The differential effects of the timing of maternal nutrient restriction in the ovine placenta on glucocorticoid sensitivity, uncoupling protein 2, peroxisome proliferator-activated receptor-γ and cell proliferation

M Yiallourides, S P Sebert, V Wilson, D Sharkey, S M Rhind, M E Symonds and H Budge

Early Life Nutrition Research Unit, Academic Child Health, School of Clinical Sciences, University Hospital, Nottingham NG7 2UH, UK and 1Macaulay Institute, Craigiebuckler, Aberdeen AB15 8QH, UK

Correspondence should be addressed to M E Symonds; Email: michael.symonds@nottingham.ac.uk

Abstract

Nutrient restriction (NR) during critical windows of pregnancy has differential effects on placento-fetal growth and development. Our study, therefore, investigated developmental and metabolic adaptations within the ovine placenta following NR at different critical windows during the first 110 days of gestation (term = 147 days). Thus, the effects of NR on cell proliferation, glucocorticoid sensitivity, IGF1 and 2 receptor, peroxisome proliferator-activated receptor γ (PPARG), and uncoupling protein (UCP)2 gene expression in the placenta were examined. Singleton bearing sheep (n = 4–8 per group) were fed either 100% of their total metabolizable energy requirements throughout the study or 50% of this amount between 0–30, 31–65, 66–110, and 0–110 days gestation. A significant reduction in cell proliferation and increased gene expression for the glucocorticoid and IGF2 receptors, PPARG, and UCP2 were detected in placentae sampled from mothers who were nutrient restricted between days 66 and 110 of gestation, only, relative to controls. This window of gestation coincides with the maximum placental growth and the start of exponential growth of the fetus when there are substantially increased metabolic demands on the placenta compared with earlier in gestation. Consequently, increased glucocorticoid sensitivity and suppressed IGF2 action could contribute to a switch in the placenta from proliferation to differentiation, thereby improving its nutrient transfer capacity. Upregulation of PPARG and UCP2 would promote placental fatty acid metabolism thereby limiting glucose utilization. These compensatory placental responses may serve to maintain fetal growth but could result in adverse adaptations such as the early onset of the metabolic syndrome in later life.


Introduction

The placenta’s capacity to transport nutrients is essential for the growing fetus and is dependent on its size, morphology, endocrine, and metabolic function (Fowden et al. 2008). Maternal nutrient restriction (NR) during early-to-mid-gestation significantly affects placental growth and morphology (Clarke et al. 1998, Heasman et al. 1998a), causing a reduction in the weight of the fetal component of the placentome (Clarke et al. 1998). At the same time placental sensitivity to glucocorticoids is raised in conjunction with reduced cell proliferation, downregulation of insulin-like growth factor (IGF1) receptor gene expression while that of uncoupling protein (UCP)2 is upregulated (Gnanalingham et al. 2007). These placental adaptations to maternal NR, would be expected to alter fetal nutrient supply (Fowden et al. 2008) and therefore potentially predispose the offspring to cardiovascular and metabolic health problems in later life (McMillen & Robinson 2005).

Glucocorticoids play a pivotal role in placento-fetal development, promoting tissue maturation and differentiation (Fowden et al. 1998) and suppressing cell proliferation (Fernandes et al. 1999). Placental sensitivity to glucocorticoids is regulated by the glucocorticoid receptor (GR) together with the activity of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) that inactivates cortisol to cortisone (Edwards et al. 1996). IGF2 also has a key role in placental development by regulating proliferation, differentiation, and fetal nutrient supply (Fowden 2003). The action of IGF2 is modulated by both the IGF1 receptor, which promotes the biological activities of IGF2, and the IGF2 receptor, which binds to IGF2 resulting in its intracellular lysosomal degradation (Fowden 2003).

