UNIVERSITY OF ALBERTA

EFFECT OF NITRIC OXIDE SYNTHASE INHIBITION ON BLOOD VOLUME REGULATION DURING PREGNANCY IN THE RAT

BY

YUNLONG ZHANG

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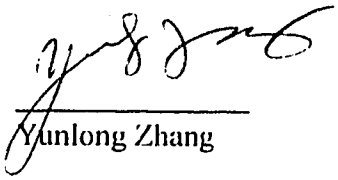

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Effect of Nitric Oxide Synthase Inhibition on Blood Volume Regulation during Pregnancy in the Rat submitted by Yunlong Zhang in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

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Date : 13 Aug '96

DEDICATED TO:

MY PARENTS

for their leading me to medical research

MY WIFE

for her **LOVE** to her family

MY SON

for making everything worthwhile

ABSTRACT

Pregnancy is associated with profound changes in the function and morphology of the cardiovascular, renal, reproductive, and endocrine systems. Among these changes, one of the most remarkable is the 50% increase in plasma volume. The mechanism by which blood volume increases in pregnancy is still unclear. Preeclampsia is a clinical syndrome characterized by impaired plasma volume expansion and an abnormal hormonal profile. Recent studies have shown that NO synthesis increases in pregnancy, and that inhibition induces changes characteristic of preeclampsia. We have investigated the effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME) on blood volume, and on plasma ANF, ET and PRA. We observed that NOS inhibition reduced blood volume at both 21-days of pregnancy (8.8 ± 0.6 ml/100g control; 6.0 ± 0.5 ml/100g L-NAME) and 7-days postpartum (7.2 ± 0.5 ml/100g control; 5.5 ± 0.4 ml/100g L-NAME). It also reduced blood volume in virgin rats, but to a much smaller extent than during pregnancy (6.6 ± 0.2 ml/100g control; 5.9 ± 0.1 ml/100g L-NAME). NOS inhibition had no effect on plasma ANF levels or PRA in either virgin or 21-day pregnant rats. However, it did decrease plasma ET levels in 21-day pregnant rats (19.6 ± 1.6 pg/100µl control; 11.6 ± 2.5 pg/100µl). Our results show that NOS inhibition mimics preeclampsia with respect to changes in blood volume, but not hormones. This affirms that the primary lesion in preeclampsia is probably not a deficit in NO biosynthesis.

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ABBREVIATIONS

ANF	Atrial Natriuretic Factor
Ang II	Angiotensin II
cGMP	Guanosine 3', 5'-cyclic Monophosphate
CO	Cardiac Output
D-NAME	D-ω-L-Arginine Methyl Ester
ECF	Extracellular Fluid
ECFV	Extracellular Fluid Volume
ERPF	Effective Renal Plasma Flow
ET	Endothelin
FF	Filtration Fraction
GC-S	Soluble Guanyly cyclase
GFR	Glomerular Filtration Rate
HLA	Human Leucocyte Antigen
IFGR	Intrauterine Fetal Growth Retardation
L-NAME	N-ω-L-Arginine Methyl Ester
MAP	Mean Arterial Pressure
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PGI_2	Prostacyclin
PRA	Plasma Renin Activity
PRC	Plasma Renin Concentration
RAA	Renin-Angiotensin-Aldosterone

CHAPTER ONE

INTRODUCTION TO THE THESIS

AND

A BRIEF REVIEW

Pregnancy is associated with profound changes in the function and morphology of the cardiovascular, renal, reproductive, and endocrine systems. All of these changes enable the pregnant woman to accommodate the growing products of conception. These changes have been extensively described but never completely understood. Preeclampsia, one of the main causes of maternal and fetal death during pregnancy, is characterized by decompensation in all systems of these normal physiological changes. Therefore, an understanding of the physiological processes in normal pregnancy is necessary in order to provide a basis for comparison with the pathophysiological processes of preeclampsia.

Physiological changes in normal pregnancy and volume homeostasis

During pregnancy, the cardiovascular system is mobilized. Cardiac output (CO), which increases by approximately 40% to 50%, reaches a peak in the mid-trimester and remains stable thereafter until term (Clark et al. 1989). Plasma volume is increased by 50% over that seen in non-pregnant woman (Brown and Gallery 1987) and plasma volume expansion is accompanied by a 24% increase in red blood cell volume. (Chesley 1972, Thomsen et al. 1993). Because plasma volume expansion exceeds the increase in red blood cell volume, the hematocrit falls. This results in the so-called physiological anemia of pregnancy (Friedman et al. 1991). This increased volume is of both physiological and clinical significance. Research has already shown a significant correlation between the plasma volume increase and fetal growth (Pirani et al. 1973). Despite increases in plasma volume and cardiac output, blood pressure in pregnancy is actually reduced. This fall in blood pressure occurs in early pregnancy, reaches a nadir in mid-trimester, and then

gradually returns toward pre-pregnant levels at term (Christianson 1976). The blood pressure decrease in pregnancy is mainly attributable to a significant reduction in peripheral vascular resistance (Clark et al. 1989), due to an increased production of vasodilatory substances (Gant et al. 1987, Lewis et al. 1983, Paller 1987, and Weiner et al. 1989). Currently, this is believed to be most likely due to an increase in prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), and nitric oxide (NO) synthesis and release. The blood pressure decrease occurs in the face of elevated levels of plasma angiotensin II (Ang II); there is much evidence that pregnant women are resistant to the pressor effects of many vasoconstrictive agents including Ang II (Gant et al. 1973).

In order to provide an adequate blood supply to the growing gestational products, the uterine vasculature undergoes marked alteration. The spiral arteries supplying the placenta dilate, due to remodeling of their walls, reaching 30 times their pre-pregnant diameter (Bieniarz et al. 1969). This dilatation is achieved by two waves of trophoblast invasion of the spiral arteries. The first wave of trophoblast invasion occurs in the 10th to 16th week of pregnancy. The muscular walls and endothelium of the decidual portions of the spiral arteries are replaced by trophoblasts. This is followed by a second wave of trophoblast invasion in the 16th to 22nd week of pregnancy which intrudes as far as the inner third portion of the myometrium (Pijnenborg et al. 1983, and Robertson et al. 1975). The consequence of trophoblast invasion is dilatation of the spiral arteries of the decidual and inner third myometrial portions resulting in a loss of contractility and increased placental perfusion. Thus, the resulting hemodynamic situation in normal

pregnancy is one of high volume, high-flow, low pressure, and low resistance in both the general circulation and the uteroplacental circulation.

The increase in blood volume is one of the most dramatic hemodynamic changes seen during normal pregnancy. Volume homeostasis is defined as the 'continuous adjustment of blood volume to the changing size of the vascular bed so that at all times an adequate fullness of the blood stream is available to the left ventricle' (Gauer and Henry 1976). In physiological conditions, blood volume is tightly controlled. Pregnancy dramatically modifies the mechanics of this control system. During pregnancy both vascular capacitance and blood volume increase to meet the growing needs of mother and fetus. The mechanisms behind these dramatic and complex alterations in volume homeostasis in pregnancy are still unclear. Extracellular fluid volume (ECFV) is mainly determined by sodium content. Therefore regulation of renal sodium excretion would appear to play a significant role in volume homeostasis. This may involve a change in the balance of natriuretic and antinatriuretic hormones (Brown and Gallery 1994), and/or a resetting of the high and/or low pressure baroreceptors (Leduc et al. 1991) and/or osmoreceptors (Davison et al. 1988, Barron et al. 1988), and/or changes in vascular reactivity.

Pregnancy-induced changes in renal function are equally remarkable. Effective renal plasma flow (ERPF) increases by 75% (Dunlop 1981), and glomerular filtration rate (GFR) increases by 50% (Davison and Hytten 1974, Dunlop 1981) above non-pregnant levels. ERPF rises more than GFR early in pregnancy but, unlike GFR, ERPF falls

slightly in the last few weeks of pregnancy (Davison and Dunlop 1984). This means that the filtration fraction (FF, FF = GFR/ERPF) is lower in mid-pregnancy and increased in the last trimester. Changes in FF affect peritubular colloidal osmotic pressure and therefore are positively correlated to proximal tubular sodium reabsorption (Seely and Levy 1981). It is expected that FF changes in pregnancy would favor sodium loss in mid-pregnancy and sodium retention in late pregnancy.

The mechanisms of the increased GFR are complicated and these changes precede the increase in blood volume (Shrier and Briner 1991). Several candidates, such as prostaglandins (Dunn and Scharschmidt 1987), Ang II (Edwards 1983, Yuan et al. 1990), NO (Ito et al. 1993), and endothelin (ET) (Clavell and Burnett 1994) may be involved in local mediation of hemodynamics by influencing afferent and/or efferent arterioles of the glomeruli. Recent studies have shown that the sensitivity of afferent and efferent arterioles are different (Edwards 1983, Yuan et al. 1990). Efferent arterioles are more sensitive to Ang II than afferent ones. Since Ang II concentration increases in pregnancy, the resultant efferent arteriolar constriction causes an increased hydrostatic pressure in glomeruli, which, in turn, increases GFR. Although this large (50%) increase in GFR might be expected to reduce ECFV, there is nevertheless net sodium retention, the mechanism of which is unclear. However, one surprising finding is that during pregnancy, the proximal tubule increases in length (Atherton and Pirie 1981, Garland et al. 1978). Thus, it is possible that an increase in reabsorptive surface area could be the mechanism which offsets the natriuretic effect of increased filtered load.

In addition to increased levels of reproductive hormones, pregnancy is also associated with changes in the secretion and regulation of several cardio-active or volume-related hormones, such as atrial natriuretic factor (ANF) and hormones in the renin-angiotensin-aldosterone (RAA) system. Both reproductive and classical cardio/renal hormones may be involved in volume homeostasis. These hormones can be simply divided into those with a natriuretic and those with an anti-natriuretic effect on the kidney.

