acute atherosis resulting in reduced perfusion of the placenta. Chen et al.

(2007) reported that activated endothelial cells are more resistant to displacement by

trophoblast cells than resting endothelial cells. It was suggested that endothelial cells

could contribute to shallow invasion of the spiral arteries by trophoblast cells during

preeclampsia. Activation of endothelial cells due to magnesium deficiency could play a

role in the defective transformation of spiral arteries and acute atherosis and contribute to maternal symptoms of preeclampsia.

Pregnancy is a state of relative insulin resistance with insulin concentration increasing throughout gestation with highest concentration in the third trimester which returns to normal after delivery (Yen, 1973 and Catalano et al. 1993). During preeclampsia, insulin resistance is exaggerated with the increase being more in the third trimester in comparison to normal pregnancy and predates the onset of clinical disease (Malek-Khosravi and Kaboudi, 2004). Insulin is an important regulator of cellular magnesium and low concentration of intracellular magnesium present during hyperinsulinemia is the effect of insulin on magnesium efflux via the Na^+/Mg^{2+} exchange activation (Ferreira et al. 2004). It takes around a year of magnesium supplementation to increase magnesium status. Insulin resistance prevents magnesium entry into the cell and is an additional problem for improving magnesium status. On the other hand, magnesium is an important regulator of insulin and glucose metabolism and a decrease in iMg++ can directly promote insulin resistance and altered vascular tone, magnesium being the cause of, rather than merely the result of, peripheral insulin resistance. Any alteration of magnesium concentration has a profound effect on insulin action (Barbagallo et al. 1999). Magnesium deficiency along with an increase in TNFα blood concentration can induce insulin resistance and hypertriacylglyceroldemia by suppressing key enzymes such as lipoprotein lipase, acetyl CoA carboxylase, and fatty acid synthethase, and promote marked stimulation of glycogenolysis and increased blood concentration of FFAs. Free fatty acids have been shown to activate endothelial cells (Maier, 2003, Carr et al. 2001, Heiskanen et al. 2002, Anim-Nyame et al. 2003, Serin et al. 2002, Rinehart et al. 1999,

Williams et al. 1999, Conrad and Benyo 1997, Kaaja et al. 1999 and Sigal and Ron, 1994). In the current study, the presence of decreased plasma concentration of adiponectin, which is an insulin sensitizing hormone, and increased blood concentration of other inflammatory markers, suggests that in the presence of magnesium deficiency, insulin resistance can be induced and is an important component in the development of inflammation during preeclampsia, and magnesium plays a significant role in the process.

Conclusion

In preeclamptic pregnant women, plasma TNFα, serum ET-1, plasma leptin, serum FFA, and serum triacylglycerol concentrations increased while plasma tMg, plasma IL-10, and plasma adiponectin concentrations decreased. There was a negative correlation between serum iMg++ and plasma tMg concentration with plasma TNFα, serum ET-1, plasma leptin, serum FFA, and serum triacylglycerol. In addition, a positive correlation was noted between serum iMg++ and plasma tMg and of serum iMg++ and plasma tMg with plasma IL-10, and plasma adiponectin.

A high correlation of serum iMg++ and plasma tMg with inflammatory and antiinflammatory markers and increased plasma concentration of TNFα, serum ET-1, plasma leptin, serum FFA, and serum triacylglycerol and decreased concentration of plasma tMg, plasma IL-10, and plasma adiponectin suggests a pathophysiological role of magnesium deficiency in the development of preeclampsia that needs to be evaluated by further research. There are several abnormalities that are common between magnesium deficiency and preeclampsia such as hypertension, dyslipidemia, endothelial cell dysfunction, insulin resistance, and systemic inflammation. Magnesium deficiency

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could be suggested as a missing link or recurring factor between preeclampsia and inflammation, insulin resistance, hyperlipedimia, endothelial cell dysfunction, and hypertension and needs to be evaluated by further research.

There are certain limitations to the study. Calcium concentrations were not determined, pH of the blood samples at the time of collection was not determined, the subjects were not followed and records kept for constitutional characteristics and biochemical changes in inflammatory and antiinflammatory markers before pregnancy, during pregnancy and post pregnancy. In addition, placenta analysis was not done.

Table 4.1   Descriptive and Clinical Characteristics of Normal and Preeclamptic Pregnant Women-age, body weight before pregnancy, current body weight, and weeks of gestation*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance
Age (year)	25 ± 0.90	26 ± 0.80	N.S.¶
Body Weight Before Pregnancy (lbs)	147 ± 60	160 ± 7.0	N.S.¶
Current Body Weight[a] (lbs)	177 ± 60	193± 7.0	N.S.¶
Weeks of Gestation (wks)	38 ± 0.30	37 ±0.30	N.S.¶

[a]Bodyweight recorded at consent to participate in the study *Values are means $\pm$SEM
Significant differences (P$\leq$ 0.05) ¶No significance at P $\geq$0.05

Table 4.2   Magnesium Concentration of Normal and Preeclamptic Pregnant Women*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance	Classic Range[+]	Proposed Range[^]
Ionized Magnesium (iMg) (mg/dl)	1.15 ± 0.02	1.13 ± 0.07	N.S.¶	1.2 to 1.7	
Total Magnesium (tMg) (mg/dl)	1.85 ± 0.90	1.50 ± 0.01	P ≤ 0.001	1.8 to 2.4	≥ 2.07

*Values are means$\pm$ SEM   Significant differences (P$\leq$ 0.05) ¶No significance at P $\geq$0.05
[+]Values from Stipanuk (2000) [^]Ford (1999) and Elin (2007)

Table 4.3   Cytokine Expression of Normal and Preeclamptic Pregnant Women*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance
Tumor Necrosis Factor Alpha (TNFα) (pg/ml)	7.6 ± 0.2	9.5 ± 0.3	P ≤ 0.001
Interleukin 10 IL-10 (pg/ml)	9.5 ± 0.6	4.4 ± 0.4	P ≤ 0.001

*Values are means± SEM   Significant differences (P≤ 0.05) ¶No significance at P ≥0.05

Table 4.4   Endothelium Derived Peptide and Adipokine Expression of Normal and Preeclamptic Pregnant Women[*]

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance	Expected Range
Endothelin-1 ET-1(pg/ml)	0.1± 0.7	2.0 ± 0.1	P ≤ 0.001	
Adiponectin (ug/ml)	10.8 ± 0.9	4.8 ± 0.8	P ≤ 0.001	6 to 10[+]
Leptin (ng/ml)	30.1 ± 3.5	47.8 ± 4.2	P ≤ 0.001	6.01 to 27.61[^]

*Values are means ±SEM Significant differences (P≤ 0.05) ¶No significance at P ≥0.05
Values from [+]Koerner et al. (2005)   [^]Tamas et al. (1998)

