

## BIOCHEMICAL MARKERS OF ADVERSE PREGNANCY OUTCOMES IN FETAL BLOOD

Aleksandra Nikolić-Đorđević

*Institute of Laboratory Diagnostics, Clinical Center »Novi Sad«, Novi Sad, Serbia*

**Summary:** Within the past decade, significant progress has been made with regard to improving maternal and newborn health. Biochemical markers in fetal blood are assessed after diagnostic cordocentesis (which was primarily collected for genetic screening), or immediately prior to fetal transfusion. Concentrations of the following diagnostic markers are measured in this study: endothelin-1, leptin, beta-2-microglobulin and the inflammatory marker IL-6. Endothelin-1 is a potent vasoconstrictor, induced by rising venous pressure and rising shear stress. Subjects involved in this study included women with anti-DRh alloimmunized pregnancies, and fetal blood sampled pre- and post-transfusions. Rapid expansion of fetal intravascular volume by intravenous transfusion of packed red blood cells with a high hematocrit enhances fetal endothelin levels. Beta-2-microglobulin is a ubiquitous cell surface protein, associated with the major histocompatibility complex. It is a potential marker of Graft-versus-Host Disease. The median concentrations of beta-2-microglobulin are significantly higher in fetuses with prior transfusions compared with non-anemic fetuses. Evaluation of fetal beta-2-microglobulin might prove useful in identifying fetuses with potentially severe Graft-versus-Host or Host-versus-Graft reaction to cell transplants. Leptin is a recently discovered circulating hormone that coordinates energy intake and expenditure in adults. Leptin levels in the umbilical cord blood positively correlate with neonatal birth weight, suggesting a role in adipose homeostasis *in utero*. In this study, leptin levels were measured in fetal and paired maternal plasma in the second half of gestation, in pregnancies complicated with Down syndrome and euploid pregnancies. In euploid pregnancies, fetal leptin levels were significantly lower than in corresponding maternal values, but increased across gestation. Down syndrome was associated with significantly lower fetal leptin levels. It is possible that lower fetal leptin levels could reflect the persistent immaturity of the pattern of placental peptide hormone synthesis in fetal Down syndrome. Recent evidence strongly implicates the inflammatory response to intrauterine infection in the pathogenesis of neonatal brain and lung injury. The frequency and clinical significance of systemic inflammatory responses were defined by elevated plasma interleukin-6 concentrations in fetuses with preterm labor. A fetal plasma interleukin-6 cut-off value of 11 pg/mg was used to define the presence of a systemic inflammatory response.

**Key words:** fetal blood, endothelin-1, beta-2-microglobulin, leptin, interleukin-6

### Introduction

Within the past decade, significant progress has been made with regard to improving maternal and newborn health. Recent developments in fetal diagnostics warrant a need for *in utero* biochemical markers to:

- guide clinical interventions;
- assess fetal response to such interventions (e.g. fetal transfusions);
- identify *in utero* environmental factors that play a role in susceptibility to conditions, such as early onset of cardiovascular disease (CVD), type-2 diabetes mellitus and hypertension; and
- measure markers of metabolic derangements in fetal blood.

Clinical interventions in fetuses are often associated with stress that may compromise fetal well-being or survival. Some fetuses may be more susceptible to

*Address for correspondence:*

Aleksandra Nikolić-Đorđević  
Clinical Center – Novi Sad  
Institute of Laboratory Diagnostics  
Hajduk Veljkova 1–7  
21000 Novi Sad, Serbia

such stress. Measuring certain biochemical parameters in fetal blood may help to identify those fetuses that are at increased risk.

Fetal blood analyses are done mainly in advanced centers for obstetrics as part of research studies, after diagnostic cordocentesis (primarily collected for genetic screening), or immediately prior to fetal transfusion.