ANF is a natriuretic hormone which is synthesized, stored in, and secreted from atrial myocytes. It is released mainly in response to atrial stretch. The physiological targets of ANF are (1) the kidney (induction of natriuresis and diuresis), (2) the cardiovascular system (lowering of blood pressure, increase in capillary permeability), and (3) the RAA system (antagonism). A controversy surrounds the levels of circulating ANF in human pregnancy (Hirai et al. 1988, Fournier et al. 1991), most likely because of one or more of a combination of the following factors: cross-section study design, heterogeneity of the study population, methodological factors such as lack of standardization of blood sampling, and blood sample processing. Duvekot and Peeters (1994), after critically analyzing a large body of data, suggested that there may be little change in plasma ANF levels during human pregnancy. Thus, in spite of the pregnancy-induced increase in blood volume, plasma ANF does not seem to respond to this change. The mechanism(s) of this phenomenon is not well understood. A possible explanation may be that volume receptors are reset at a higher threshold. Previous studies have shown that, unlike the response of virgin animals, there is no change in either ANF release (Zhang, Novak and Kaufman

1995), urine output increase (Kaufman and Deng 1993), or central nervous system activation (Deng and Kaufman 1995) in response to stimulation of the atrial volume receptors in pregnant rats. Another potential mechanism which could explain the lack of ANF secretion by volume changes may involve direct changes in the bio-synthesis and/or release of ANF in atrial myocytes during pregnancy. This is evidenced by the lack of stretch-induced ANF release from isolated atria derived from pregnant rats (Kaufman et al. 1994). One other mechanism which may be involved is down-regulation of ANF receptors in pregnancy rather than decreased release of ANF (Potvin and Varma 1991), as revealed by the reduced natriuretic activity of ANF during late pregnancy (Masilamani et al. 1994).

Plasma progesterone increases to levels more than 20-fold higher than the luteal average. This hormone can cause natriuresis by at least two mechanisms: namely antagonism of the action of mineralocorticoids (Landau and Lugibihl 1958), for which there is direct tissue receptor evidence (Sharp et al. 1966), and increased proximal tubular sodium loss (Oparil et al. 1975). Although it has been claimed that there is a causal relationship between the increase in progesterone and aldosterone levels during pregnancy, stimulation and inhibition of aldosterone release by short-term changes in salt intake in pregnant women does not result in parallel changes in plasma progesterone levels (Brown et al. 1986). Thus progesterone is not involved in the day-to-day regulation of sodium balance. Nor is it intimately related to aldosterone production. However, it may exert a background effect on sodium excretion in pregnancy. Ironically, although this

"natriuretic" hormone may have a background effect on sodium excretion, it may also have an anti-natriuretic effect. Studies have shown that at high plasma concentrations, progesterone can be converted to deoxycorticosterone by extra-adrenal 21-hydroxylation, possibly in the maternal kidney (Winkel et al. 1980a and 1980b). Deoxycorticosterone is an important mineralocorticoid involved in sodium reabsorption and is found at elevated levels during pregnancy (Brown et al. 1972, Ehrlich et al. 1974).

The RAA system is markedly activated during pregnancy. Plasma levels of renin, renin substrate, Ang II, and aldosterone all increase early in pregnancy and remain elevated until term (Gordon et al. 1973, Weir et al. 1975). In spite of the higher basal activity of the RAA system during pregnancy, its response to external stimuli remains unaffected (Brown et al. 1987). Apparently, pregnancy induces a resetting of this system. The RAA system, in terms of volume homeostasis, is anti-natriuretic. Ang II has direct effects on renal function by increasing tubular sodium reabsorption and by influencing renal hemodynamics, and an indirect effect via stimulation of aldosterone secretion. Aldosterone, exerting at least 95% of the mineralocorticoid activity of adrenocorticoid secretion, promotes exchange of sodium and potassium through the renal tubular walls (Guyton 1986). Sodium/potassium exchange occurs in the epithelial cells of distal tubular, collecting tubular and collecting duct. Therefore, aldosterone causes sodium to be conserved in the extracellular fluid while potassium is excreted into the urine. When excess sodium is absorbed, an equivalent amount of water is also absorbed.

The increased estrogen production of normal pregnancy also promotes sodium retention, though it is not clear how important this hormone is in sodium and water homeostasis under these circumstances. Estrogen induces sodium retention by a direct renal action (Johnson and Davis 1976) and by increasing hepatic production of renin substrate (Klett et al. 1992). One study suggests that estrogen may be responsible for the increase in mucopolysaccharide ground substance present in the skin and subcutaneous tissue of many women in the second half of pregnancy (Fekete 1954). This allows the tissue to retain fluid to a greater extent than that seen in the non-pregnant state.

Pathophysiological changes in preeclampsia

Eclampsia is a clinical syndrome characterized by seizures in late pregnancy which usually begins to abate 24 to 48 hours postpartum. The discovery of this syndrome has been credited to the ancient Egyptians, Indians and Chinese. One of the oldest cited sources is the Kahun (Petrie) papyrus dating from about 2200 BC. The first documentation of preeclampsia as a separate disease originated from the ancient Greeks before the time of Hippocrates (Chesley 1974). Prompt termination of pregnancy was recommended in 1694 as the best treatment in cases of eclampsia. It was also stated that primigravidas were at a far greater risk of developing convulsions than multiparas (Mauriceau 1694). Depending on the diagnostic definition, preeclampsia occurs in 6-8% of pregnancies (Chesley 1978, Zuspan 1991, Pietrantoni and O'Brien 1994) and appears to be almost unique to humans, being only very rarely reported in sub-human primates (Baird 1981).

Traditionally, preeclampsia has been classified as a hypertensive disease of pregnancy presumably because of the clinical importance of hypertension. However, this emphasis on hypertension has diverted attention from other, perhaps more important, pathogenetic factors. Preeclampsia is actually a complex clinical syndrome involving a large variety of physiological systems. Preeclampsia is characterized clinically by an increase in blood pressure, proteinuria and edema. *Hormonal changes include increases in plasma levels of ANF and ET, and decreases plasma renin activity (PRA).*

Many of the pathophysiological changes in preeclampsia are essentially a failure of compensatory responses during normal pregnancy. In the cardiovascular system, variability in CO has been reported (Friedman 1991). But in severe cases, CO is decreased compared to normal pregnancy (Visser and Wallenburg 1991, Lang et al. 1991). Blood pressure, by definition, is elevated. This is easily detected in the clinic and provides the reason why preeclampsia was classified as a hypertensive disease for such a long time. The characteristic increase in blood pressure of preeclampsia is due to an elevated systemic vascular resistance (Cotton et al. 1984, Groenendijk et al. 1984, Wallenburg 1988). The pressor response to Ang II, as well as other vasoconstrictors, in preeclampsia is increased compared to normal pregnancy (Gant et al. 1973, Novak and Kaufman 1991). However, the cause of the vasospasm and increased sensitivity to circulating pressor agents is not clear. It seems to be due to decreased plasma levels of vasodilators such as PGI_2 and NO (both of which normally increase during pregnancy), or to increased plasma levels of ET (which normally does not change during pregnancy)

(Taylor et al. 1990). The vasospasm and increased sensitivity to pressor agents can also impair renal function: in preeclampsia both ERPF and GFR are decreased in comparison with normal pregnancy (Chesley 1978, Gallery and Gyory 1979).

It has been appreciated for many years that plasma volume is reduced in preeclampsia, an observation initially based on the hematocrit changes, but later confirmed by measurement of plasma volume by the Evans Blue dye dilution technique (Gallery et al. 1979, Hays et al. 1985, and Brown 1988a). The increase in plasma volume, which may be as high as 150% in normal pregnancy, is small or nonexistent in preeclampsia (Gallery et al. 1979, Hays et al. 1985). It has been recognized that this reduction of plasma volume precedes the development of the clinical syndrome (Gallery et al. 1979). The mechanism of the phenomenon is not clear, however, it does not seem to involve net sodium loss (Brown et al. 1988b). Indeed preeclamptic patients are more likely to retain sodium and water because of decreased GFR (Abraham and Schrier 1994). Therefore the reduction of plasma seems to be closely related to increased vascular permeability and redistribution of ECFV (Brown et al. 1992). There is a good correlation between the reduction of plasma volume, the severity of preeclampsia (Brown et al. 1992), and the incidence of abnormally small gestational age babies in preeclamptic patients (Soffronoff et al. 1977, Sibai et al. 1983). The decrease in plasma volume due to preeclampsia contributes, at least partly, to decreased cardiac output and ERPF, which leads to poor kidney and uteroplacental perfusion. Since preeclampsia is accompanied by amplification of the sodium retention that is a normal feature of pregnancy, the mechanism behind this

volume reduction seems to be more complex than merely a simple decompensatory effect of volume expansion seen in normal pregnancy.

I have described earlier how, in normal pregnancy, there are two waves of trophoblast invasion, the result of which is a marked loss of vascular contractility of the spiral arteries and an increase in uterine blood flow. In preeclampsia, the second wave of trophoblast invasion fails to occur (Brosens et al. 1972, Robertson et al. 1975). The myometrial segment of the spiral arteries remains muscular, undilated, and capable of contraction. This is, at least partly, responsible for the decreased uteroplacental blood flow in preeclampsia which is the earliest discernible difference between preeclamptic and normal pregnancies. Thus the hemodynamics of preeclampsia, in contrast to normal pregnancy, may be described as low volume, low flow, high pressure and high resistance.