Table 4.5   Lipid Profile of Normal and Preeclamptic Pregnant Women*

Variables	Normal Pregnant (N = 35)	Preeclamptic Pregnant (N = 35)	Significance	Expected Range
Non-Esterified Fatty Acid (NEFA) (mEq/L)	0.5 ± 0.03	0.7 ± 0.04	$P \leq 0.001$	
Triacylglycerol (mg/dl)	141.43 ± 3.9	260.60 ± 13.4	$P \leq 0.001$	44 to 148[+]

*Values are means $\pm$SEM Significant differences (P$\leq$ 0.05) ¶No significance at P $\geq$0.05
[+]Values from Rifkind et al. (1983)

Table 4.6   Pearson Correlation ($^{\circ}$R) between Variables and iMg and tMg of
             Preeclamptic Pregnant Women

Variables	Ionized Magnesium		Total Magnesium	
	$^{\circ}$R	Significance	$^{\circ}$R	Significance
Ionized Magnesium (iMg) (mg/dl)			0.68	$P \leq 0.001$
Total Magnesium (tMg) (mg/dl)	0.68	$P \leq 0.001$		
Tumor Necrosis Factor (TNFά) (pg/ml)	-0.45	$P \leq 0.001$	-0.56	$P \leq 0.001$
Interleukin 10 (IL 10) (pg/ml)	0.52	$P \leq 0.001$	0.52	$P \leq 0.001$
Endothelin-1 (ET-1) (pg/ml)	-0.41	$P \leq 0.010$	-0.34	$P \leq 0.040$
Adiponectin (ug/ml)	0.54	$P \leq 0.001$	0.59	$P \leq 0.001$
Leptin (ng/ml)	-0.33	$P \leq 0.050$	-0.53	$P \leq 0.001$
Non-Esterified Fatty Acids (NEFA) (mEq/L)	-0.38	$P \leq 0.020$	-0.53	$P \leq 0.001$
Triglyceride (mg/dl)	-0.39	$P \leq 0.020$	-0.39	$P \leq 0.020$

*Significant differences (P$\leq$ 0.05) ¶No significance at P $\geq$0.05 $^{\circ}$R Pearson correlation
N = 35

CHAPTER V

CONCLUSION

The present study is the first human study to evaluate the role of magnesium and its relationship with inflammatory response in normal and preeclamptic pregnancy. The study investigated and identified the potential role and contribution of magnesium in the inflammatory model of preeclampsia in order to further understand the role magnesium plays with preeclampsia during the last trimester of pregnancy and how pregnancy proceeds to preeclampsia and the related role of magnesium. The study was conducted in Starkville, Mississippi and included 70 pregnant women, 35 normal pregnant controls and 35 preeclamptic pregnant women from original pool of 3000 pregnant women.

Maternal gestational age, body weight, age, prepregnancy diastolic blood pressure, intake of birth control pills, supplements, miscarriage, still births, race, and employment status were found to be statistically unchanged between the two groups. While current systolic and diastolic blood pressure, number of children and pregnancies, family history of preeclampsia, urine albumin, and minimum and maximum temperature and precipitation at the time of presentation were significantly different between the two groups.

In the preeclamptic group of pregnant women, plasma concentration of TNFα ($P \leq 0.001$), serum ET-1 ($P \leq 0.001$), plasma leptin ($P \leq 0.001$) , serum FFA ($P \leq 0.001$), and serum triacylglycerol ($P \leq 0.001$) were increased, while plasma tMg ($P \leq 0.001$),

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plasma IL-10 (P ≤ 0.001) and plasma adiponectin (P ≤ 0.001) concentrations were decreased and were found to be statistically significant between normal pregnant and preeclamptic pregnant women. In addition, a significant and high positive correlation (R 0.68, P ≤ 0.001) was noted between serum iMg++ and plasma tMg concentration and of serum iMg++ and plasma tMg with plasma IL-10 (R 0.52, P ≤ 0.001) and plasma adiponectin (R 0.54, P ≤ 0.001). A significant and high inverse correlation was demonstrated between iMg++ and plasma tMg with plasma TNFα (R -0.45, P ≤ 0.001), serum ET-1 (R -0.41, P ≤ 0.001), plasma leptin (R -0.33, P ≤ 0.001), serum FFA (R -0.38, P ≤ 0.001), and serum triacylglycerol (R -0.39, P ≤ 0.001) between normal pregnant and preeclamptic pregnant women.

Temperature and precipitation, nulliparity, second pregnancy, hypertension, proteinuria, and a family history of preecalmpsia appeared to be risk factors for preeclampsia. However multiparity, age, race, weight, intake of birth control pills, supplements, miscarriage, still births, and employment status had no contribution.

Decreased plasma tMg concentration and a high positive correlation (R 0.68, P ≤ 0.001) between serum iMg++ and plasma tMg, in concert with increased plasma concentration of TNFα, serum ET-1, plasma leptin, serum FFA, serum triacylglycerol; and decreased plasma concentration of IL-10 and plasma adiponectin, together with high correlation of serum iMg++ and plasma tMg with the inflammatory and antinflammatory mediators in preeclamptic pregnant women suggest a role of magnesium in the development of the inflammatory model of preeclampsia.

Pregnancy is a state of hypomagnesaemia, systemic inflammation, and relative insulin resistance, which are further aggravated during preeclampsia. Magnesium

deficiency is also associated with inflammatory prooxidant condition, hypertension, insulin resistance, hyperlipidemia, and endothelial cell dysfunction. The current study identified an association of plasma tMg deficiency and serum iMg++ with inflammatory mediators (increased plasma concentration of TNFα, serum ET-1, plasma leptin, serum FFA, serum triacylglycerol) and antiinflammatory mediators (decreased plasma concentration of IL-10, adiponectin) and preeclampsia. The data supports the hypothesis that magnesium may have a role in the pathphysiology of preeclampsia. In the current study, magnesium deficiency was associated with an increase in proinflammatory mediators and a decrease in anti-inflammatory mediators creating a proinflammatory environment consequently leading to inflammation. Magnesium is an important regulator of insulin and magnesium deficiency can directly promote insulin resistance, which is reflected in the decreased plasma concentration of adiponectin an insulin sensitizing hormone. An increase of serum triacylglycerol and serum FFA suggests an altered lipid state with an increase in serum ET-1 resulting in endothelial cell activation and dysfunction, altered vascular tone, and hypertension. Increased plasma concentration of TNFα, plasma leptin, serum ET-1, serum FFA and serum triacylglycerol and corresponding decrease in plasma IL-10 and adiponectin promote an inflammatory environment. There are several abnormalities that are common between magnesium deficiency and preeclampsia such as hypertension, dyslipidemia, endothelial cell dysfunction, insulin resistance, and systemic inflammation. Magnesium deficiency could be described as the missing link or recurring factor between preeclampsia and inflammation, insulin resistance, hyperlipedimia, endothelial cell dysfunction, and hypertension and needs evaluation by further research.