Placental metabolism is primarily dependent on glucose and oxygen availability (Meschia et al. 1980,
Reproduction or the fetal to placenta ratio at day 110 (Table 1). At the Maternal NR during the specific stages of gestation had Results

Hay 1995). Following maternal NR, maternal glucose concentrations can be reduced (Heasman et al. 1998a) and plasma non-esterified fatty acids (NEFA) increased (Heasman et al. 1998a, Bispham et al. 2007). While placental transfer of NEFA to the fetus in sheep is normally limited (Hull et al. 1979), when nutrient supply is constrained they may play a role in placental metabolism in conjunction with increased uptake of ketone bodies (Chandler et al. 1985). One factor that could regulate oxidative metabolism in the placenta is the mitochondrial UCP2 by promoting NEFA oxidation in preference to carbohydrate (Pecqueur et al. 2008). Any change in placental metabolism could be further influenced by the action of peroxisome proliferator-activated receptors (PPARs; Nunn et al. 2007) of which PPARγ (PPARG) is highly expressed in the placenta (Schaiff et al. 2006).

Maternal NR between days 28 and 80 of gestation promotes gene expression of both UCP2 and the GR in the placenta, but the extent to which this adaptation may be dependent on the timing of nutritional intervention is unknown. Both epidemiological and animal studies demonstrate that the timing of maternal NR has differential long-term effects on the health of the offspring (Roseboom et al. 2006, Symonds et al. 2007). In this regard, the placenta is an important modulator of the intrauterine environment and has a key role to play in mediating the programming effects of maternal NR (Fowden et al. 2008). Placental development in sheep comprises three distinct critical windows: embryogenesis occurring within the first 30 days of mating; a period of rapid placental growth with peak weight occurring between days 75 and 80 (Ehrhardt & Bell 1995); and a period of extensive vascular remodeling and a decline, of up to 30%, in weight for the remainder of the pregnancy (Schneider 1996). We hypothesized that maternal NR during these critical windows would result in differential effects on placental development and metabolism. Specifically, the primary aim of the study was to investigate the effects of maternal NR on modulators of placental growth and nutrient supply. In view of the potential switch in placental metabolism during maternal NR, the secondary aim of the study was to investigate the effects of maternal NR on genes involved in placental lipid metabolism.

Results

Maternal NR during the specific stages of gestation had no effect on the total weight of the placenta, fetal weight, or the fetal to placenta ratio at day 110 (Table 1). At the end of each period of maternal NR, there was a decline in maternal weight in all groups compared with their body weight at the start of the period of NR with the exception of the group of animals that were nutrient restricted between days 66 and 110 (NR 0–30 days, -7.2±2.1%, P<0.05; NR 31–65 days, -11.2±2.0%, P<0.05; NR 0–110 days, -24.8±2.0%, P<0.001). However, there was only a significant difference in maternal body weight between conception and day 110 of gestation in those animals nutrient restricted throughout gestation (Table 1).

Proliferating cells within the placenta as determined by proliferating cell nuclear antigen (PCNA) staining indicated these were present in both the maternal caruncular and fetal cotyledon components primarily around the luminal epithelium and glandular endothelium (Fig. 1A–C). Following maternal NR from 66 to 110 days of gestation, placental cell proliferation had decreased in both the maternal caruncular (P<0.05, Fig. 1D and E) and the fetal cotyledonary components of the placentome (Fig. 1F).

Placental gene expression of the GR was increased only following maternal NR between days 66 and 110 (Fig. 2A) and this was not accompanied by any change in mRNA abundance for HSD11B2 (Fig. 2B). IGF2R (Fig. 2C), PPARG, and UCP2 mRNA abundance were also specifically upregulated following NR between days 66 and 110 (Fig. 3). There was no effect of maternal NR at any time point on gene expression for IGF1, its receptor, or IGF2 (data not shown).

Discussion

The aim of this study was to investigate the endocrine and metabolic adaptations within the ovine placenta following maternal NR during different critical windows of placento-fetal development. We have, therefore, established that significant effects were only seen when maternal NR commenced at day 66 of gestation and was continued to the end of the study, i.e. day 110 of gestation. This 45-day period of NR reduced placental cell proliferation, without any effect on total placental mass. At the same time, there was an increase in glucocorticoid sensitivity (as indicated by increased GR mRNA abundance), whereas IGF2 activity was suppressed, following upregulation of the IGF2R. These adaptations were accompanied by the upregulation of a number of genes involved in lipid sensing and metabolism. Importantly, placental responses to maternal NR were coincident with the time of maximal placental growth (Ehrhardt & Bell 1995) and the beginning of the period of placental growth. Such adaptations to NR may be beneficial for the survival of the fetus during a time of high metabolic demands but could alter the intrauterine environment to reset fetal organ growth and predisposing the offspring to metabolic disease in later life (McMillen & Robinson 2005, Sebert et al. 2009, Sharkey et al. 2009). The comparative importance of our findings is emphasized by the close similarity in the growth curves of the placenta and the fetus between sheep and humans. In both species, placental growth is most rapid between early-to-mid-gestation and is then
followed by an exponential period of fetal growth in late gestation (Heasman et al. 1998b).