Generally there is no evidence for progesterone deficiency in preeclampsia, although in some cases there is estrogen deficiency (Brown and Gallery 1994). However, other cardio-active hormones are dramatically altered. Plasma ANF levels increase in preeclampsia (Thomsen et al. 1987, Cowan et al. 1988, and Miyamoto et al. 1989) in contrast to the small changes in plasma ANF levels observed during normal pregnancy. Poulsen et al. (1993) hypothesized that this increase in plasma ANF is responsible for the hemoconcentration characteristic of preeclampsia; increased plasma ANF may reduce the intravascular volume by direct natriuresis and diuresis via the kidneys and by increasing capillary permeability (Huxley et al. 1987), thereby enhancing transcapillary

movement of water from the vascular compartment to the interstitial compartment. Furthermore, ANF may antagonize the RAA system (Maack et al. 1984, and Goodfriend et al. 1984).

The majority of studies have found that PRA and plasma renin concentration (PRC) are lower in preeclamptic patients in comparison with normal pregnant women (Broughton Pipkin 1988). Plasma aldosterone concentration is also reduced in preeclampsia (Broughton Pipkin 1988, Pedersen et al. 1984). However, the precise role of the RAA system in the pathogenesis and volume homeostasis of preeclampsia is still not well established. The suppressed RAA system in preeclampsia is either a compensatory mechanism to the increase in blood pressure, or the result of pathological inhibition by some neurohormonal factors, such as pathologically increased plasma ANF and/or ET.

Another prominent cardioactive hormone which is increased during preeclampsia is ET (Taylor et al. 1990, Floriji et al. 1991). ET, an endothelium-derived 21-residue peptide vasoconstrictor, was first isolated, cloned and sequenced from porcine aortic endothelium (Yanagisawa et al. 1988). Subsequently, three members in this family, ET-1, ET-2, and ET-3 were found. ET-1 is a peptide synthesized in and secreted from endothelial cells and is a very potent vasoconstrictor. In normal physiological conditions, the plasma ET-1 concentration is very low (Vane and Botting 1993). During pregnancy, plasma ET-1 concentration does not change appreciably (Ihara et al. 1991), but during preeclampsia, its concentration increases (Taylor et al. 1990, Floriji et al. 1991, Ihara et al. 1991). This

increase in ET-1 concentration implies that endothelial cells are damaged or dysfunctional. ET-1 is likely to be involved in the increase in blood pressure seen during preeclampsia due to its vasoconstrictive effect. Preeclamptic volume homeostasis may also be affected by ET-1 due to its effect on the kidney, RAA system and capillary permeability.

Pathogenetic theory of preeclampsia

Despite intensive and extensive research in this area, the mechanisms underlying the development of preeclampsia and the reduction in blood volume remain an enigma. One of the reasons for this is that preeclampsia is unique to human pregnancy and there is a lack of a good animal model. Attempts to create an animal model of preeclampsia in rabbits (Abitbol et al. 1976a), dogs (Abitbol et al. 1976b), sheep (Thatcher and Keith 1986), and primates (Cavanagh et al. 1985) have met with varying degrees of success. Recently, endothelial dysfunction has been proposed as a major factor in the pathogenesis of preeclampsia (Roberts et al. 1989). The essence of this theory is that systemic arterial endothelial cell damage/dysfunction, which is caused by some still unidentified endothelial toxins derived from the poorly perfused placenta, leads to a decrease in the production of endogenous vasodilators, an increase in the production of vasoconstrictors, an activation of intravascular coagulation and fibrin deposition, and an increase in vascular permeability. All these factors are characteristic of preeclampsia.

There are several lines of evidence supporting the fact that there is endothelial cell damage/dysfunction in preeclampsia. Morphologically, the glomerular endotheliosis characteristic of preeclampsia, specifically involves endothelial cells (McCartney 1964), an occurrence only seen in preeclampsia, not in other hypertensive diseases (Fisher et al. 1980). Studies also indicate that the endothelial cells in kidney (Sparogo et al. 1976), spiral artery (Robertson et al. 1967), and umbilical vessels (Dadak et al. 1984) are damaged during preeclampsia. Further evidence that supports the theory that the endothelial cells are damaged or dysfunctional in preeclampsia is the increase in pressor response to Ang II (Gant et al. 1973), the increase in vascular permeability (Brown et al. 1989), and the activation of the coagulation cascade (Fournie et al. 1981, Scholtes et al. 1983, Roberts and May 1976). Accumulated biochemical data show that circulating factor VIII antigen (Fournier et al. 1981) and fibronectin levels (Lockwood and Peters 1990), which are known to be related to endothelial activation, increase in preeclampsia. Also, production of PGI_2 is reduced in several vessels (Friedman 1988) whereas ET increases (Taylor et al. 1990, Floriji et al. 1991); all of these are possible markers of endothelial cell damage during preeclampsia.

The mechanism(s) underlying endothelial cell damage is still unclear. Several possibilities have been studied recently. Tulenko et al. (1987) reported that perfusing rabbit aorta with serum from preeclamptic patients results in an increased sensitivity of the preparations to Ang II and norepinephrine. Rodgers et al. (1988) demonstrated that the predelivery serum from preeclamptic women causes an increased release of

chromium^{51} (Cr^{51}) from prelabeled human umbilical vein endothelial cells compared to that released in response to treatment with serum from either the same women after delivery or normal pregnant women. The cytotoxicity index defined by the authors is positive in predelivery serum from preeclampsia patients and negative from postdelivery serum of the same patients or normal pregnant women. They proposed that poorly perfused trophoblasts secrete a substance toxic to endothelial cells. The nature of this substance remains unknown. Lipid peroxides, which cause vasoconstriction and inhibition of PGI_2 synthesis, are found at elevated concentrations in the blood of normal pregnant women and at significantly higher levels in preeclamptic women (Wickens et al. 1981). Also, the balance of oxygenation and anti-oxygenation during preeclampsia is disturbed (Hubel et al. 1989, Wang et al. 1991, Davidge et al. 1992). So the free radical-mediated oxidation from uncontrolled lipid peroxidation may be potential mechanism which cause the endothelial cell damage. Another possible mechanism of endothelial cell damage is antibody mediated. Rappaport et al. (1990) reported preeclamptic women possess higher titers of autoantibodies directed against endothelial cells. These antibodies are not directed against human leucocyte antigen (HLA) antigens and have no cross reactivity with platelets, meaning that they are specific anti-vascular antibodies. But the source and specificity of the anti-vascular endothelial cell antibodies in preeclampsia is unclear. These autoantibodies, which may be induced by endothelial cell damage caused by more primary factor(s), might then enhance the ongoing endothelial damage. Recently it was found that the concentration of the endogenous nitric oxide synthase (NOS) inhibitor is higher in preeclampsia than in normal pregnancy (Fickling et al. 1993). All

these data imply that the cause of endothelial cell damage in preeclampsia might be multi-factorial, with both primary and secondary factors.

Role of NO in pregnancy and preeclampsia

The endothelium is no longer believed to be simply a passive barrier; it can secrete a multitude of bioactive materials which are involved in many physiological and pathophysiological processes. NO is one of these endothelium-derived mediators and is synthesized in endothelial cells. NO synthesis is an enzymatic process which involves a specific enzyme, namely NOS. There are three kinds of isoforms of NOS according to their gene cloning: an endothelial NOS (eNOS), a neuron NOS (nNOS) and an inducible NOS (iNOS). Endothelial NOS is the isoform existing in endothelial cells. NOS converts L-arginine into NO and L-citrulline in the presence of oxygen (Fig 1-1). NO diffuses into both lumen and smooth muscle of blood vessels. The NO signal transduction mechanism involves NO binding to the soluble guanylyl cyclase (GC-S), thereby activating the enzyme. GC-S then catalyzes GTP into guanosine 3',5'-cyclic monophosphate (cGMP) which serves as a second messenger to activate different bioactive processes. Recent studies have shown that NO is involved in blood pressure regulation by relaxing smooth muscle, in blood coagulation by preventing platelet adhesion, in central and peripheral neurons system by acting as a neurotransmitter, and in the immune system by acting as a cytotoxic agent (Vallance and Collier 1994).

NO may also play an important role in cardiovascular adaptation to pregnancy. During pregnancy, systemic endogenous NO biosynthesis increases in gravid rats (Conrad et al. 1993). Uterine NO production also increases during pregnancy, but decreases during labor and postpartum (Yallampalli et al. 1994). Other studies have shown that NOS exists in the umbilical vessels (Voorde et al. 1987, Chaudhuri et al. 1991) and in the syncytiotrophoblast (Myatt et al. 1993). This increased NO synthesis may serve as a vasodilator to decrease the peripheral resistance, which may well account for the blood pressure decrease, refractoriness to pressor agents seen in pregnancy and it also can reduce platelet aggregation. It has been found that NO release in response to bradykinin stimulation is diminished in the umbilical vessels ex vivo from infants of preeclamptic patients in comparison to those from a normal pregnancy (Pinto et al. 1991). Since NO synthesis is an enzymatic process, any inhibition or activation of NOS will regulate NO production. NOS inhibitors, the analogues of L-arginine, are very useful tools in the study of the physiological or pathological role of NO. Data now available show that NOS inhibition induces an increase in mean arterial pressure (MAP) (Molnar et al. 1992, Yallampalli and Garfield 1993) in pregnant animals. The latest data even show that NOS inhibition induces proteinuria, a decrease in plasma volume and intrauterine fetal growth retardation (IFGR) (Molnar et al. 1994). This implies that NO synthesis may play an important role in normal pregnancy and that inhibition of NO production may be involved in the pathogenesis of preeclampsia. Thus, it seems that inhibition of NO synthesis in pregnant animals may have potential as an animal model for studying preeclampsia.