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APPENDIX A

LETTER OF APPROVAL

MISSISSIPPI STATE UNIVERSITY (MSU) INSTITUTIONAL REVIEW BOARD

(IRB)

Mississippi State
UNIVERSITY

August 17, 2005

Fauzia Khan
Nutrition
Mailstop 9745

Re: IRB Docket #04-209: An evaluation of magnesium status and inflammatory response during
   the third trimester of normal pregnancy and preeclampsia

Dear Ms. Khan:

Our office has received the item listed as a contingency on your IRB approval. Our records show
that you have completed the requirements for approval. You may proceed with your project.

Thank you for your cooperation and good luck to you in conducting this research project. If you
have questions or concerns, please contact me at 325-5220 or at jmiller@research.msstate.edu.

Sincerely yours,

Jonathan E. Miller
IRB Coordinator

Cc: Brian Rude

Office for Regulatory Compliance
P. O. Box 6223 • 8A Morgan Street • Mailstop 9563 • Mississippi State, MS 39762 • (662) 325-3294 • FAX (662) 325-8776

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APPENDIX B

LETTER OF APPROVAL

MISSISSIPPI STATE UNIVERSITY (MSU) INSTITUTIONAL BIOSAFETY

COMMITTEE

(IBC)

Mississippi State University

August 3, 2005

To: Brian Rude, PI
From: F. R. Champlin, BSO
Regarding: IBC Application No. 009-05

Project Title: An Evaluation if Magnesium Status and Inflammatory Respond During the
Third Trimester of Normal Pregnancy and Preclampsia

Project Period: June 15, 2005 thru December 31, 2006

Funding Source: Internal

I am writing to inform you that your application to register the above-referenced infectious disease study has been reviewed and approved by the Institutional Biosafety Committee which determined it to come under Section III-C of the *NIH Guidelines*. Please find attached a photocopy of the completed application, the original which will remain on file in the Regulatory Compliance Office in a manner compliant with IBC policies.

Please note that I have attached the summary of the IBC review and am in agreement with it. I would add that all practices, equipment, and the facility itself should be conducted or configured in a manner consistent with BSL-2 containment principals as described in the CDC-NIH *Biosafety for Microbiological and Biomedical Laboratories*.

I wish you the best with your research. Please feel free to contact me should you require assistance with regard to any aspect of biosafety, including regulatory compliance.

ATTACHMENT

Cc: Gary Jackson, Unit Head
Terry Kiser, Unit Head
Todd Pharr, IBC Chair
Tracy Arwood, ORC Director

Office of Regulatory Compliance
P. O. Box 6223 • 8A Morgan Street • Mailstop 9563 • Mississippi State, MS 39762 • (662) 325-3294 • FAX (662) 325-8776

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APPENDIX C

LETTER FROM THE DIRECTOR OF NURSING SERVICES OKITEBBEHA

COUNTY HOSPITAL (OCH) STARKVILLE, MS INDICATING

PERMISSION TO CONDUCT STUDY

Oktibbeha County Hospital

P. O. Drawer 1506 • Starkville, Mississippi 39760-1506 • Telephone (662) 323-4320

November 11, 2004

Institutional Review Board
Office of Regulatory Compliance
P. O. Box 6223
Mississippi State, Mississippi 39762

To Whom It May Concern:

It is our pleasure to be a part of this worthwhile study and project. This research project has been discussed with my immediate supervisor, the CEO (Chief Executive Officer) of the hospital. We have reviewed the criteria and these are acceptable.

The person who will be Dr. Fauzia Khan's contact is Mrs. Karen Tiffin, our Maternal-Child Nursing Supervisor. She has met with Dr. Khan several times and will be available to assist the nursing staff in completing the project.

We are aware that the findings outside the criteria may confound the results of the research. However, we, remain committed in our participation because of the possibility of improving the care for future mothers and babies.

If I can be of further assistance with the matter, please feel free to call me.

Sincerely,

Martha Fulcher, RNC

Martha Fulcher, RNC, BSN, IBCLC
Director of Nursing Services

APPENDIX D

ANNOUNCEMENT OF RESEARCH: BRIEF DESCRIPTION OF RESEARCH

INITIALLY PRESENTED TO POTENTIAL VOLUNTEERS

Date: August 15, 2005

To: Patients of Oktibbeha County Hospital
Subject: **Volunteers for research**
From: Fauzia A. Khan MD

I am a doctoral candidate in Nutrition at Mississippi State University and conducting a research project to understand the causes of preeclampsia. Preeclampsia is a condition during pregnancy characterized by high blood pressure, presence of protein in the urine and edema. We need volunteers who are in the third trimester of pregnancy, do not use tobacco and alcohol, and have no chronic or acute infection or disease (hypertension, diabetes, etc).

The volunteers need to:
  (1) Donate 15 ml (2 teaspoon) of her blood (a blood draw is part of routine care, this 15 ml of blood sample will be collected at that time. Therefore, no additional blood draw will be required).
  (2) Answer some questions such as age, number of pregnancies, and use of nutrient supplementation.
  (3) Have her blood pressure taken.
  (4) Give permission to see medical chart
  (5) Allow placental sample to be collected (only in case that you are presently delivering your baby).

Your identity will be kept confidential; your name will never appear on any sample.

This research may not have immediate benefit for you but it offers hope and benefit for future mothers and infants.

Your participation is greatly appreciated.

Sincerely,

Fauzia A. Khan
School of Human Sciences
Mississippi State University
Tel: (662) 325- 5173

APPENDIX E

CONSENT FORM

Informed Consent
(Fauzia A. Khan, MD, Doctoral Candidate in Nutrition)

Title of Study: An evaluation of magnesium status and inflammatory response during the third trimester of normal pregnancy and preeclampsia
(A study of the response of your magnesium blood levels and how this effects your last 12 weeks of pregnancy)

Study Site: School of Human Sciences/ Mississippi State University

Name of Researcher(s) & University affiliation: Fauzia A. Khan MD, Doctoral Candidate in Nutrition Mississippi State University

What is the purpose of this research project?
The purpose of the study is to examine the blood level of magnesium in normal and pregnant women who have high blood pressure in their last 12 weeks of pregnancy (that is between 28 to 40 weeks of pregnancy). Preeclampsia is a condition during pregnancy in which there is high blood pressure, presence of protein in the urine and edema (retention of water and swelling of hands and feet. This study will help us to understand the problems some women encounter during pregnancy.