In previous studies, maternal NR during early-to-mid-gestation (30–80 days) resulted in the reduction of weight of the fetal component, but not the total placenta, immediately after the period of NR (Clarke et al. 1998). Previously, when nutrient-restricted sheep were subsequently fed to requirements from mid-gestation (80 days to term), the weights of the fetal component of the placenta, as well as that of the whole placenta weight, increased compared with adequately fed controls (Clarke et al. 1998, Heasman et al. 1998a). In the present study, we did not detect a difference in the total placental weight following NR at each of the different gestational windows but found a marked loss of cell proliferation throughout the placenta following maternal NR between days 66 and 110. In addition, fetal weight and fetal-to-placental weight ratio, a measure of placental efficiency, were unaffected by any of the periods of maternal NR in the present study. The most probable explanation for the lack of any effect on placental weight in the current study is that not only were the periods of nutritional intervention of shorter duration (with the exception of those mothers who were nutrient restricted through out the entire 110-day study period), but placent al sampling was undertaken at day 110 gestation, instead of day 80 and is thus within the period in which the placental weight starts to decline (Stegmann 1974, Ehrhardt & Bell 1995).

Glucocorticoids are important modulators of placentofetal growth and excessive exposure during later stages of pregnancy results in a reduction in placental and fetal weight with an overall increase in fetal-to-placenta weight ratio (Jensen et al. 2002). Maternal NR between days 66 and 110 increased placental sensitivity to glucocorticoids by upregulation of GR mRNA abundance, but had no effect on HSD11B2 gene expression. By contrast, in previous studies maternal NR during early-to-mid-gestation resulted in a persistent upregulation of GR, whereas the abundance of HSD11B2 mRNA was reduced (Whorwood et al. 2001, Gnanalingham et al. 2006). One factor that appears to determine the magnitude of change in placenta glucocorticoid action following maternal NR is the reduction in plasma cortisol (Whorwood et al. 2001, Jaquiery et al. 2006, Rumball et al. 2008). Consequently, reduced maternal cortisol with NR appears to be amplified in early, but not later, gestation, thereby explaining the absence of any reduction in placental HSD11B2 when food intake is reduced later in gestation. This could also explain the lack of any effect on fetal plasma cortisol with maternal NR, irrespective of its timing (Brameld et al. 2000, Edwards & McMillen 2001, Jaquiery et al. 2006). The main outcome of increased GR abundance appears to be a suppression of cell proliferation (Fernandes et al. 1999) that in the placenta may act to promote placental remodeling and differentiation (Fowden et al. 1998). It is also established that, in the

![Table 1 The effect of maternal nutrient restriction (NR; 50% of metabolizable energy) during different gestational windows on maternal, placental, and fetal weights at day 110 of pregnancy in the sheep.](image)

<table>
<thead>
<tr>
<th>Maternal nutrient group (days of gestation of NR)</th>
<th>Control</th>
<th>0—30 days</th>
<th>31–65 days</th>
<th>66–110 days</th>
<th>0–110 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal weight change (%)</td>
<td>1.7 ± 2.2</td>
<td>3.7 ± 3.1</td>
<td>−1.9 ± 2.6</td>
<td>−7.2 ± 0.7</td>
<td>−22.8 ± 2.0*</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>632 ± 59</td>
<td>670 ± 40</td>
<td>636 ± 38</td>
<td>639 ± 17</td>
<td>697 ± 63</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>2048 ± 57</td>
<td>2198 ± 47</td>
<td>2226 ± 80</td>
<td>2030 ± 41</td>
<td>1961 ± 99</td>
</tr>
<tr>
<td>Fetal/placental ratio</td>
<td>3.4 ± 0.4</td>
<td>3.4 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td>2.9 ± 0.3</td>
</tr>
</tbody>
</table>

Data represents means ± S.E.M.; n=4–8 animals/group. *P<0.001 compared to controls. NR, nutrient restriction.