### Placenta

Placenta is a specialized organ that connects the mother and fetus, and has various functions: it provides the fetus with oxygen and nutrients, and removes waste, such as carbon dioxide, via the umbilical cord. Moreover, it also has a complex endocrine function: it produces many hormones, cytokines, growth factors and other substances able to either inhibit or stimulate placental activity. For example, much like the hypothalamus and other parts of the brain, as well as endocrine tissues, the placenta produces gonadotropin-releasing hormones (GnRH), corticotropin-releasing hormone (CRH), and thyrotropin-releasing hormone (TRH), somatostatin, neuropeptide Y, dinorphin, interleukin-1, tumor necrosis factor alpha (TNF-alpha), epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). In place of gonads, it produces progesterone and estrogens; and it also produces human chorionic gonadotropin (hCG), human placental lactogen (hPL), and adrenocorticotrophic hormone (ACTH) instead of the pituitary gland. Furthermore, the placental production of some pregnancy-associated proteins, such as specificity protein-1 (SP-1), pregnancy-associated plasma protein-A (PAPP-A) and placental protein-5 (PP5), has been demonstrated (1). These proteins circulate in the maternal and fetal bloodstream and are considered markers for the risk of miscarriage, preterm birth and pregnancy complications. The placenta is also a selective barrier that protects against some microorganisms, although other viruses or parasites (e. g. rubella, cytomegalovirus and toxoplasma) can pass via unknown mechanisms and injure the fetus (2).

### Sample collection

Fetal umbilical blood samples described in this study were obtained during diagnostic cordocentesis (which was primarily collected for genetic screening), or immediately prior to transfusion in anemic fetuses. Cordocenteses were performed under strict aseptic conditions using a 15 cm spinal needle, which was guided via sonogram to the umbilical cord at the placental insertion. After satisfactory umbilical vein access was achieved, 3-4 mL of pure fetal blood were obtained for biochemical, hematological and genetic analysis. Samples of fetal blood were centrifuged at 2000g for 10 min (at +4 °C for the cytokine assay and leptin

assay). A 1 mL aliquot of fetal serum (EDTA-plasma in some studies) was obtained and stored at -70 °C. Endothelin was assayed by ELISA, beta-2-microglobulin was assayed by ELISA, leptin was assayed by RIA, interleukin-6 was also assayed by ELISA. As indicated, measurements in some studies were done in paired maternal blood.

Statistical analyses were performed using the Statgraphics (version 3.0) statistical software package (STSC, Rockville, USA) and the JPM statistical software package (version 4.0.4., SAS Institute, Cary, NC, USA) for leptin.

### Preliminary findings

Analysis of fetal blood encompassed a number of biochemical tests. In case of Rh alloimmunization and fetal transfusion, besides hematological fetal parameters, serum iron, ferritin and total and direct bilirubin were measured. Beta-2-microglobulin was analyzed in cases of fetal transfusions, before and after the transfusions, as a potential marker for Graft-versus-Host Disease (GVHD). The hormones cortisol and ACTH, as well as markers for oxidative stress, were monitored in studies that focused on fetal responses to particular clinical interventions. Inflammatory markers (cytokines, interleukin-1 alpha, interleukin-6, interleukin-8 and TNF) were measured in studies examining the prognostic relevance of biochemical parameters in pregnancy outcomes—preterm labor, as pulmonary and neurological complications of premature babies.

A special challenge in analyzing fetal markers is the prevention of Intrauterine Growth Retardation (IUGR), in view of the complexity of causative pathophysiological mechanisms leading to IUGR. Common problems in all studies trying to determine fetal blood metabolites are the relatively small number of available samples and the inability to define reference values, especially considering that the samples are collected during various points of gestation (between the 20<sup>th</sup> and 37<sup>th</sup> week).

Values of fetal blood metabolites are expressed, in comparison to control:

- in cases of fetal transfusion, before and after the transfusion,
- in cases when ultrasonogram or genetic testing identifies fetal abnormalities, in comparison to healthy fetuses,
- in cases when fetal blood samples are received during the last trimester of gestation, by comparing fetal and neonatal metabolites.

Concentrations of the following diagnostic markers were measured in this study:

- endothelin,
- leptin,
- beta-2-microglobulin,
- inflammatory markers IL-1-beta, IL-6, TNF.

### Endothelin in fetal transfusions

Endothelin is a potent vasoconstrictor, induced by rising venous pressure and rising shear stress. The relative potency and interrelationship among vasoactive and natriuretic mediators are thought to be important in the transition from fetal to neonatal life. Cord levels of endothelin-1 were considerably higher comparing with normal adults' values (3).

Subjects involved in this study included women with anti-DRh alloimmunized pregnancies, and fetal blood sampled before and after transfusions. Endothelin-1 was assayed by ELISA (Figure 1).