How will the research be conducted?
●In a routine check up in 3rd trimester (28 to 40 weeks of pregnancy), you will be asked questions about your
1. Date of birth
2. Race
3. Weight (how much do you weigh)
4. Current blood pressure (your present blood pressure)
5. Number of alive births, that is babies that were born alive (Parity)
6. Number of pregnancies, that is how many times were you pregnant (Gravida)
7. Loss of pregnancy or baby (Miscarriage)
8. Babies that were not alive at the time of birth (Still births)
9. Number of children
10. Do you take any vitamin or mineral before or during your pregnancy (Vitamin and mineral supplements?)
11. Did you smoke or drink alcohol before or during your pregnancy (Smoking and alcohol use)
12. Do you take any medicine regularly?
13. Take birth control pills to keep from getting pregnant
14. Occupation (your job or work)
15. Are you working yes or no?
16. City/County (the name of the city and county where you live)

●After the interview, less than 1 tablespoon of your blood will be drawn by

a member of the hospital staff. Collection of your blood will not require any additional prick. Blood will be collected at the same time your other lab work is being done.
●After you deliver your baby and placenta, a small piece of your placenta will be collected.
●In addition pre pregnancy weight (your weight before you got pregnant), current weight (your weight now), pre pregnancy blood pressure (your blood pressure before you got pregnant) and current blood pressure (your blood pressure now) and pregnancy/medical history will be obtained from your medical chart. All information obtained from your chart will be completely private and the medical chart will be consulted for only one time
●All of your answers will remain private
●Your name will never appear on the samples and data.
●Your taking part in this study is completely voluntary and you can quit at any time. You may refuse to answer any question on the questionnaire or asked verbally-for any reason
●Your taking part or not taking part in the study will not be noted in your medical chart.
●The study requires about 20 minutes of your time.

Are there any risks or discomforts to me because of my participation? There is some discomfort when blood is drawn and there is a risk of bruise i.e. redness. Again, no additional blood draw will be required; the blood sample used for this study will be collected at the same time your other lab work is being done. Qualified hospital staff will draw your blood and you will have no additional risk over those of normal medical care during pregnancy.

Other than filling out the questionnaire you will not need to do anything specific apart from what is required during a routine checkup for pregnancy. Your OB doctor at the time of delivery will deliver the baby and placenta as is required at completion of pregnancy and beginning of contractions and is a part of normal medical care during delivery.

Taking part in this study causes no additional stress or harm over and beyond normal care to either you or your baby.

Does participation in this research provide any benefits to others or me?
This research may not have any immediate benefit for you but it offers hope and benefit for future mothers and infants

Will this information be kept confidential?
Your name will never appear on any sample or data. All samples and data will be numbered with a confidential code number. The key to the code number will be kept in a locked cabinet in an office at MSU. At no time will samples and data be linked with your name. All of the information given and collected from the medical chart will remain confidential and your participation is completely voluntary.
*Also please note that these records will be held by a state body and therefore are subject to disclosure if required by law

Whom do I contact with research questions?
If you should have any questions about this research project, please feel free to contact Fauzia A. Khan at (662)325-5173 or Dr. Brain J Rude at (662)325-2933. For additional information regarding your rights as a research subject, please feel free to contact the MSU Regulatory Compliance Office at 662-325-3294.

What do I do if I am injured at a result of this research?
In addition to reporting an injury to Fauzia Khan (662)325-5173 or Dr. Brain J Rude at (662)325-2933 and to the Regulatory Compliance Office (662)325-3294), you may be able to obtain limited compensation from the State of Mississippi if the injury was caused by the negligent act of a state employee where the damage is a result of an act for which payment may be made under §11-46-1, et seq. Mississippi Code Annotated 1972. To obtain a claim form, contact the University Police Department at *MSU UNIVERSITY POLICE DEPARTMENT, Stone Building, Mississippi State, MS 39762, and (662) 325-2121.*

What if I do not want to participate?
Please understand that your taking part in the study **is your choice and you may quit at any time**, there will be **no penalty or loss** of benefits.

You will be given a copy of this form for your records.

The approximate number of subjects in the study: There will be approximately 60 subjects in this study

_____   _____

Patient Signature   Date

_____   _____

Investigator Signature   Date

_____   _____

Witness (OCH Nurse)   Date

APPENDIX F

QUESTIONNAIRE (SELF-ADMINISTERED)

An Evaluation of Magnesium Status and Inflammatory Response during the Third
Trimester of Normal Pregnancy and Preeclampsia
Fauzia A. Khan

(A study of the response of your magnesium blood levels and how this effects your last 12 weeks
of pregnancy)

Demographic and pregnancy/medical history

Date:
Subject Code:

1.What is your age?:……....

2. What is your date of birth? ……………..

3. What is your current weight? ………..   Your weight before you got pregnant?……….

4. How far along (weeks) are you in your pregnancy?………

5. How many times have you been pregnant?:…………

6. How many children do you have?:………..

7. Have you ever lost a baby before completion of pregnancy?………..if so how many?……..

8. Have you ever had any babies that were not alive at the time of birth? …..if so how many?…

9. Did you take any vitamin or mineral before your pregnancy? Yes………..No…………

10. Did you take any vitamin or mineral during your pregnancy? Yes………..No…………

11. Have you ever taken birth control pills to keep from getting pregnant? Yes……No……

12. Do you take any medication regularly? Yes…...No……if so what?……………..

13.What is your race?………..

14.What is your Occupation (your job or work)?……………

15. Are you still working? Yes………..No………..

16. What is the name of the city and county where you live?…………

17. What is your current blood pressure?:…………

18. What was your blood pressure before you got pregnant?……….

19. Has anyone in your family had preeclampsia? Yes…………. No……….

APPENDIX G

SELECTION OF SUBJECTS AND RESEARCH DESIGN

SELECTION OF SUBJECTS AND RESEARCH DESIGN

Normal pregnant women (third trimester) and preeclamptic pregnant women
(third trimester) were matched according to the eligibility criteria:

Thirty five healthy pregnant women (third trimester) and thirty five preeclamptic women,

patients of Oktibbeha County Hospital (OCH), Starkville Mississippi (MS) volunteered

to participate. The study was approved by Mississippi State University (MSU)

Institutional Review Board (IRB), IRB Docket #04-209 and Institutional Biological

Safety Program (IBC), IBC Protocol #009-05. Selected information was collected from

the subject's medical chart after they had given their written informed consent.

Pregnant and preeclamptic subjects were paired by age, parity and gestation.

Number of subjects to have 80 % CI was determined with the assistance of a statistician.

Assuming a significance level of $P \leq 0.05$ and a power of 0.8, the number of subjects in

each group was determined by the statistician to be a minimum of 18 normal and 18

preeclamptic pregnant women, by the use of "statistical power analysis". Based on prior

studies done in our laboratory (Borazjani & Dodson, 2000, Khan et al. 2000) a total

number of 35 normal and 35 preeclamptic pregnant women were tested out of total pool

of 3000 pregnant women.