![Figure 1 Quantitative immunohistochemistry of proliferating cell nuclear antigen (PCNA) in the ovine placenta. (A–C) PCNA positive nuclei (stained brown) are localized in both the maternal caruncular (MC) and fetal cotyledon (FC) components of the placentome particularly surrounding the maternal luminal epithelium (LE) and endothelial glands (EG). (D) The effect of maternal nutrient restriction during different gestational windows on PCNA expression in the maternal caruncular component of the placentome. (E and F) Representative placentome micrographs (×40) from sheep that were nutrient-restricted from 66 to 110 days of gestation. MS, maternal stroma.](image)
We found no effect on placenta IGF1 or 2 mRNA abundance which is in accord with other studies (Osgerby et al. 2002, McMullen et al. 2005). In the present study, NR between days 66 and 110 gestation enhanced IGF2R mRNA abundance, whereas no effect was seen on IGF1R. Gene expression of IGF1R was raised in the fetal kidney in the absence of any difference in kidney weight or nephron number (Brennan et al. 2005). It is, therefore, proposed that these placenta-fetal adaptations within the IGF axis could ensure that fetal growth and development are maintained despite a reduction in maternal food intake. The overall placental response to a period of maternal NR commencing later in pregnancy is a switch from cell proliferation to differentiation while placenta nutrient transfer capacity is maintained (Dandrea et al. 2001, Fowden et al. 2006). Of further interest is the absence of any significant effects within the placenta when maternal NR was applied from the time of mating until day 110 of gestation. Taken together, these findings indicate that if the level of food intake is kept constant through pregnancy and the mother is able to meet any energy deficit from mobilization of her own body reserves, placenta responses may not be required. However, an increase

placenta, gene expression of the GR is positively correlated with UCP2 and may thus be one underlying mechanism by which changes in placental metabolism are mediated (Gnanalingham et al. 2007).

Placental adaptations could be further modulated by changes in IGF action, of which IGF2 is the dominant factor regulating placento-fetal growth (Fowden 2003). During maternal NR, maternal and fetal IGF1 concentrations are either decreased (Bauer et al. 1995, Osgerby et al. 2002, Bispham et al. 2003) or unchanged (Quigley et al. 2008, Sosa et al. 2009), while maternal and fetal IGF2 concentrations remain unchanged (Bauer et al. 1995, Quigley et al. 2008).
in fetal fat mass is seen in this group (Brennan et al. 2005), that may be indicative of other adaptations within the placento-fetal unit that ultimately contribute to a marked change in adipose tissue function in later life, especially following obesity (Sharkey et al. 2009).

Placenta metabolism is primarily dependant on the availability of oxygen and glucose (Hay 1995). During maternal NR in sheep, maternal glucose concentrations are either unchanged (Edwards & McMillen 2001, Bispham et al. 2003, Sosa et al. 2009) or reduced (Heasman et al. 1998a, Osgerby et al. 2002, Quigley et al. 2008), whereas plasma NEFA concentrations are consistently raised (Chandler et al. 1985, Bauer et al. 1995, Heasman et al. 1998a, Brameld et al. 2000, Bispham et al. 2003, Quigley et al. 2008, Sosa et al. 2009). Fetal plasma NEFA concentrations, however, remain unchanged (Bauer et al. 1995) as transfer of NEFA across the ovine placenta is limited (Hull 2009). If raised, maternal NEFA were accompanied by reduced utero-placental oxygen uptake (Chandler et al. 1979), that may be indicative of other adaptations that would be detrimental to the long-term metabolic health of the offspring (Sebert et al. 2009, Sharkey et al. 2009).