It was with these facts in mind, that we wished to determine whether, in addition to the above-described changes in the cardiovascular and renal systems, NOS inhibition would also mimic preeclampsia with respect to perturbations in volume homeostasis and the hormonal profile. We hypothesized that administration of the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), to pregnant rats would reduce blood volume, would increase plasma ANF and ET, and would decrease PRA. We chose to investigate these particular parameters because they seem to play such a crucial role in the physiology of normal pregnancy and in the pathophysiology of preeclampsia. All these three hormones, being both cardio-active and renal-active, are involved in volume homeostasis. The high concentration of ANF in preeclampsia has been proposed to be responsible for the hemoconcentration of preeclampsia, by its natriuretic and diuretic effect on kidney and by its antagonism of RAA. Increased levels of ET, serving as a marker of endothelial cell damage, also influence the volume regulation by affecting renal hemodynamics and such volume-related hormones as ANF and PRA. Importantly, both ANF and ET increase the permeability of capillaries; this is one of the most significant pathological changes associated with preeclampsia, and another mechanism by which blood volume reduction occurs. Knowing that the NOS system is activated during pregnancy, we postulated that NOS inhibition would reduce blood volume to a greater extent in the pregnant rats than in the virgin animals. The question we wished to address in this project was whether NOS inhibition would precipitate the physiological changes in pregnant rats analogous to the pathophysiological perturbations associated with human preeclampsia. If this is proved to be the case, not only would our results advance our understanding of the underlying

etiology of preeclampsia, but we would have made an important contribution to the validation of the NOS-inhibited pregnant rat as a model of human preeclampsia.

Our preliminary results showed that the blood volume of rats increases significantly during pregnancy, and returns to the prepregnant level postpartum (Fig 1-2). These results are in agreement with other studies (see review Baylis 1994). The similarity of blood volume changes during pregnancy in the rat with those known to occur in humans provides the basis for our current study on **the effect of nitric oxide synthase inhibition on blood volume regulation during pregnancy in rats.**

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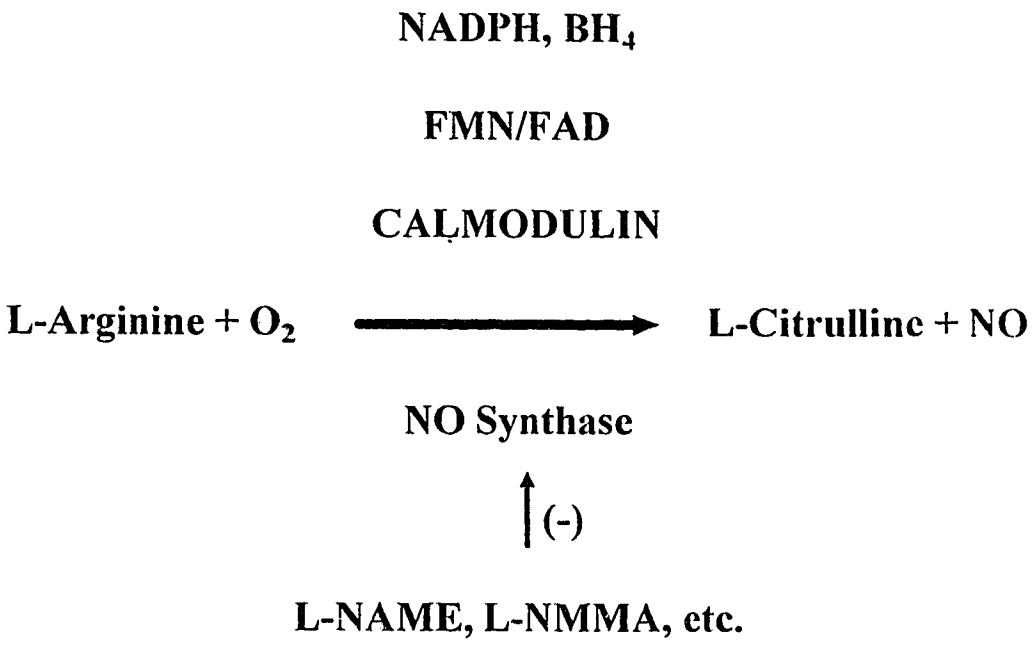

Fig 1. Biosynthesis of NO and its co-product L-citrulline from L-arginine and molecular oxygen. All isoforms of NO synthase known so far are heme proteins, utilize L-arginine as the substrate and require the cofactors, such as NADPH, BH_4, and FMN/FAD, as well as calmodulin. All isoforms of NO synthase are inhibited by L-arginine analogs, such as L-NAME, L-NMMA.

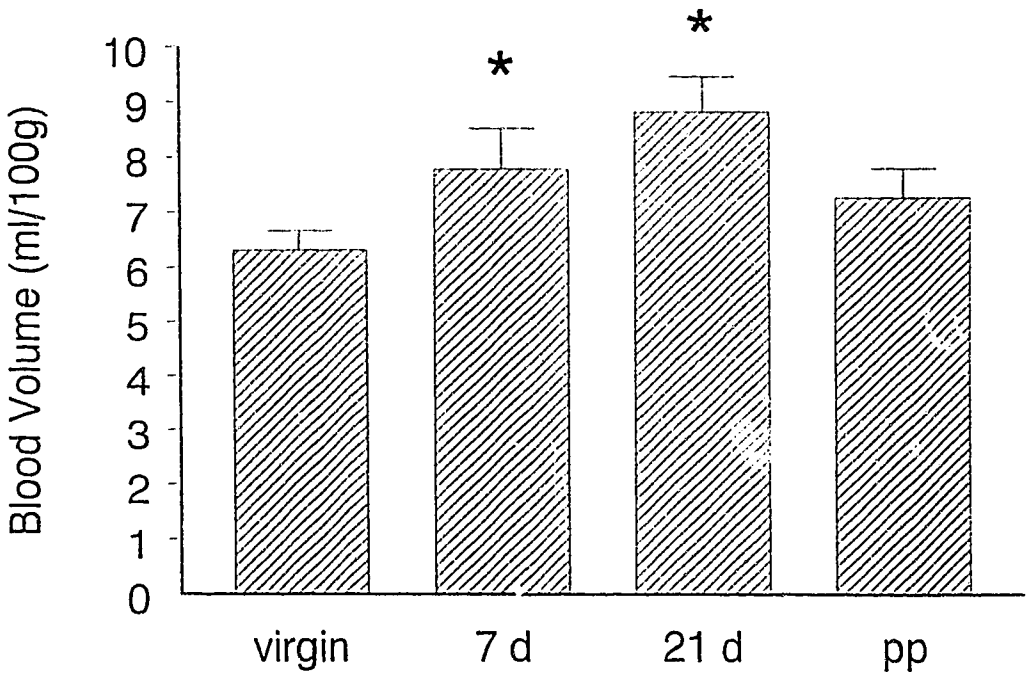

Fig 2. Blood volume changes in virgin, at 7-day (7d) and 21-day (21d) pregnancy, and at 7-day post-partum (pp) in rats. Blood volume is measured by Evans blue dilution technique (Appendix I) and expressed as ml/100g. The vertical bars delineate standard error of the mean. *, $p<0.05$ (7d pregnancy vs. virgin, and 21d pregnancy vs. virgin,).

CHAPTER TWO

EFFECT OF NITRIC OXIDE SYNTHASE INHIBITION ON BLOOD VOLUME REGULATION DURING PREGNANCY IN THE RAT

(This paper has been submitted to American Journal of Obstetrics and Gynecology. 1996)

INTRODUCTION

Pregnancy is associated with profound changes in the cardiovascular, renal, and endocrine systems, all of which enable the pregnant woman to accommodate the growing products of conception. Among these changes, one of the most remarkable is the 50% increase in plasma volume (1). The mechanism by which blood volume increases in pregnancy is still unclear. It may involve a change in the balance of natriuretic and antinatriuretic hormones (1), or resetting of the volume receptors (2,3).

Preeclampsia, which complicates 5-10% of pregnancies, is still one of the major causes of maternal and fetal death during gestation. It is characterized by increased blood pressure, proteinuria, and edema. Hormonal changes include increases in plasma levels of atrial natriuretic factor (ANF) and endothelin (ET), and a decrease in plasma renin activity (PRA) (4,5,6). Preeclampsia is also associated with a significant reduction in plasma volume, which precedes development of the clinical syndrome (1). Despite extensive research in this area, the mechanisms underlying the development of preeclampsia and the reduction in blood volume remain obscure. One reason for this has been lack of a good animal model. However, endothelial dysfunction has been proposed as a major factor in the pathogenesis of preeclampsia (7).

The endothelium is no longer believed to be simply a passive barrier; it can secrete many bioactive materials which are involved in a multitude of physiological and

pathophysiological processes. Nitric oxide (NO) is one of these endothelium-derived mediators that may play an important role in cardiovascular adaptation to pregnancy. During pregnancy, systemic endogenous NO biosynthesis increases in gravid rats (8). Uterine NO production is also increased during pregnancy, but decreases during labor and postpartum (9).

It has been demonstrated that, during pregnancy, NOS inhibition induces an increase in mean arterial pressure (MAP), proteinuria, a decrease in plasma volume, and intrauterine fetal growth retardation (10,11,12). This implies that NO biosynthesis may play a role in normal pregnancy and that inhibition of NO production may be involved in the pathogenesis of preeclampsia. It has seemed thus that inhibition of NO synthesis might provide a potential animal model for studying preeclampsia. The hypothesis of the current study was that administration of Nω-nitro-l-arginine methyl ester (L-NAME) to pregnant rats would reduce blood volume and would change the profile of plasma ANF, ET and PRA to resemble that found in preeclampsia, i.e., L-NAME would increase plasma ANF and ET and decrease PRA.

MATERIALS AND METHODS

The experiments described in this paper were examined by the local Animal Welfare Committee, and found to be in compliance with the guidelines issued by the Canada Council on Animal Care.