CRITERIA FOR SELECTION OF SUBJECTS

(Pregnant and preeclamptic subjects were paired by age, parity and length of gestation).

Control	Experimental
Normal pregnant woman in 3rd trimester Oktibbeha County Hospital	Preeclamptic pregnant woman in 3rd trimester Oktibbeha County Hospital
35 Normal pregnant subjects. Selected according to criteria for selection.	35 Preeclamptic subjects selected according to criteria for selection.
Age 18-40 years	Age 18-40 years
●Women with a history of diabetes; thyroid, liver, or chronic renal disease, HIV, HBV, HCV, HDV, HEV, STD or any other disease or infection or pathology or preexisting chronic hypertension (defined as blood pressure greater than or equal to 140/90 or need for antihypertensive medications before pregnancy or before 20 wk gestation) or any acute or chronic infection or disease were excluded. ●No subject testing positive on these tests were selected. ●Blood samples were only collected from women who had been screened for HIV, HBV, HCV, HDV, HEV, and STD. ●Non-smoker, not taking any medication including contraceptives or alcohol.	●Preeclampsia was defined by a blood pressure elevation of $\geq$140/90 mm Hg after 20 weeks gestation in association with proteinurea, either 1+ or more by dipstick or 300 mg/24 h urine collection or more in the absence of urinary tract infection Women with a history of diabetes; thyroid, liver, or chronic renal disease, HIV, HBV, HBC, STD or any other disease or infection or pathology or preexisting chronic hypertension (defined as blood pressure greater than or equal to 140/90 or need for antihypertensive medications before pregnancy or before 20 wk gestation) or any acute or chronic infection or disease were excluded. ●No subject testing positive on these tests were selected ●15 ml blood samples was collected from women who had been screened for HIV, HBV, HCV, HDV, HEV, and STD. ●Non-smoker, not taking any medication including contraceptives or alcohol

COLLECTION OF DATA

The subject did not need to do anything specific apart from what was required during a hospital checkup (once and for approximately 20 minutes). Blood samples were collected at the same time when the subject's laboratory work was done. The participants were free to withdraw from the study at any time with no repercussion. The participants were also verbally informed and in writing that no penalty or loss of benefits would occur as a result of either not participating or withdrawing from the study at any time.

In addition to the written informed consent (Appendix E) the subjects were asked verbally if they had any questions about the study by the researcher. The OCH nursing staff was available to help confirm subject understanding of what was required of them. The following procedures were applied:

Brief description of study (appendix D)

Consent form (appendix E)

Questionnaire (self-administered) (appendix F)

Obtain permission to collect pertinent data from medical record (medical chart) and immediately examine medical history: any past or present history of any infectious disease such as TB, Hepatitis A, B, C, D, and E, HIV, STD, or any other chronic or acute disease. Demographic: age, date of birth, weight, race, occupation, currently employed place of residence, city/county. Pregnancy history: number of pregnancies, number of miscarriages, still birth, family H/O preeclampsia, weeks of pregnancy, number of children, current blood pressure, intake of vitamins/minerals, and contraceptives.

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Routine care: Blood pressure, weight, urine analysis was carried out by a hospital staff. In addition to routine blood draw, the laboratory technologist of OCH drew approximately a total of 15 ml of subject's whole blood into three vacutainer tubes (two for serum and one for plasma, containing sodium citrate or heparin or no additive). The following was observed in the transport of blood samples from OCH to MSU. The specimens were kept on ice. The specimen container was watertight, leak proof, tightly caped and sealed and was placed in a rack to maintain it in an upright position. The specimen container and rack was placed in a solid, leak proof plastic or metal transport box with secure tight fitting cover. The transport box was secured in the transport vehicle. Each transport box had the biohazard label. Specimen identification and data form accompanied each transport box. A spill kit containing absorbent material, a chlorine disinfectant, and a leak proof waste disposal container and heavy duty gloves were kept in the transport vehicle. The serum and plasma were separated by centrifugation at 1500 x g for 15 minutes. After separation plasma and serum were stored in the laboratory initially in a -20 degree Celsius freezer for a month and than in -80 degrees Celsius till the time of analysis.

APPENDIX H

ESTIMATION OF SERUM IONIZED AND PLASMA TOTAL MAGNESIUM

DETERMINATION OF TOTAL AND IONIZED MAGNESIUM

Total magnesium was determined at Biochemical and Toxicology Analytical Laboratory, College of Veterinary Medicine, Mississippi State University

PROCEDURE

Total magnesium assay was performed by flame atomic absorption spectrometry (Perkin Elmer Analyst 800, Norwalk, Connecticut, USA), according to manufacturer instructions, (Cox and Campbell 1991). Absorbance was read at 285.2 nm. The instrument was programmed to project an average of three readings taken 0.3 seconds apart. Concentration of magnesium in the sample was determined by linear regression analysis based on an average of several standard curves. For the determination of total magnesium the plasma sample was diluted 1:50 with 0.1 % (w/v) lanthanum chloride diluents. A 0.1 % (w/v) lanthanum chloride solution was used as a blank. Standards (Fisher Scientific) were prepared by diluting the stock solution with a 0.1 % (w/v) lanthanum chloride solution. Standards used were 0.01, 0.05, 0.10, 0.25, 0.50 ppm.

Ionized magnesium assay was performed by ion select electrode (AVL Scientific Cooperation 988-4 Roche, Indianapolis, Indiana USA), Department of Laboratory Medicine, National Institute of Health (NIH), Bethesda, Maryland USA. Care was taken to collect samples in a non-silicone tube to avoid interference as it can lead to falsely elevated results. Binding of magnesium to proteins and ligands in the plasma and blood is pH dependent. The average pH of the samples was 7.4.

APPENDIX I

TUMOR NECROSIS FACTOR ALPHA ASSAY

DETERMINATION OF TUMOR NECROSIS FACTOR ALPHA

ELISA was read by use of ELISA Microplate Reader (Molecular Devices Thermomax, Sunnydale, California USA). TNFα assay was performed by Quantikine Human ELISA kit (R&D Systems DTA00C, Minneapolis, Minnesota, USA), according to manufacturer instructions (Cesari et al. 2003). The intra and inter-assay coefficient of variation was 4.6 and 5.8 %, respectively. The minimum detection limit of TNFα was 0.5 to 5.5 pg/ml; the mean detection limit was 1.6 pg/ml. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNFα has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNFα present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNFα is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color developed in proportion to the amount of TNF α bound in the initial step. The color development is stopped and the intensity of the color was measured.

REAGENTS

Bring all reagents to room temperature before use.

Wash Buffer. 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives was used. In the case of crystals formation in the concentrate, The wash buffer was warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution. Color Reagents A, 12.5 mL/vial of stabilized hydrogen peroxide and Color Reagents B, 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine) should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200μL of the resultant mixture is required per well.