Materials and Methods

Animals

A total of 59 mature Scottish Blackface sheep of similar body condition scores (range 2.0–3.0, on a scale of 0–5; Russel 1984) were randomly allocated to one out of five nutritional treatment groups at mating. They were housed individually under normal lighting conditions. At day 80 of gestation, fetal number was determined by ultrasound. Only singletons (n=30) were included in the present study. All sheep had weight and body condition score assessments at regular intervals throughout the study as previously reported (Rae et al. 2001). Pregnant sheep were killed by barbiturate overdose (Euthatal, 500 mg/ml, 30 ml i.v.; Rhone Merieux, Harlow, UK) on day 110 gestation. The total placental mass was calculated by dissecting and weighing individual placenomes. Representative placentomes from each sheep were randomly selected, frozen immediately in liquid nitrogen and stored at −80°C for later analysis. Additional placentomes were dissected and placed into 10% formalin and embedded in paraffin wax for histological analysis. All experimental procedures had the required Home Office approval as designated by the Animals (Scientific Procedures) Act (1986) and were performed under UK legislation.

Maternal diet

Sheep were fed diets estimated to provide the metabolizable energy (ME) requirement for live weight maintenance (∼8.0 MJ/ME per day) or 50% of that amount. The diet comprised concentrated pelleted feed (Green Keil, Harbro Ltd, Turriff, UK) and hay, with access to water ad libitum. The animals were fed, initially, as follows: control animals (n=6) received 100% of requirements throughout the study, whereas nutrient restricted groups received 50% (4.0 MJ/ME per day) of this amount from 0 to 30 (n=8), 31 to 65 (n=6), 66 to 110 (n=4), and 0 to 110 (n=6) days of gestation respectively. From day 80 of gestation, the rations were increased to match the increasing nutrient requirements of...
Table 2 Oligonucleotide primer sequences used for quantification of target and endogenous control gene cDNA by real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>Forward: GAT GCC GCG GCG TTA TTC C</td>
<td>Reverse: CTC CTG GTG GTG CCC TCC C</td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>Forward: ACT GCC CCA AGT GAA AAG AGA</td>
<td>Reverse: ATG AAC AGA AAT GGC AGA AGA CAT TTT ATT</td>
<td></td>
</tr>
<tr>
<td>HSD11B2</td>
<td>Forward: AGC AGG AGA CAT GCC GCT TC</td>
<td>Reverse: GCA ATG CCA AGG CTG CT</td>
<td></td>
</tr>
<tr>
<td>IGF1</td>
<td>Forward: TCT TAG TCC CTG CCT TCT</td>
<td>Reverse: GCC TGC TGA ATG AAT GTC</td>
<td></td>
</tr>
<tr>
<td>IGF1R</td>
<td>Forward: TCT AAG TTT GTC TTT GCA AGA A</td>
<td>Reverse: TCA CTG GCC CAG GAA ATG TC</td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>Forward: TCA CAG CAG GAA AGT CGAG T</td>
<td>Reverse: GCC ACA GTA AGT CTC CAG CA</td>
<td></td>
</tr>
<tr>
<td>IGF2R</td>
<td>Forward: GAG ACT CCT TGT TGT ACA CTG CGG</td>
<td>Reverse: CGG GCA CTT GGC TTC CTC</td>
<td></td>
</tr>
<tr>
<td>PPARG</td>
<td>Forward: AGC GGA AAG ACG ACA GAC AAA</td>
<td>Reverse: GAC GGA GCG AAA CTG ACA CC</td>
<td></td>
</tr>
<tr>
<td>UCP2</td>
<td>Forward: ATG ACA GAC GAC CCT CTC TG</td>
<td>Reverse: GGG CAT GAA CCC TTT GTA GA</td>
<td></td>
</tr>
</tbody>
</table>

18S, 18S ribosomal RNA; GR, glucocorticoid receptor; HSD11B2, 11β-hydroxysteroid dehydrogenase type 2; IGF, insulin-like growth factor; R, receptor; PPARG, peroxisome proliferator-activated receptorγ; UCP, uncoupling protein.

the developing fetuses (Robinson et al. 1983). Fetal gender was determined at delivery and was as follows: controls (three males and three females); NR 0–30 days (four males and four females); NR 31–65 days (four males and two females); NR 66–110 days (two males and two females); NR 0–110 days (two males and four females).