Animals. Female Long-Evans rats (body weight 225 - 250g), were obtained from Charles River Canada (St. Foy, Quebec). They were held in a temperature- and humidity-controlled room with 12 hr light (0700-1900) and 12 hr dark (1900-0700) for at least one week before surgical preparation. They were maintained on a 0.28% sodium diet (PMI Feeds Inc., St. Louis, MO) and water ad libitum

Surgery. Under isoflurane anesthesia (Solvay Animal Health, Inc. Kitchener, Ont.), the left femoral vein (Silastic tubing 0.020 in. ID × 0.037 in. OD, Dow Corning Corporation, Midland, MI), the right femoral vein (Micro-renathane tubing, 0.012 in. ID × 0.025 in. OD, Braintree Scientific, Inc. Braintree, Mass.), and the left femoral artery (Micro-renathane tubing, 0.012 in. ID × 0.025 in. OD) were cannulated. An osmotic minipump (model 2ML1, ALZA Corporation, Palo Alto, CA), which contained L-NAME or D-NAME 120mg (60mg/ml, 10μg/min; Sigma, St. Louis, MO), was implanted under the skin on the back of the rat. For the control animals, a similar size piece of tubing was implanted.

Experimental protocol. Vaginal smears were taken and, at proestrus, the animals destined for the pregnant groups were mated. Fourteen days later, if the body weight had increased by 40g or more, the indwelling cannulae were implanted. (Note: during the surgery, the fetuses could be palpated to confirm the pregnancy). On day 19 of pregnancy, the rats were put into metabolic cages. On day 20, 24hr water intake, urine

output and sodium excretion were monitored. Blood volume was measured early the next day (day 21 of pregnancy). The animals were then returned to their home cages, where they delivered their pups spontaneously the following day. Seven days postpartum, blood volume was measured again. A separate group of animals was designated as virgin controls. Surgery, as described above, was performed at random times during the estrus cycle. As before, osmotic minipumps containing L-NAME were implanted in the experimental animals. Blood volume was measured 7 days later. Further sets of animals (virgin and pregnant) were treated in the same manner. However, instead of measuring blood volume, blood samples (1.6ml) were taken to assay plasma ANF, PRA and ET concentrations.

During the experiments, mean arterial pressure (MAP) was monitored and recorded via the femoral arterial cannula using a Statham pressure transducer and a Gould physiological recorder.

Measurement of blood volume. Plasma volume was determined by means of the Evan's blue dye dilution method. In short, initial blood samples (0.25ml) were taken. A solution (0.3ml, 0.5 w/v in sterile isotonic saline) of Evan's blue (Baker Chemical Co., Phillipsburg, NJ) was injected via the smaller indwelling venous cannula. The line was flushed with 0.2 ml saline. At 10, 20, 30, 40, and 60 min, blood samples (0.15ml) were taken from the larger venous cannula, rapidly transferred to heparinized Fisherbrand Caraway tubes (Fisher Scientific, Edmonton) and centrifuged. The hematocrit was

measured and the plasma was separated from the red blood cells. Meanwhile the blood sample was replaced with the same volume of saline. The plasma samples (50 µl) were diluted in 950 µl saline and absorbance was measured at 605 µm on a Spectrophotometer (LKB Biochrom, model 4049, Cambridge, England). The readings were compared with standards obtained by adding 0, 1, 2 µl of the 0.5 % Evan's blue solution to 50 µl initial plasma plus 950 µl saline. The plasma volume and blood volume were calculated by extrapolation back to time zero.

Blood samples and radioimmunoassay (RIA). The blood samples for the hormone assays were mixed with EDTA (80µl Sequester-sol) plus aprotinin (40 kallikrein inhibition units; Trasylol, Bayer AG, Leverkusen, Germany), and centrifuged (14,000 rpm for 10 min at 4°C). The plasma was separated from the cells and stored at -43°C. Before radioimmunoassay, the plasma samples for ANF and PRA, but not those for ET, were extracted using C_{18}-columns. Rat ANF and ET were assayed using materials and methods supplied by Peninsula Laboratories, Belmont, CA. Each set of plasma samples for ET and ANF was measured within one assay. Assay sensitivity for the ANF and ET assays [half maximal displacement (IC_{50})] were 8.5 pg/100µl and 18 pg/100µl respectively. Intra-assay variability was 5% for each assay. PRA was assayed using materials and methods supplied by Dupont Canada Ltd., Mississauga, Ont. Assay IC_{50} for was angiotensin I was C.315ng/ml. Intra-assay variability was 4%. Inter-assay variability was 13.8% (the samples were measured in two assays).

Statistics. All data are expressed as mean ± SE of mean. The data for mean arterial pressure, blood volume, ANF, PRA and ET were compared using Student's t-test. The data for hematocrit were analyzed using ANOVA, followed by Student-Newman-Keuls method for multiple pairwise comparisons. Levels of statistical significance are indicated at $p<0.05$.

RESULTS

Chronic infusion of L-NAME, but not D-NAME, induced a significant increase of MAP in both virgin and 21-day pregnant animals (Fig 1). L-NAME also reduced blood volume significantly in virgin rats, at 21-days of pregnancy and at 7-days postpartum, compared with the equivalent untreated control groups (Fig 2). However, the degree of blood volume reduction was more obvious in pregnant than in virgin rats (31% vs. 9.9%). D-NAME had no effect on blood volume in any of the groups. Hematocrit was increased in the L-NAME-treated animals compared with the controls (Fig 3). There was no difference in the slope of the Evan's Blue dye-decay curves between the experimental and control groups (not shown).

L-NAME had no effect on plasma ANF levels or PRA in either virgin or 21-day pregnant animals (Figs 4, 5). By contrast, L-NAME did lower plasma ET levels in 21-day pregnant rats, but not in virgin animals (Fig 6). There were no statistically significant differences in water intake, urine output or sodium excretion between the 21-day pregnant control-

and L-NAME-treated groups (50.1±3.28 vs. 39.7 ± 5.52 ml/day; 35.6 ± 5.03 vs. 28.3 ± 5 ml/day; 224 ± 41 vs. 157 ± 26 μEq, respectively, $p>0.05$, $n=7$).

COMMENT

There is much evidence that blood volume increases during pregnancy in rats (13) and in humans (1). Our results demonstrate that this increase is blunted by L-NAME, an NOS inhibitor, whereas D-NAME, a non-bioactive stereo isoform of L-NAME, has no such activity. The effects on MAP were similar; L-NAME, but not D-NAME, increased MAP. Our data, which are in agreement with those of Molnar et al (10) and Salas et al (12), suggest that NO plays an important role in blood volume and pressure homeostasis during pregnancy. Since L-NAME reduced the blood volume also in virgin rats, its effect on volume regulation does not seem to be pregnancy-specific. However, pregnant rats appear to be more sensitive to the inhibition of NO synthesis than do virgin rats. An explanation for this phenomenon may be that NO plays a much more important role in volume homeostasis during pregnancy. Thus, any disturbance of its synthesis during pregnancy will cause more pronounced changes than in virgin rats.

We chose to express our blood volumes as ml/100g body weight since it is virtually impossible to estimate maternal body weight at the different stages of pregnancy. Had we expressed the data as ml/100g initial body weight, or as absolute blood volume, the results would have been the same, only more pronounced. Similarly, we chose to

administer the L-NAME as a fixed dose (10μg/min) so that the dose was, if anything, even lower in the pregnant than in the virgin animals.

The mechanisms by which L-NAME reduces blood volume are still unknown. Volume homeostasis is a complex process which consists of sensor and effector components. Changes in blood volume, or more generally extracellular fluid volume (ECFV), are sensed by stretch receptors in the atria, the great veins, and perhaps in the interstitial space (1). The most important effector of volume homeostasis is the kidney, which regulates ECFV principally by controlling sodium reabsorption. Both sensors and/or effectors may be affected by the neural and hormonal changes associated with pregnancy. We found no significant effect of L-NAME on water intake, urine volume or sodium excretion of the pregnant animals in our study. This is consistent with the results from a similar study done on male rats (14). It is therefore unlikely that L-NAME decreases blood volume directly by interfering with water intake and urine output. Nor did NOS inhibition appear to influence vascular permeability, since the loss of Evan's Blue (conjugated to plasma protein) was no greater in the L-NAME-treated animals. Although Molnar et al (10) found that L-NAME did increase dye clearance, their dose (5mg/kg/hr) was considerably higher than that used in our studies.

Our data also show that L-NAME has no effect on two hormones intimately involved in volume homeostasis, namely ANF and renin. One study has shown that administration of L-NAME (bolus injection of a dose 10mg/kg) increases basal plasma ANF levels and

enhances the secretory response to volume loading in vivo in male rats (15). However, this effect was only found with the highest dose of L-NAME; lower doses (1-3mg/kg) had no such activity. Yamamoto et al. (16) also failed to find any effect of L-NAME (bolus injection of a dose 2mg/kg) on plasma ANF of male rats. The reason for the anomalous activity of higher doses of L-NAME may be that elevated concentrations of alkyl esters of arginine, such as L-NAME, have muscarinic receptor antagonist activity (17). Moreover, blocking muscarinic receptors by methylatropine has been shown to enhance ANF release in response to volume loading (18). It is likely therefore that the increase in ANF release in response to high doses of L-NAME, may have resulted from the muscarinic effects of L-NAME rather than from its inhibition of NO synthesis. In our study, L-NAME (chronic infusion of 10μg/min, or between about 20μg/kg pregnant rat/min to 30 μg/kg virgin rat/min) failed to induce ANF secretion in vivo. All these data are thus in agreement that L-NAME, at low doses, has no effect on plasma ANF levels.