TNFά Conjugate. 21 mL/vial of polyclonal antibody against TNFά conjugated to horseradish peroxidase, with preservatives.

TNF ά Standard. 10 ng/vial of recombinant human TNFά in a

buffered protein base with preservatives, lyophilized.

Reconstitute the TNFά Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 500 μL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series. 100 μL of the standard into tube one. Then 500 μL fron each successive tube into the next. Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. The calibrator diluent serves as the zero standard (0 pg/mL).

Assay Diluent RD1F. 6 mL/vial of a buffered protein base with preservatives. Contains a precipitate. Mix well before and during use.

Stop Solution. 6 mL/vial of 2 N sulfuric acid.

PROCEDURE

Dilution series: 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, and 15.6 pg/ml.

Bring all reagents and samples to room temperature before use.

1. Add 50 μL of Assay Diluent RD1F to each well. Assay Diluent RD1F will have a precipitate present. Mix well before and during use.

2. Add 200 μL of Standard, sample, or control per well. Cover with the adhesive strip and Incubate for 2 hours at room temperature.

3. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 200 μL of TNFά conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

5. Repeat the aspiration/wash as in step 3.

6. Add 200 μL of substrate solution to each well. Incubate for 20 minutes at room temperature. Protect from light.

7. Add 50 μL of stop solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

8. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

To calculate results, concentration of standards were plotted against their corresponding optical density and concentration in each sample was read directly from the standard curve by using optical density values and the best fit line was determined by regression analysis.

APPENDIX J

INTERLEUKIN-10 ASSAY

DETERMINATION OF INTERLEUKIN-10 (IL-10)

ELISA was read by use of ELISA Microplate Reader (Molecular Devices Thermomax, Sunnydale, California USA). Interleukin-10 assay was performed by Quantikine Human ELISA kit (R&D Systems D1000B, Minneapolis, Minnesota, USA), (Freeman et al. 2004) according to manufacturer instructions. The intra and inter-assay coefficient of variation was 3.6 and 6.9 %, respectively. The minimum detection limit of IL-10 is less than 3.9 pg/ml. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-10 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-10 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for IL-10 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-10 bound in the initial step. The color development is stopped and the intensity of the color is measured.

REAGENTS

Bring all reagents to room temperature before use.

Wash Buffer. 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives. If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD6P. 21 mL/vial of animal serum with preservatives.

Substrate Solution. Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well. Color Reagent A. 12.5 mL/vial of stabilized hydrogen peroxide. Color Reagent B. 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).

IL-10 Conjugate. 21 mL/vial of mouse monoclonal antibody against IL-10 conjugated to horseradish peroxidase with preservatives.

IL-10 Standard. 5.0 ng/vial of recombinant human IL-10 in a buffered protein base with preservatives, lyophilized. Reconstitute the IL-10 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 5000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Use polypropylene tubes. Pipette 900 μLof calibrator diluent RD6P into the 500 pg/mL tube. Pipette 500 μL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series. 100 μL of the standard into tube one. Then 500 μL from each successive tube into the next. Mix each tube thoroughly before the next transfer. The 500 pg/mL standard serves as the high standard. The appropriate calibrator diluent serves as the zero standard (0 pg/mL).

Stop Solution. 6 mL/vial of 2 N sulfuric acid

PROCEDURE

Dilution series: 5000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml,15.6 pg/ml and 7.8 pg/ml.

Bring all reagents and samples to room temperature before use.

1. Add 50 μL of Assay Diluent RD1W to each well.

2. Add 200 μL of Standard, control, or sample per well. Cover with the adhesive strip. Incubate for 2 hours at room temperature.

3. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 200 μL of IL-10 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

5. Repeat the aspiration/wash as in step 3.

6. Add 200 μL of Substrate Solution to each well. Protect from light.
 Incubate for 30 minutes at room temperature.

7. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

18. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate. To calculate results, concentration of standards were plotted against their corresponding optical density and concentration in each sample was read directly from the standard

curve by using optical density values and the best fit line was determined by regression

analysis.

APPENDIX K

ENDOTHELIN-1 ASSAY

DETERMINATION OF ENDOTHELIN-1

Endothelin-1 assay was read at LSBI (Life Sciences and Biotechnology Institute) Mississippi State University, by use of L-max microplate Luminometer, (Molecular Devices, ID # 35333-27, Part number 0112-0073, Berthold Technologies, 75323 Bad Wildbad, Germany) according to manufacturer instructions. Endothelin-1 (ET-1) assay was performed by QuantiGlo Human ELISA kit (R&D Systems QET00B, Minneapolis, Minnesota, USA), according to manufacturer instructions, (LaMarca et al. 2005). The intra and inter-assay coefficient of variation was 3.1 and 6.7 %, respectively. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for ET-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ET-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for ET-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of ET-1 bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted.

REAGENTS

Bring all reagents to room temperature before use.

Wash Buffer. 100 mL of a 10-fold concentrated solution of

buffered surfactant with preservatives. If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 100 mL of wash buffer concentrate into deionized or distilled water to prepare 1000 mL of wash buffer.

Working Glo Reagent. Glo Reagent A. 4 mL of stabilized enhanced luminol. Glo

Reagetn B 8 mL of stabilized hydrogen peroxide. 1 part Glo Reagent A (4 mL) and 2

parts Glo Reagent B (8 mL) should be mixed together 15 minutes to 4 hours before use.

Store in a capped plastic container protected from light. 100 μL of the resultant mixture is

required per well.

Calibrator Diluent RD5-13. 21 mL of buffered protein base with preservatives.

Calibrator Diluent RD5-13.   21 mL of buffered protein base with preservatives.

Assay Diluent RD1-19. 11 mL of a buffered protein base with preservatives.

ET-1 Conjugate. 21 mL of a mouse monoclonal antibody against ET-1

conjugated to horseradish peroxidase, with preservatives.

Standard. 2.5 ng of synthetic human ET-1 in a buffered protein base with

preservatives, lyophilized. Reconstitute Standard with 1.0 mL of deionized or distilled

water. This reconstitution produces a stock solution of 2500 pg/mL. Allow the standard

to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette

900 μL of calibrator diluent RD5-13 into the 250 pg/mL tube. Pipette 600 μL of

calibrator diluent RD5-13 into the remaining tubes. Use the stock solution to produce a

dilution series. 100 μL of the standard into tube one. Then 500 μL from each successive

tube into the next. Mix each tube thoroughly and change pipette tips between each

transfer. The 250 pg/mL standard serves as the high standard. calibrator diluent RD5-13

serves as the zero standard (0 pg/mL).

PROCEDURE

Dilution series: 2500 pg/ml, 250 pg/ml, 83.3 pg/ml, 27.8 pg/ml, 9.26 pg/ml, 3.09

pg/ml,1.03 pg/ml and 0.34 pg/ml.