Fetal endothelin-1 concentrations are significantly higher after transfusion if the transfusion is done before the gestational age of 28 weeks; between 28-31 gestational weeks it is significantly lower, while between weeks 32-35 it remains in the same range as before transfusion (4). A study (Radunović et al.) done on 34 transfused fetuses claims that post-transfusion endothelin levels correlated significantly with the volume of transfused blood and with post-transfusional increases in umbilical vein pressure. Rapid expansion of fetal intravascular volume by intravenous transfusion of packed red blood cells with a high hematocrit enhances fetal endothelin levels (5).

### Beta-2-microglobulin in fetal blood

Beta-2-microglobulin is a ubiquitous cell-surface protein, associated with the major histocompatibility complex. It is a potential marker of Graft-versus-Host Disease (GVHD) (6). Beta-2-microglobulin is detectable on the surface of nearly all cell types with the exception of erythrocytes and trophoblasts (6). Intrauterine transfusions for rhesus alloimmunization lead to alterations in circulating T-cell populations. Given that elevations in circulating beta-2-microglobulin are a marker of T-cell mediated transplant rejection, the effect of intrauterine transfusions on beta-2-microglobuline was evaluated.

Subjects used for this study included blood collected from fetuses in D-Rh alloimmunized women vs. those from normal pregnancies. Umbilical venous samples were obtained immediately prior to initial transfusions and in fetuses with prior transfusions. Control groups were nonanemic fetuses and healthy neonates. Beta-2-microglobulin was measured by ELISA (Figure 2 and 3).

The median concentrations of beta-2-microglobulin are significantly higher in fetuses with prior transfusions, compared with nonanemic fetuses. Among anemic and transfused fetuses, beta-2-microglobulin levels showed a negative correlation with the fetal hematocrit. Intrauterine transfusion as a treatment for fetal anemia is associated with increased beta-2-microglobulin levels, suggesting that short-term immunomodulatory effects of intrauterine

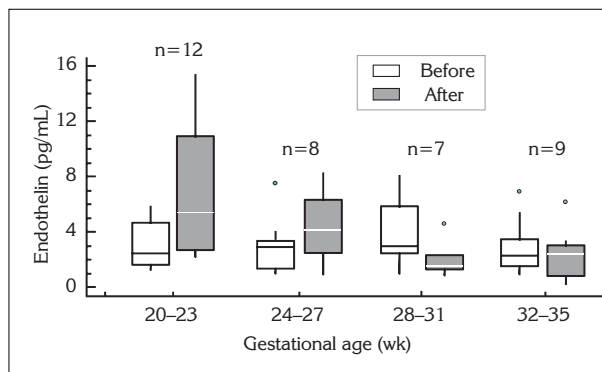


Figure 1 Fetal endothelin concentrations pre- and post-transfusion across gestation (from reference 4)

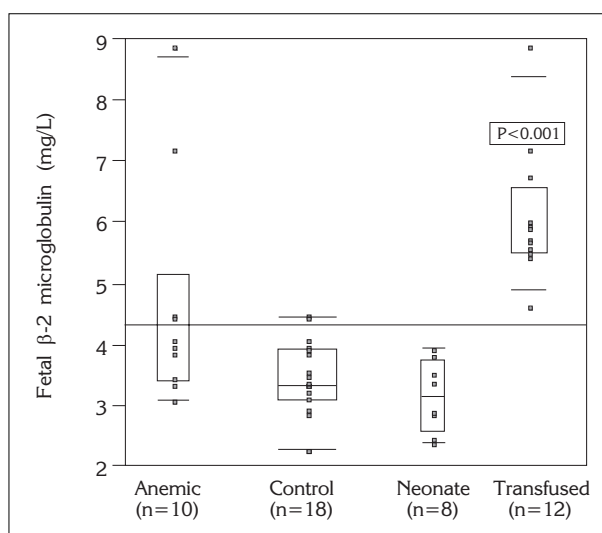


Figure 2 Fetal beta-2-microglobulin concentration in anemic, normal and transfused pregnancies and in neonates (from reference 7)