The results from studies on the effects of L-NAME on PRA have been inconsistent. L-NAME suppresses PRA acutely in anesthetized male rats at a bolus dose of 75 μmol/kg (19). Navarro et al (14) demonstrated that oral administration of L-NAME to male rats influenced PRA only at the highest dose tested (30mg/100ml drinking water) and at the longest administration period (4 weeks). Manning and Hu (20) have found that chronic infusion of L-NAME (10μg/kg/min) induces hemodynamic changes in both the general and renal circulation of dogs, but has no effect on PRA. This is in agreement with our results showing that L-NAME (20-30μg/kg/min), administered by subcutaneous osmotic

minipump for one week, has no effect on PRA in either virgin or pregnant rats. In the only other study to have been published using female rats, Salas et al (12) found that L-NAME (in the drinking water) reduced PRA. However, the doses of L-NAME used by these investigators were at least as high as those used by Navarro et al (14). On the other hand, in vitro studies have revealed that NO decreases renin secretion (21). Moreover, when renal hemodynamics and β-adrenergic activity are controlled, or when the dose is so low that mean arterial pressure is unaltered, L-NAME increases PRA (22,23). This suggests that, in addition to the dose and the length of administration of L-NAME being important, the results may be confounded by reflex inhibition of PRA secondary to hypertension. In our case, the presumed primary stimulatory activity of L-NAME on PRA was probably counteracted by reflex suppression caused by the increased blood pressure.

The endothelium can release both vasodilators (e.g. NO) and vasoconstrictors (ET). During pregnancy, plasma ET-1 levels normally do not change appreciably (27). However, they do increase in preeclampsia (5), and it has been proposed that the high concentration of ET in preeclampsia may contribute to the observed increases in blood pressure and vascular permeability. Under these circumstance the increased ET levels may reflect the endothelium damage that is characteristic of this disease (7). NO can inhibit thrombin-induced ET release (25) and attenuate the effects of ET. However, neither NO donors nor L-NAME affect basal ET release (25). With regard to in vivo studies, Navarro et al (14) have found that long term oral administration of L-NAME in a

stepped increasing manner does not affect plasma ET-1 levels I male rats. However, apart from our own study, there are no published data on the effect of NO on ET release during pregnancy. Our study showed that, after chronic administration of L-NAME to virgin and 21-day pregnant rats, plasma ET concentration was not changed in the virgin group, but did decrease in the pregnant group. This is unlike the situation found in preeclampsia, where both ET (5) and ANF (6) increase. Since ET is a secretagogue of ANF (26), the increase in plasma ANF in preeclampsia may be secondary to the elevated levels of ET. This suggestion is consistent with the failure of ANF levels to increase in our NOS-inhibitor-treated animals.

Although this and other studies (10,11,12) have demonstrated that deficient NO biosynthesis may be one of the pathogenetic factors involved in the etiology of preeclampsia, it does not appear to be the primary lesion. L-NAME blocks NO production, which in turn causes an increase in blood pressure, a decrease in blood volume, proteinuria, and fetal growth retardation (10,11,12). However, inhibition of NO synthesis does not induce the *hormonal* changes characteristic of preeclampsia, i.e. increased plasma ANF and ET levels, and decreased PRA. Our results serve to affirm that the primary lesion in preeclampsia is probably endothelial cell damage. This would cause not only a decrease NO production, but would also increase secretion of the vasoconstrictive hormone ET. ET is a potent agonist of ANF secretion, and both ET and ANF are antagonists of renin secretion. Taken together, the result of endothelial cell

damage would thus be decreased NO biosynthesis, increased ET and ANF and decreased PRA, i.e., the classical profile of preeclampsia.

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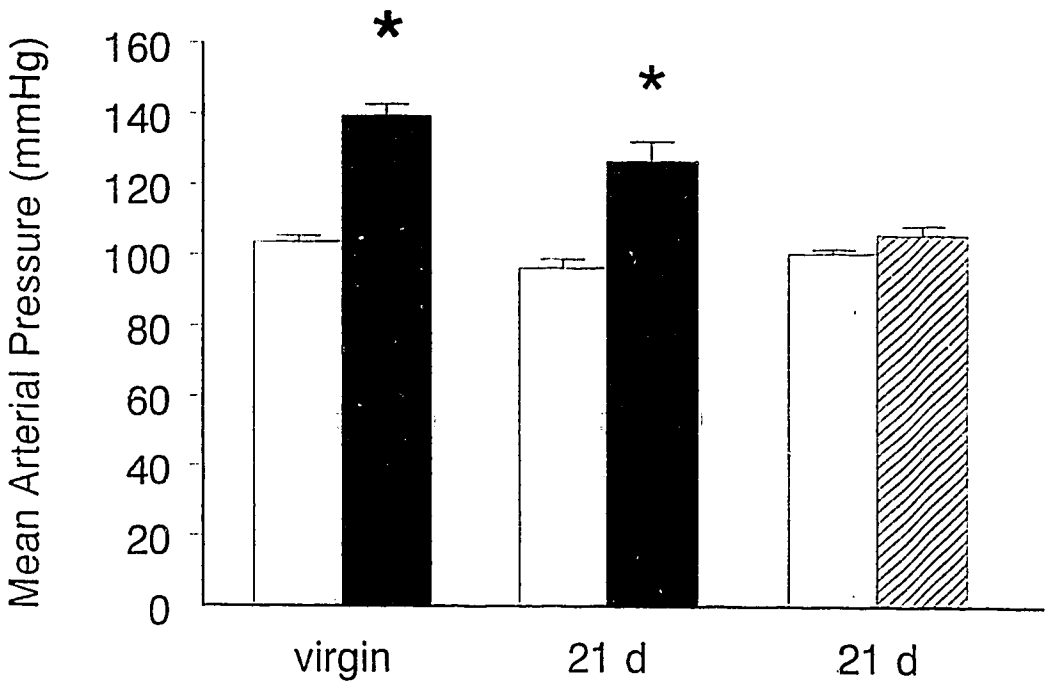

Fig 1. Effect of L-NAME and D-NAME on mean arterial pressure in virgin and 21-day pregnant (21d) rats. L-NAME or D-NAME was infused by osmotic minipump subcutaneously for 7 days in the test animals. Open bars = control groups, virgin (n=14) and 21d pregnant (n=11 and n=8); solid bars = L-NAME-treated animals, virgin (n=8) and 21d pregnant (n=11); hatched bar = D-NAME-treated animals, 21d pregnant (n=10). The vertical bars delineate standard error of the mean. *, $p<0.05$ (test vs. control)

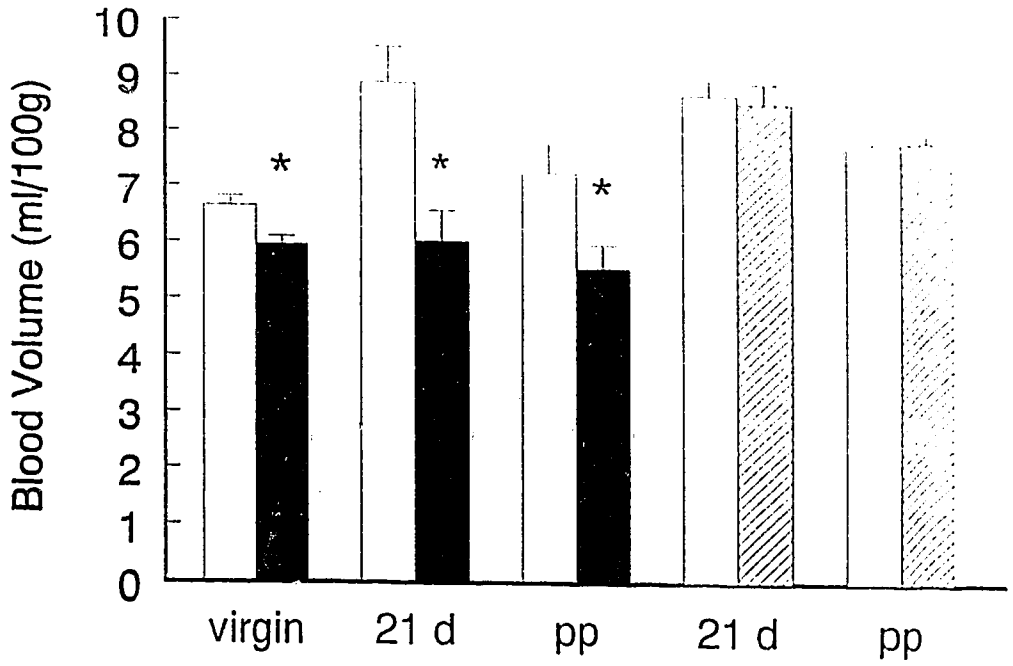

Fig 2. Effect of L-NAME and D-NAME on blood volume changes in virgin rats (vir), at 21-days of pregnancy (21d) and at 7-day post-partum (pp). L-NAME or D-NAME was infused by osmotic minipump subcutaneously for 7 days in the test animals. Open bars = control groups, virgin (n=6), 21d pregnant (n=7 and n= 8) and 7d post-partum (n=7 and n= 8); solid bars = L-NAME-treated animals, virgin (n=6), 21d pregnant (n=7) and 7d post-partum (n=7); hatched bar = D-NAME-treated animals, 21d pregnant (n=9) and 7d post-partum (n=9). The vertical bars delineate standard error of the mean. *, $p<0.05$ (test vs. control).