Bring all reagents and samples to room temperature before use.

1. Add 100 μL of assay diluent RD1-19 to each well.

2. Add 100 μL of standard, control, or sample per well. Cover with the adhesive strip. Incubate for 1.5 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

3. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 200 μL of ET-1 conjugate to each well. Cover with a new adhesive strip. Incubate for 3 hours at room temperature on the shaker.

Note: Prepare Working Glo Reagent at this time.

5. Repeat the aspiration/wash as in step 3.

6. Add 100 μL of Working Glo Reagent to each well. Incubate for 5 - 20 minutes at room temperature on the bench top. Protect from light.

7. Determine the RLU of each well using a luminometer set with the following parameters: 1.0 min. lag time; 0.5 sec/well read time; summation mode; auto gain on. To calculate results, concentration of standards were plotted against their corresponding optical density and concentration in each sample was read directly from the standard curve by using optical density values and the best fit line was determined by regression analysis.

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APPENDIX L

ADIPONECTIN ASSAY

DETERMINATION OF ADIPONECTIN

ELISA was read by use of ELISA Microplate Reader (Molecular Devices Thermomax, Sunnydale, California USA). Adiponectin assay was performed by Quantikine Human ELISA kit (R&D Systems DRP300, Minneapolis, Minnesota, USA), according to manufacturer instructions, (Donghong et al. 2006). The intra and inter-assay coefficient of variation was 3.5 and 6.5 %, respectively. The minimum detection limit of adiponectin was 0.079 –0.891 ng/ml, the mean detection limit was 0.246 ng/ml. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for the Adiponectin globular domain has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Adiponectin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for the Adiponectin globular domain is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Adiponectin bound in the initial step. The color development is stopped and the intensity of the color is measured.

REAGENTS

Bring all reagents to room temperature before use.

Wash buffer. 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives. If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of wash buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate solution. Color Reagent A. 12.5 mL/vial of stabilized hydrogen peroxide.

Color reagent B. 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).

Color reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

Adiponectin standard. 500 ng/vial of recombinant human Adiponectin in a buffered protein base with preservatives, lyophilized. Reconstitute the Adiponectin standard with 2.0 mL of calibrator diluent RD6-39. This reconstitution produces a stock solution of 250 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of15 minutes with gentle agitation prior to making dilutions. Pipette 200 μL of calibrator diluent RD6-39 into each tube. Use the stock solution to produce a dilution series. 200 μL of the standard into tube one. Then 200 μL from each successive tube into the next. Mix each tube thoroughly before the next transfer. The 250 ng/mL standard serves as the high standard. The appropriate calibrator diluent serves as the zero standard (0 ng/mL).

Adiponectin conjugate. 21 mL/vial of mouse monoclonal antibody against the adiponectin globular domain conjugated to horseradish peroxidase with preservatives.

Calibrator diluent RD6-39. 21 mL/vial of a buffered protein base with preservatives.

Assay diluent RD1W. 11 mL/vial of a buffered protein base with preservatives.

Stop solution. 6 mL/vial of 2 N sulfuric acid.

PROCEDURE

Dilution series: 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.2 ng/ml, 15.6 ng/ml, 7.8 ng/ml, 3.9 ng/ml.

Bring all reagents and samples to room temperature before use.

1. Add 100 μL of assay diluent RD1W to each well.

2. Add 50 μL of standard, control, or sample per well. Cover with the adhesive strip. Incubate for 2 hours at room temperature.

3. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with wash buffer (400 μL) using a squirt bottle. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 200 μL of Adiponectin conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

5. Repeat the aspiration/wash as in step 3.

6. Add 200 μL of substrate solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

7. Add 50 μL of stop solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

8. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

To calculate results, concentration of standards were plotted against their corresponding optical density and concentration in each sample was read directly from the standard curve by using optical density values and the best fit line was determined by regression analysis.

APPENDIX M

LEPTIN ASSAY

DETERMINATION OF LEPTIN

ELISA was read by use of ELISA Microplate Reader (Molecular Devices Thermomax, Sunnydale, California USA). Leptin assay was performed by Quantikine ELISA kit (R&D Systems DLP00, Minneapolis, Minnesota, USA), according to manufacturer instructions, (Rizos et al. 2002). The intra and inter-assay coefficient of variation was 3.1 and 4.4 %, respectively. The minimum detection limit of leptin was 7.8 pg/ml, the mean detection limit was 0.064 pg/ml. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Leptin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Leptin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Leptin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Leptin bound in the initial step. The color development is stopped and the intensity of the color is measured.

REAGENTS

Bring all reagents to room temperature before use.

Wash buffer. 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of wash buffer concentrate into deionized or distilled water to prepare 500 mL of wash buffer.

Substrate solution. Color Reagent A. 12.5 mL/vial of stabilized hydrogen peroxide.

Color Reagent B. 12.5 mL/vial of stabilized chromogen

(tetramethylbenzidine). Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

Calibrator diluent RD5P (1X). 21 mL/vial of a concentrated buffered protein base with preservatives. .Dilute 20 mL of Calibrator Diluent RD5P Concentrate into deionized or distilled water to yield 100 mL of Calibrator Diluent RD5P (1X).

Assay diluent RD1-19. 11 mL/vial of a buffered protein base with preservatives.

Leptin standard. 10 ng/vial of recombinant human Leptin in a

buffered protein base with preservative, lyophilized. Reconstitute the Leptin standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a

minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 μL of calibrator diluent RD5P (1X) into the1000 pg/mL tube. Pipette 500 μL of calibrator diluent RD5P (1X) into each of the remaining tubes. Use the stock solution to produce a dilution series. 100 μL of the standard into tube one. Then 500 μL from each successive tube into the next. Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator diluent RD5P (1X) serves as the zero standard (0 pg/mL).

Leptin conjugate. 21 mL/vial of mouse monoclonal antibody against leptin conjugated to horseradish peroxidase, with preservatives.

Assay diluent RD1-19. 11 mL/vial of a buffered protein base with preservatives.

Color reagent A. 12.5 mL/vial of stabilized hydrogen peroxide.

Color reagent B. 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine) .

Stop solution. 6 mL/vial of 2 N sulfuric acid

PROCEDURE

Dilution series

10000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5pg/ml, 31.2 pg/ml and

15.6 pg/ml.

Bring all reagents and samples to room temperature before use.

1. Add 100 μL of assay diluent RD1-19 to each well.

2. Add 100 μL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.

3. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with wash buffer (400 μL) using a squirt bottle. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 200 μL of leptin conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.

5. Repeat the aspiration/wash as in step 3.

6. Add 200 μL of substrate solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

7. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

8. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate. To calculate results, concentration of standards were plotted against their corresponding optical density and concentration in each sample was read directly from the standard curve by using optical density values and the best fit line was determined by regression analysis.