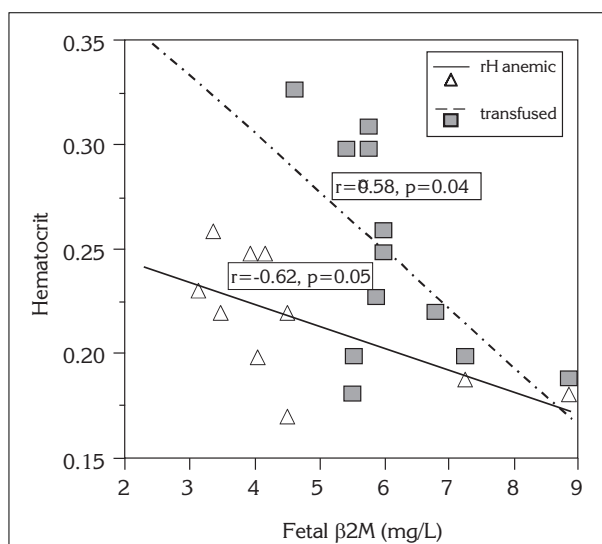


Figure 3 Correlation of beta-2-microglobuline with hematocrit in anemic and transfused fetuses (from reference 7)

transfusion on host immune responses donor leukocyte antigens. This beta-2-microglobulin increase was not of the same magnitude in all transfused fetuses, probably as a result of variations in the number of leukocytes transfused, HLA-DR sharing between donor and fetus, or other antigenic differences related to the transfusion. Since the elevation of beta-2-microglobulin has been suggested as a potential marker of transplant rejection, evaluation of fetal beta-2-microglobulin might prove useful in identifying fetuses with potentially severe Graft-versus-Host or Host-versus-Graft reaction to cell transplants (7, 8).

### Leptin in fetal blood

Leptin is a recently discovered circulating hormone that coordinates energy intake and expenditure in adults through its interaction with specific receptors located in the central nervous system and peripheral tissues (9). It is a regulator of lipid and glucose homeostasis and a growth regulator. It is produced in the white adipose tissue, stomach, muscle and placenta. Maternal plasma leptin levels rapidly decrease after the delivery of placenta, suggesting that the placenta is a significant source of circulating leptin. Leptin levels in the umbilical cord blood positively correlate with neonatal birth weight, suggesting a role in adipose homeostasis *in utero*.

Fetal Down syndrome has been associated with abnormal lipid metabolism. Obese adults with Down syndrome have lower leptin levels, suggesting that deficient leptin expression contributes to obesity in these individuals. The hypothesis was that fetuses with Down syndrome would similarly display lower leptin levels, and that it could serve as a potential biochemical marker.

In this study, leptin levels were measured by the RIA method in fetal and paired maternal plasma in the second half of gestation, in pregnancies complicated with Down syndrome (n=9) and euploid pregnancies (n=30) (Figure 4 and 5).

In euploid pregnancies, fetal leptin levels were significantly lower than in corresponding maternal values, but increased across gestation. This finding may reflect either rising fetal and maternal adipose content or increasing placental mass. However, Hoggard et al demonstrated that only 15% of circulating maternal leptin levels was derived from placental leptin production (11). Down syndrome was associated with significantly lower fetal leptin levels. It is

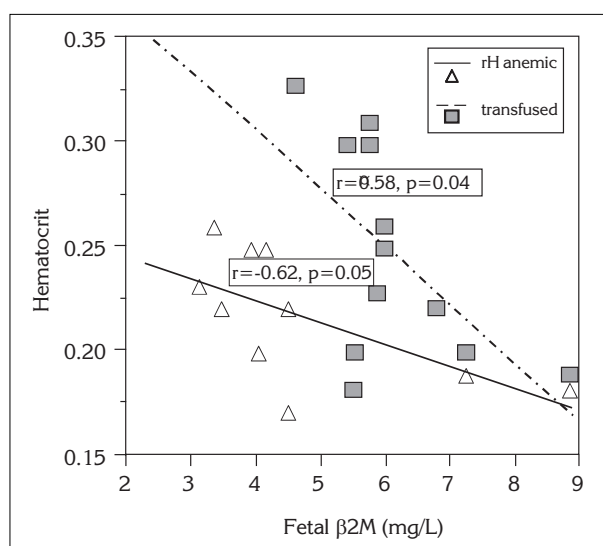


Figure 5 Correlation of fetal leptin concentrations across gestation in Down syndrome and normal pregnancies (from reference 10)