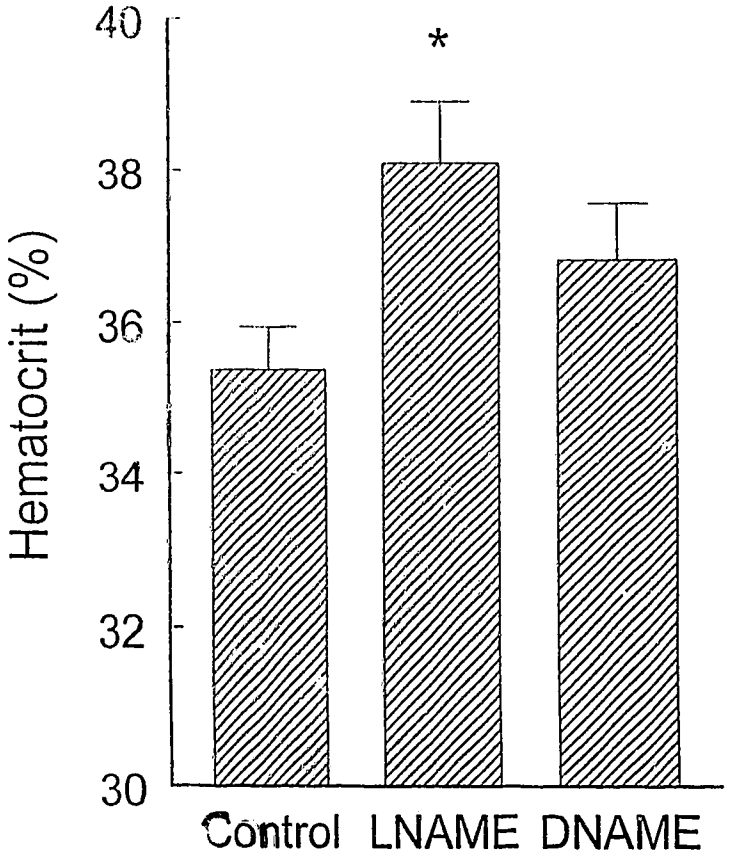

Fig 3. Effect of L-NAME and D-NAME on hematocrit of 21-day pregnant rats. L-NAME or D-NAME was infused by osmotic minipump subcutaneously for 7 days in the test animals. Control = untreated 21-day pregnant rats (n=15). L-NAME, D-NAME = 21-day pregnant rats treated with L-NAME (n=7) or D-NAME (n=8) respectively. The vertical bars delineate standard error of the mean. *, $p<0.05$ (test vs. control)

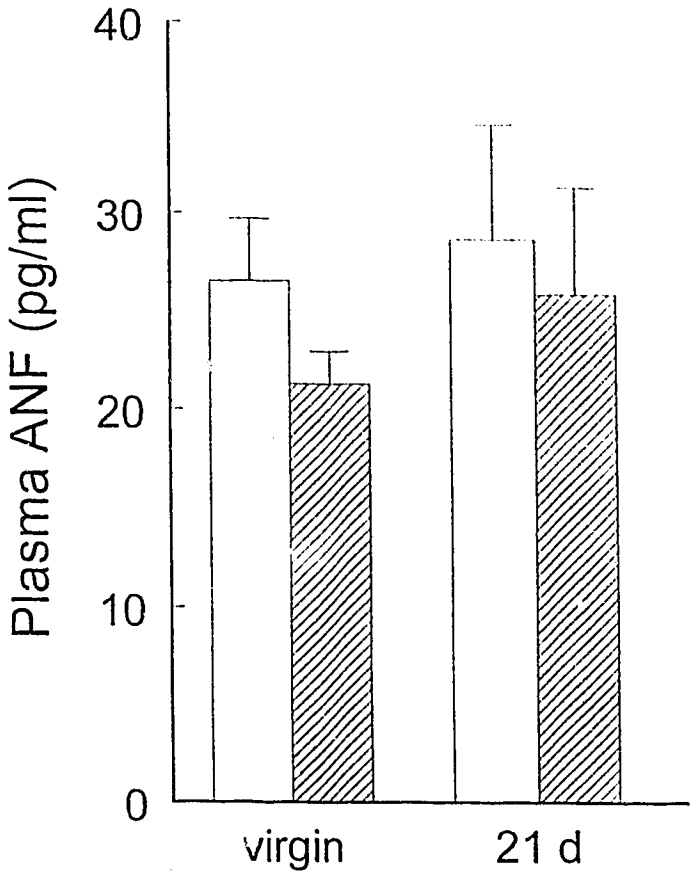

Fig 4. Effect of L-NAME on plasma atrial natriuretic factor in virgin and 21-day pregnant (21d) rats. L-NAME was infused by osmotic minipump subcutaneously for 7 days in the test animals. Open bars = control group, virgin (n=8) and 21d pregnant (n=8); hatched bars = L-NAME-treated animals, virgin (n=8) and 21d pregnant (n=10). The vertical bars delineate standard error of the mean.

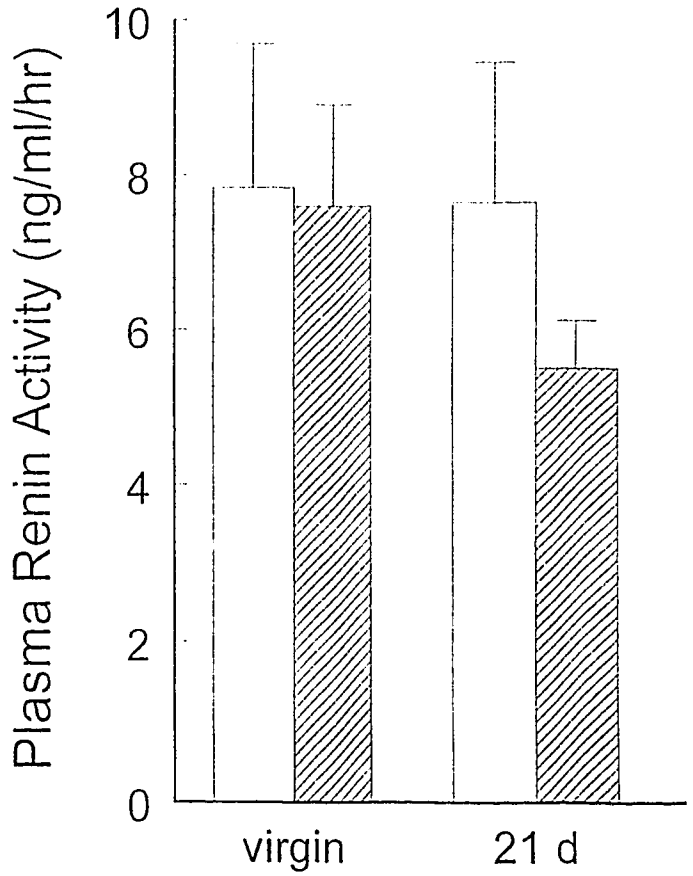

Fig 5. Effect of L-NAME on plasma renin activity in virgin and 21-day pregnant (21d) rats. L-NAME was infused by osmotic minipump subcutaneously for 7 days in the test animals. Open bars = control group, virgin (n=6) and 21d pregnant (n=8); hatched bars = L-NAME-treated animals, virgin (n=10) and 21d pregnant (n=7). The vertical bars delineate standard error of the mean.

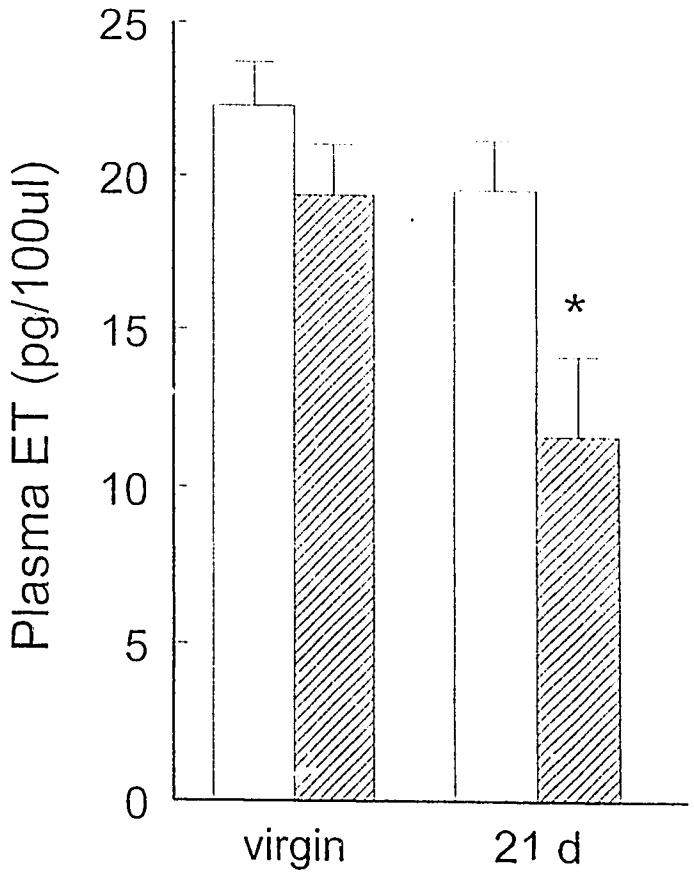

Fig 6. Effect of L-NAME on plasma endothelin in virgin and 21-day pregnant (21d) rats. L-NAME was infused by osmotic minipump subcutaneously for 7 days in the test animals. Open bars = control group, virgin (n=8) and 21d pregnant (n=8); hatched bars = L-NAME-treated animals, virgin (n=8) and 21d pregnant (n=8). The vertical bars delineate standard error of the mean. * $p<0.05$ (test vs. control).

CHAPTER THREE

SIGNIFICANCE OF THE THESIS

AND

FUTURE RESEARCH DIRECTION

The etiology and pathogenesis of preeclampsia have puzzled both clinicians and researchers for many years. This is because preeclampsia is a disease unique to human pregnancy, and lacking of good animal model. Although, for decades, researchers have endeavored to describe the etiology of this disease, many steps of the pathogenesis are still uncertain. However, there are two steps in this pathogenetic process which are currently accepted. One is the inadequate trophoblast invasion of the maternal spiral arteries, which leads to a poor placental perfusion. The other is systemic arterial endothelial cell damage, which is caused by some still unknown placenta-derived endothelial "toxins". This leads to a decrease in the production of endogenous vasodilators, an increase in the production of endogenous vasoconstrictors, activation of intravascular coagulation, increase in vascular permeability, etc. (Fig 3-1, also see review of Friedman et al. 1991).