APPENDIX N

FREE FATTY ACID ASSAY

DETERMINATION OF FREE FATTY ACIDS

Free fatty acid assay was performed by NEFA-HR (2) kit (Wako Pure Chemicals, Richmond, Virginia USA), (Hubel C A et al. 1996), according to manufacturer instructions, and samples read using a Perkin Elmer 552A UV/VIS Spectrophotometer (Norwalk, Connecticut USA). The within run precision was 0.68 % and total precision was 0.029 %. The minimum level was 0.0014 mEq/L. The assay enzymatic method relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (POD), permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple colored product which can be measured colorimetrically at 550 nm.

REAGENTS

Color Reagent

When reconstituted

0.53 U/mL Acyl-coenzyme A synthetase (ACS) (Pseudomonas sp.)

0.31 mmol/L Coenzyme A (CoA, Candida)

4.3 mmol/L Adenosine triphosphate (ATP) (Bacterium sp.)

1.5 mmol/L 4-aminoanitipyrine

2.6 U/mL Ascorbate oxidase (pumpkin)

0.062% Sodium azide (as Color Reagent A Solution)

Store at 2-10°C.

Solvent A

50 mmol/L phosphate buffer, pH 7.0

0.05% Sodium azide

Store at 2-10°C

Color Reagent B

When reconstituted

12 U/mL Acyl-coenzyme A oxidase (ACOD) (Arthrobacter sp.)

14 U/mL Peroxidase (POD) (horseradish)

Store at 2-10°C

Color reagent A solution. Add one bottle of solvent A to one vial of color reagent A. Mix gently by inverting the vial until the contents are completely dissolved. Reconstituted solution is stable for ten days at 2-10 degree Celsius

Color reagent B solution. Add one bottle of solvent B to one vial of color reagent B. Mix gently by inverting the vial until the contents are completely dissolved. Reconstituted solution is stable for three week at 2-10 degree Celsius

1. Prepare working color reagent solutions A and B.

2. Accurately pipette 25 μL of sample, each level of calibrator and blank (saline or DI water) into each tube. Add 1.0 mL of color reagent solution A.

3. Mix well and incubate at 37°C for 5 minutes.

4. Measure the absorbance of each tube at 550 nm (Sub: 660 nm). This reading serves as a 'sample blank.'

5. Add 0.5 mL of color reagent solution B.

6. Mix well and incubate at 37°C for 5 minutes.

7. Measure the absorbance of each tube at 550 nm (Sub: 660 nm).

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8. To get the final absorbance value for each sample, subtract the absorbance of the first reading (step 4) from that obtained in the second reading (step 7). 9. Plot the final absorbance vs. concentration of the calibration curve. A linear calculation model should be used.

10. To calculate sample concentration by calculation use the following formula:

Sample Con. = $\underline{\text{Sample Absorbance}}$ X Standard Conc.
              Standard Absorbance

(Use the final absorbance obtained in step 9 for the calculations).

APPENDIX O

TRIACYLGLYCEROL ASSAY

DETERMINATION OF TRIACYLGLYCEROL

Triacylglycerol assay was performed by Triacyglycerol (GPO) Pointe Scientific, Inc. kit (Canton, Michigan USA), (Bodnar et al. 2005), according to manufacturer instructions and samples read using Perkin Elmer 552A UV/VIS Spectrophotometer (Norwalk, Connecticut USA). Precision was 1.6 %. Sensitivity was calculated as absorbance and 1mg/dl of triglyceride gave absorbance of 0.001. The triacylglycerol (GPO) method is based on the enzymatic determination of glycerol using the enzyme glycerol phosphate oxidase (GPO) after hydrolysis by lipoprotein lipase. Serum triacylglycerol are hydrolyzed to glycerol and free fatty acids by lipase. In the presence of ATP and glycerol kinase (GK), the glycerol is converted to glycerol-1-phosphate. The glycerol-1-phosphate is then oxidized by glycerol phosphate oxidase (GPO) to yield hydrogen peroxide. The condensation of hydrogen peroxide with 4-cholorphenol and 4-aminophenazone (4-AA) in the presence of peroxidase (POD) produces a red colored quinonimine dye which absorbs at, or near 500nm. The intensity of the colored complex formed is directly proportional to the triglycerides concentration of the sample.

REAGENTS

Good's buffer (pH 7.4 ± 0.1) 50mM, 4-Chlorophenol 3.5mM, ATP 0.5mM, Magnesium salt 12 mM, 4-Aminophenazone 0.3mM, Glycerol Kinase (microbial) >250 U/L, Glycerol Phosphate Oxidase (microbial) >4500 U/L, Peroxidase (horseradish) > 2000 U/L, Lipase (microbial) >200,000 U/L, Surfactants, Stabilizers, and Preservatives, including sodium azide (0.1%).

PROCEDURE

1. Label test tubes: "Blank", "Calibrator/Standard", "Patient", "Control", etc.

2. Pipette 1.0 ml of reagent into the appropriate tubes and pre-warm to 37°C. 3. Add

0.010ml (10ul) of the appropriate sample to their respective tubes. Swirl gently to mix.

 4. Incubate all tubes for five (5) minutes.

5. After incubation, zero the spectrophotometer with "Blank" tube, at 500nm. (500-520

nm is acceptable). Read and record the absorbance (Abs.) of all the tubes. The final

color is stable for at least 60 minutes.

Limitations

The procedure is linear to 1000 mg/dl (11.3 mmol/L)

Calculations

Triglycerides results are expressed as mg/dl or mmol/L

Triglycerides = $\dfrac{\text{Absorbance Unknown}}{\text{Absorbance Standard}}$ x Conc. Std

To convert results into SI units (mmol/L), multiple the result (mg/dl) by 0.0113.

APPENDIX P

DATA ANALYSIS

DATA ANALYSIS

PART-I

Data were analyzed by analysis of variance (ANOVA), Tukey's test, and Chi-Square. Differences in continuous variables between groups were tested by ANOVA and Tukey's test and reported as mean ± SEM. Chi square test was used for categorical data and reported as percentages. Means were separated by Fisher's protected least significant difference method (at $\acute{\alpha} = 0.05$). The Statistical Analysis Software (SAS) was used to analyze data (SAS, version 9.1.3, 2006).

PART-II

Data were analyzed by analysis of variance (ANOVA), Tukey's test, and Pearson Correlation analysis. Differences in continuous variables between groups were tested by ANOVA and Tukey's test and reported as mean ± SEM. Relationship in continuous variables between groups was tested by Pearson Correlation and reported as R and ± P value. Means were separated by Fisher's protected least significant difference method (at $\acute{\alpha} = 0.05$). The Statistical Analysis Software (SAS) was used to analyze data (SAS, version 9.1.3, 2006).