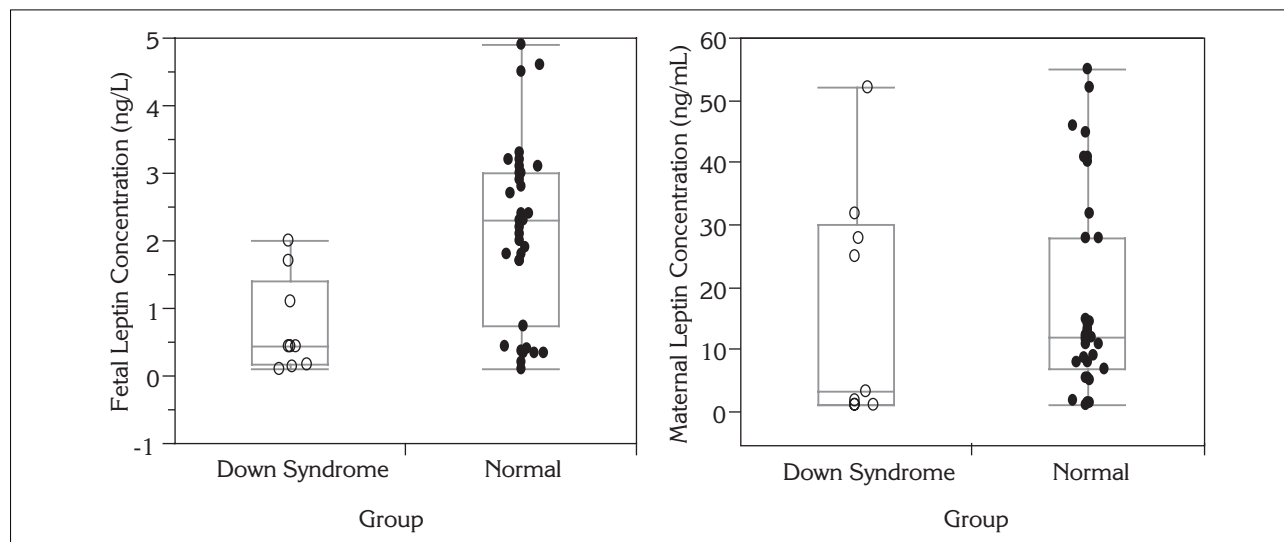


Figure 4 Fetal and maternal leptin concentrations in normal and Down syndrome fetuses (from reference 10)

possible that lower fetal leptin levels could reflect the persistent immaturity of the pattern of placental peptide hormone synthesis in fetal Down syndrome. Unfortunately, since placental leptin contributes only 15% to the maternal pool, this reduced Down syndrome-associated placental production does not result in enough change in maternal plasma leptin to make it an effective predictor of Down syndrome fetuses.

### **Inflammatory markers in fetal blood**

Premature birth is the leading cause of perinatal morbidity and mortality in the world, and the second leading cause of infant mortality in the USA (12). There is accumulating evidence that intrauterine infection is a major factor in preterm labor and preterm premature rupture of the membranes, particularly in gestations of <30 wk duration (12, 13).

Intrauterine infection has also been implicated in the pathogenesis of adverse neonatal sequelae. Recent evidence strongly implicates the inflammatory response to intrauterine infection in the pathogenesis of neonatal brain and lung injury (14).

The study of Gomes et al (12) determined the frequency and clinical significance of a systemic inflammatory response as defined by an elevated plas-

ma interleukin-6 concentration in fetuses with preterm labor. In this study, cordocenteses were performed together with amniocenteses in 157 patients with premature labor and PROM (Premature Rupture of Membranes). Fetal plasma interleukin-6 concentrations were measured by ELISA, and amnion was cultured for aerobic and anaerobic bacteria. The overall prevalence of severe neonatal morbidity was 34 (5%). A fetal plasma interleukin-6 cut-off value of 11 pg/mg was used to define the presence of a systemic inflammatory response. Fetuses with fetal plasma interleukin concentrations >11 pg/mL had a higher rate of severe neonatal morbidity than those with fetal plasma interleukin-6 levels ≤11 pg/mL (14). A systemic fetal inflammatory response, as determined by an elevated fetal plasma interleukin-6 value, is an independent risk factor for the occurrence of severe neonatal morbidity. The study of Viscardi et al (15) claims that elevated fetal IL-6 of >17 pg/mL was associated with abnormal cranial ultrasound in infants >28 wk GA. The study claims that in preterm infants <28 systemic inflammatory responses may involve variations by the brain injury. Because intrauterine infection/inflammation is a major factor in preterm birth and adverse sequelae in gestation, intervention strategies should focus on the early identification of women at high risk and the development of drugs to modulate the inflammatory response (15).