**PCNA staining**

Cell proliferation was determined by immunoreactive PCNA expression (Waseem & Lane 1990). Placentome sections (5 μm) were stained with PCNA-specific antibody using a mouse MAB to PCNA (PC10) (Alexis Biochemicals, Nottingham, UK) at a 1:200 dilution. Immunohistochemical analysis was performed on a computerized bond automated immunohistochemistry system (Leica Biosystems Ltd, Newcastle-upon-Tyne, UK) using a bond polymer refine detection kit. At the end of the automated immunostaining, the sections were dehydrated in alcohol and xylene and mounted in resinous medium. Image acquisition was carried out using a Nikon Eclipse 90i microscope and camera Micropublisher 3.3 RTM (Q imaging, Surrey, BC, Canada). Volocity Acquisition and Quantification Software (Version 4.2.1, Improvision Ltd, Coventry, UK) was used for image analysis. A total of 10 fields from each section were randomly selected from the maternal caruncular section of the placentome and photographed at ×100 magnification (0.370277 mm² per field). Positive (brown) and negative (blue) cells were identified by the software using red-green–blue ranges. The index of cell proliferation was assessed as a ratio of positively stained cells over total number of cells per field (Correia-da-Silva et al. 2004).

**mRNA analysis**

Total RNA was extracted from 100 mg of maternal caruncular placentome tissue using Qiazol lysis reagent and RNaseasy extraction kit (Qiagen). RNA was treated with DNase I digestion using RNase-free DNase set (Qiagen). Two microgram RNA was used to generate 20 μl cDNA using 200 units of Superscript II reverse transcriptase (Invitrogen Ltd) and 125 ng random primers (GE Healthcare Life Sciences, Amersham, UK) according to the enzyme manufacturer’s guidelines. Negative control RT samples lacking Superscript II (−RT) were included for each sample. We performed real-time PCR using 15 μl reactions consisting of 4.5 μl diluted 1:10 cDNA, 3.0 μl (final concentration of 250 nM) gene specific primers (Table 2, Sigma–Aldrich), and 7.5 μl SYBR Green mastermix (Qiagen). Duplicate samples were run for 40 cycles with negative controls in 96-well plates using the Techne Quantica thermocycler (Techne Inc., Barloword Scientific, Stone, UK). 10-fold serial dilutions of cDNA for each gene were used to generate standard curve analysis and only experiments with R² > 0.985 were included. 18S ribosomal RNA was used as a housekeeping gene and relative mRNA expression was calculated by 2−ΔΔCt method (Livak & Schmittgen 2001). Data for each NR group were expressed as a ratio to the control animals.

**Statistical analysis**

All statistical evaluations were performed by using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Maternal NR effects were assessed using general linear model multivariate analysis with least significant difference post hoc tests. The study was not designed or powered to look at the effect of fetal gender on the placenta and this was analyzed as a covariate. All values presented are means with their standard errors. P value < 0.05 was deemed to represent statistical significance.

**Declaration of interest**

None of the authors have any interest to declare.

**Funding**

The research was funded by the European Union Sixth Framework for Research and Technical Development of the European Community – The Early Nutrition Programming Project (FOOD-CT-2005-007036), the Institute of Clinical Research, University of Nottingham and the Rural Environment Research and Analysis Department of the Scottish Government.

**References**


Hull D, Elphick MC & Broughton Pipkin F 1979 The transfer of fatty acids across the sheep placenta. Journal of Developmental Physiology 1 33–45.

Jaqiery AL, Oliver MH, Bloomfield FH, Connor KL, Challis JR & Harding JE 2006 Fetal exposure to excess glucocorticoid is unlikely to explain the effects of periconceptional undernutrition in sheep. Journal of Physiology 572 109–118.


Nunn AV, Bell J & Barter P 2007 The integration of lipid-sensing and anti-inflammatory effects: how the PPARs play a role in metabolic balance. Nuclear Receptor S 1.


Downloaded from Bioscientifica.com at 05/22/2019 10:50:12AM via free access


Received 10 February 2009
First decision 10 March 2009
Revised manuscript received 18 May 2009
Accepted 12 June 2009