There have already been several lines of evidence to support this cascade. A family predisposition has been observed (Chesley et al. 1968). Simon et al. (1988) and Kilpatrick et al. (1989) suggested that HLA DR4 may be an antigen link to the development of preeclampsia. Increased histocompatibility between mother and fetus is also found to be associated with the development of the disease (Jenkins et al. 1980). These findings, together with the fact that preeclampsia is more common in the first pregnancies, is more frequent in pregnancies by a new consort (Feeney 1980), and is increased following barrier contraception (Klonoff-Cohen et al. 1989), all suggest that an immunogenetic factor is involved in the pathogenetic process of preeclampsia.

Preeclampsia is also found to be more commonly associated with twin pregnancies (Bulfin and Lawler 1957), with increased placental mass secondary to fetal hydrops (Scott 1958), with the trophoblastic tumor, hydatidiform mole (Page 1939), and with several medical conditions such as, hypertension, diabetes mellitus, and collagen vascular disease (Roberts 1989). These obstetrical and medical risk factors related to increased occurrence of preeclampsia suggest that a direct and/or an indirect decrease in placental perfusion plays an important role in the development of preeclampsia. Furthermore, all attempts to establish an animal model of preeclampsia have involved experimental reduction in uterine perfusion (Berger and Cavanagh 1963, Hodgkinson et al. 1967, Abitbol 1977).

How does this poor placental perfusion cause a multisystem disease? Recent research suggested that it is mediated by systemic endothelial cell damage (Roberts et al. 1991), although the placenta-derived endothelial toxin has not been identified. The evidence for endothelial cell damage and the possible mechanisms mediating it, have been reviewed in Chapter One. Insight into the role of endothelial function and NO in physiological processes has stimulated a surge of studies on **the role of endothelium and NO in the pathogenesis of preeclampsia.**

Our results show that chronic infusion of L-NAME induces an increase in MAP. We also found that, whereas blood volume increases during normal pregnancy, chronic infusion of

L-NAME reduces blood volume in both pregnant and virgin rats. However, pregnant animals are more sensitive to the L-NAME. This decrease in blood volume is unlikely to be related directly to water intake, urine output or sodium excretion since L-NAME had no effect on these parameters. Finally, we demonstrated that L-NAME has no effect on plasma ANF and PRA levels in either pregnant or virgin rats, and decreases plasma ET levels during pregnancy. Our studies thus support the contention that inhibition of NO synthesis is involved in several pathophysiological processes of preeclampsia, such as the increase in blood pressure and the decrease in blood volume (Molnar et al. 1992, 1994; Yallampalli and Garfield 1993). However, it fails to reproduce the characteristic hormonal profile of preeclampsia. The controversial results in this field serve to highlight the need for further studies to identify the primary lesion in preeclampsia. However, given these limitations, abnormal pregnancy induced by NOS inhibition, may nevertheless serve as a useful animal model of preeclampsia.

Further studies will focus on following aspects of this research. Firstly, we are going to address the question as to **how the volume receptors are reset during pregnancy. Is the mechanism(s) also dependent upon endothelial function and NO synthesis?** In these series of animal experiments, the effect of chronic administration of L-NAME on the responsiveness of volume receptors to volume loading or direct atrial distention will be performed. Secondly, if inhibition of NO synthesis does indeed play a role in the second common pathogenetic step of preeclampsia, (systemic endothelial damage or dysfunction), it is quite possible that **NO synthesis may be involved in the regulation**

of trophoblast invasion (the first common pathogenetic step of preeclampsia) (Fig 3-1). The effect of L-NAME on trophoblast invasion will be studied both in vitro and in vivo.

It is becoming increasingly apparent that NO is intimately involved, not only in the physiological adaptations to normal pregnancy, but also in the pathophysiological changes associated with preeclampsia. This research, our recently obtained results presented in my thesis, and the experiments for the future, offer new and exciting insights into this most important field of physiology.

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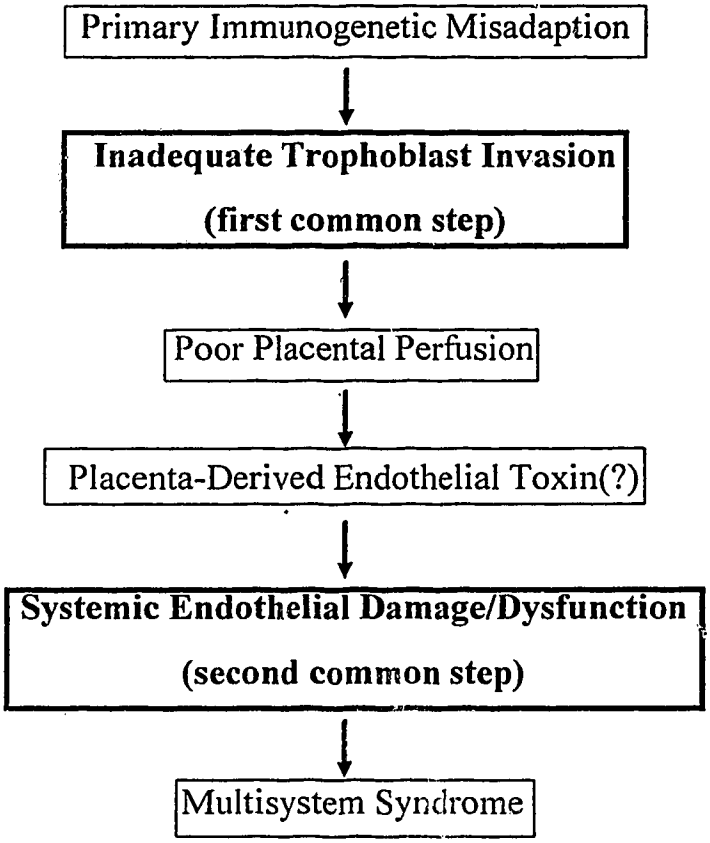

Fig 1. Pathogenetic Cascade of preeclampsia.

APPENDIX I

THEORY OF EVANS BLUE DYE DILUTION TECHNIQUE AND OUR PROCEDURES

The volume of fluid in the body can be measured by placing a substance into the body fluid compartment, allowing it to disperse evenly throughout the fluid in that compartment, and then measuring and calculating the concentration of the substance which has been diluted by the fluid in that compartment. This is based on the total mass of the substance after dispersion in the fluid will not change the amount. The volume of the tested compartment can be calculated by the equation:

Volume (ml) = quantity of test substance/concentration per ml of dispersed fluid.

Two groups of substances satisfy these conditions: radioisotope such as radioactive chromium (Cr^{51}) or radioactive idiom (I^{131} or I^{125}) and dye such as Evans Blue when they bind to either red blood cells or plasma proteins. Two most important properties of Evans Blue as for an indicator of dilution technique are: (1) it binds the plasma proteins immediately when it is injected into blood stream; (2) the concentration of it can be easily measured by spectrophotometer. Evans Blue dye dilution technique has been employed in pregnancy for almost half a century (Freis and Kenny 1948). Overall and Williams (1959) found this method to be as accurate as I^{125}-albumin, the accepted standard, but variability

may occur with the Evans Blue method, probably as a result of altered optical density in serum due to lipids and inadequate mixing time in circulation (Chesley 1978). We find that Evans Blue dye dilution technique is still the easiest, cheapest way to measure the plasma volume. It is practicable and reliable with a careful performance. Brown and his colleagues (1992) even employed a two-optical density method to overcome the short point of this method.

The procedures of this technique employed in this thesis are as follows:

Initial blood sample:

- Blood of 0.2 ml is drawn up into clean syringe via PE 50 line which is connected to the pedestal on the animal and set aside.
- A 0.3 ml blood sample is drawn with a clean dry needle/syringe.
- The original needle/syringe is replaced and the blood is reinjected.
- The blood sample is replaced with 0.3 ml heparinized saline.
- The blood is put into a heparinized Caraway tube, capped and centrifuged (11700 rpm for 5 min.).
- Hematocrit is measured in the tube. The tube is then cut at the interface of red blood cell and plasma.

Evans Blue dye injection:

- Solution of 0.5% weight/volume Evans Blue in normal saline.

- **Exactly** 0.3 ml of dye solution is injected through right femoral vein cannula (Do **not** rinse syringe).
- The dye needle/syringe is removed.
- The line is flushed with 2 ml of heparinized saline.

Timed blood samples for blood volume determination:

- All blood samples are taken from Silastic left femoral vein line at 10, 20, 30, 40 and 60 min. after injection of dye.
- Technique for sampleing is the same as initial sample except that:
    a. The actual sample is 0.15 ml instead of 0.3 ml.
    b. The line is cleaned with 0.15 ml of saline to replace the volume removed.
- Blood samples are placed in Caraway tubes, capped and numbered. Then samples are centrifuged.
- The tubes are treated as described above.

Analysis and calculations:

- Plasma analysis is done by spectrophotometry. Wave length is set at 605 nm.
- The standards curve is obtained by adding 0, 1 and 2 µl of the 0.5% Evans Blue solution to the initial plasma sample (50 µl) diluted in 950 µl saline.
- Plasma (50 µl) from the timed samples is diluted in 950 µl saline. Readings are plotted, extrapolated back to zero point and compared with standards.
- Plasma and blood volumes are calculated by following equations.

Plasma volume = (50 µl × 300 µl)/dye in sample

Blood volume = plasma volume/(1-corrected hematocrit)

Corrected hematocrit = hematocrit × 0.91 × 0.98

Note: In our study, the blood volume is calculated from the plasma volume and the hematocrit. Since the ratio of the blood cells to the plasma is about 10% higher in IVC than in the whole circulatory system, and blood trapping of the order of 2% occurs in the centrifuge (5 min. at 10,000g), a corrected hematocrit has been made by means of appropriate factors (Heimpel 1984).

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