## **BIOHEMIJSKI MARKERI ISHODA TRUDNOĆE U FETALNOJ KRVI**

*Aleksandra Nikolić-Đorđević*

*Institut za laboratorijsku medicinu, Klinički centar – Novi Sad, Srbija*

*Kratak sadržaj:* Tokom poslednjih deset godina postignut je značajan napredak u oblasti poboljšanja maternalno-fetalnog zdravlja. Biohemijska i hematološka ispitivanja fetalne krvi obuhvataju veliki broj parametara i vrše se iz umbilikalne krvi nakon dijagnostičke kordocenteze (radi genetskog ispitivanja) ili iz pre-transfuzionog uzorka fetalne krvi. U ovom radu je dat prikaz nekoliko dijagnostičkih markera analiziranih u fetalnoj krvi: endotelina-1, leptina, beta-2-mikroglobulina i inflamatornog markera interleukin-6. Endotelin-1 je vazokonstriktor na čiju indukciju utiču povećanje venskog krvnog pritiska i stanje stresa. Studija je obuhvatila uzorke pre i posle transfuzije fetalne krvi u slučajevima intrauterine transfuzije kod Rh-aloimunizovanih hemolitičkih fetalnih anemija. Povećanje fetalnog intravaskularnog volumena nakon fetalne transfuzije eritrocita sa visokim hematokritom podiže vrednosti nivoa endotelina-1 u fetalnoj krvi nakon inicijalne ali ne i nakon ponovljene transfuzije. Beta-2-mikroglobulin je proteinski molekul prisutan na ćelijskoj površini, povezan sa glavnim histokompatibilnim kompleksom. Nivo beta-2-mikroglobulina je takođe praćen u slučajevima intrauterine transfuzije kod Rh-aloimunizovanih fetalnih anemija pre i posle transfuzije. Koncentracije beta-2-mikroglobulina značajno su više kod fetusa sa prethodnom transfuzijom u odnosu na neanemične fetuse. Određivanje beta-2-mikroglobulina može se pokazati korisnim u identifikaciji fetusa sa potencijalno ozbiljnijim efektom Graft-versus-Host reakcije na ćelijske transplantate. Povećan nivo beta-2-mikroglobulina nakon transfuzije sugerše imunomodulatorni efekat intrauterine transfuzije (leukocitnih antigena donora) na fetalni imuni odgovor. Leptin je cirkulišući hormon koji koordinira unos i potrošnju energije u organizmu. U ovoj studiji, leptin je praćen u fetalnoj i maternalnoj krvi nakon genetskog »skrininga« u normalnoj euploidnoj trudnoći i trudnoći sa Daunovim

sindromom. U normalnoj trudnoći, fetalne vrednosti leptina su značajno niže u odnosu na majčinu krv, ali se nivo tokom gestacije povećava. Daunov sindrom povezan je sa značajno nižim vrednostima u fetalnoj krvi. Moguće je da niže vrednosti leptina odražavaju postojeću nezrelost hormonske sinteze na nivou placente koja je prisutna u fetalnom Daunovom sindromu. Novije studije ukazuju da antiinflamatorni odgovor na intrauterinu infekciju predstavlja najznačajniji razlog prevremenog porođaja, te oštećenja fetalnih organa. Inflammatory citokin interleukin-6 praćen je kao potencijalni marker prevremenog porođaja kod prisutne infekcije amnijske tečnosti kao najznačajnijeg faktora rizika. Vrednost IL6 > 11 pg/mL označena je kao *cut-off* vrednost povezana sa povećanim rizikom prisustva sistemskog inflamatornog odgovora.

*Cljučne reči*: fetalna krv, endotelin-1, beta-2-mikroglobulin, leptin, interleukin-6

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*Received: July 15, 2006*

*Accepted: August 23, 2006*