cAMP-dependent regulation of ovulatory response genes is amplified by IGF1 due to synergistic effects on Akt phosphorylation and NF-κB transcription factors

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Abstract

Granulosa cells play a crucial role as mediator of the LH-dependent ovulatory response. The intraovarian factor IGF1 is produced by ovarian somatic cells of healthy follicles during the ovulatory response. The objective of this study was to identify mechanisms by which IGF1, alone or in combination with LH, regulates the expression of genes in granulosa cells, which are crucial for ovulation. To achieve this objective, short-term, primary murine granulosa cell cultures were treated for 2–8 h with 1 mM 8-bromoadenosine 3',5'-cAMP to mimic the LH surge and/or 100 ng/ml IGF1. While cAMP induced significant increases in the expression of important ovulatory response genes including amphiregulin (Areg), epiregulin (Ereg), betacellulin (Btc), or interleukin 6 (Il6), IGF1 alone had no effect. However, co-treatment of cells with IGF1 and cAMP had a synergistic effect on Areg, Ereg, Btc, and Il6 mRNA abundance. Pretreatment of granulosa cells with the MEK1/2 inhibitor U0126 demonstrated that cAMP-dependent increases in Areg, Ereg, Btc, and Il6 were mediated by extracellular regulated kinase 1/2 phosphorylation. However, western blot analyses coupled with pretreatment of cells with the PI3K inhibitor LY294002 indicated that the synergistic effect of cAMP and IGF1 on transcript levels was due in part to cooperative increases in Akt phosphorylation. Western blot analyses also demonstrated that IGF1 and the combined treatment of cAMP and IGF1 decreased NF-κB p65 phosphorylation and increased NF-κB p52 levels. Together, these data indicate that IGF1 may amplify cAMP-dependent regulation of ovulatory response gene expression above an important threshold level and therefore represents a novel role for IGF1 during ovulation.

Reproduction (2012) 144 595–602

Introduction

Ovulation is a complex process whereby the oocyte undergoes cytoplasmic and nuclear maturation and is ultimately released from the ovary. This process is dependent, in part, on intraovarian factors expressed by granulosa cells. For example, the epidermal growth factor (EGF)-like factors amphiregulin (Areg), epiregulin (Ereg), and betacellulin (Btc) that are expressed by mural and cumulus granulosa cells stimulate oocyte meiotic resumption and the expression of genes associated with cumulus expansion and the physical expulsion of the oocyte during ovulation (Park et al. 2004, Ashkenazi et al. 2005, Hsieh et al. 2007). Interleukin 6 (Il6), which is an inflammatory response gene, is also expressed by mural and cumulus granulosa cells and promotes cumulus cell expansion and oocyte germinal vesicle breakdown (Liu et al. 2009). The coordinated actions of these factors make a significant contribution to the successful ovulation of an oocyte competent for fertilization and embryonic development.

The expression of these and other granulosa cell genes associated with the ovulatory response is regulated by the LH surge. LH binds to its Gα protein-coupled receptor located predominately on theca and mural granulosa cells resulting in increased cAMP synthesis. Subsequently, cAMP activates protein kinase A (PKA), p38 MAPK, extracellular regulated kinase 1/2 (Erk1/2), and PKB (Akt) signaling to bring about changes in gene expression (Gonzalez-Robayna et al. 2000, Richards et al. 2002a, Hunzicker-Dunn & Maizels 2006). Studies by Liu et al. (2009) and Hsieh et al. (2011) have demonstrated that the activation of p38 MAPK and Erk1/2 rapidly upregulates Areg, Ereg, Btc, and Il6 mRNA abundance in mural granulosa cells. Intraovarian factors produced by the oocyte and follicular cells modify and complement the LH-dependent ovulatory program. Among these factors are the insulin-like growth factor (IGF) family that is synthesized by somatic cells of the ovary and includes ligands (IGF1 and IGF2), receptors (IGF1R and IGF2R), and binding proteins (IGFBP1–6) (Kwintkiewicz & Giudice 2009, 2012 Society for Reproduction and Fertility DOI: 10.1530/REP-12-0225 Online version via www.reproduction-online.org Downloaded from Bioscientifica.com at 11/23/2018 01:20:14PM via free access

The actions of IGF1 have been intimately linked to, and in some cases require, gonadotropin signaling (Poretsky et al. 1999, Kwinkiewicz & Giudice 2009). Furthermore, a multitude of studies have demonstrated that the IGF family makes a significant contribution to follicular growth and steroidogenesis (deMoura et al. 1997, Devoto et al. 1999, LaVoie et al. 1999, Eimerl & Orly 2002, Hu et al. 2004, Mani et al. 2010). However, the contribution of IGF1 during the ovulatory response and the underlying mechanisms of IGF1 actions to promote cumulus expansion and ovulation remain unclear. In the current study, we have analyzed the ability of IGF1 either alone or in combination with cAMP to regulate Areg, Ereg, Btc, and Il6 mRNA abundance in murine primary granulosa cell cultures. The signaling pathways and transcription factors that are activated by cAMP and IGF1 either alone or in combination in the granulosa cell cultures were also examined to determine the mechanisms of this potential synergy on ovulation.

Results

**IGF1 has a synergistic effect on cAMP-dependent regulation of Areg, Ereg, Btc, and Il6**

Quantitative real-time RT-PCR (qPCR) analyses were carried out to determine the acute effects of IGF1 and cAMP on the regulation of mRNAs associated with the ovulatory response. Murine granulosa cells were cultured in the absence or presence of cAMP, IGF1, or a combination of cAMP and IGF1 for 2, 4, or 8 h. As expected, cAMP increased the mRNA abundance of *Areg*, *Ereg*, *Btc*, and *Il6* transcripts (Fig. 1). Specifically, the expression of *Areg*, *Ereg*, and *Btc* was significantly increased 2, 4, and 8 h post-cAMP treatment with a peak in mRNA abundance 2 h posttreatment. While IGF1 alone had no effect on *Areg*, *Ereg*, or *Btc* mRNA levels at any time point, there were significant synergistic effects of cAMP and IGF1 on the abundance of these transcripts 2 h (*Areg*), 4 h (*Areg*, *Ereg*, and *Btc*), and 8 h (*Ereg* and *Btc*) after treatment. In addition to the EGF-like factors, the mRNA abundance of *Il6* was also increased by cAMP alone at 2 and 8 h posttreatment (Fig. 1). Furthermore, the combined treatment of cAMP and IGF1 had a modest but significant synergistic effect on *Il6* levels 2 and 8 h posttreatment.

**Akt but not Erk1/2 phosphorylation was regulated by IGF1 in the primary murine granulosa cell cultures**

Given the synergistic effect of IGF1 on cAMP-dependent increases in *Areg*, *Ereg*, *Btc*, and *Il6* mRNAs, the regulation of signaling factors by cAMP and IGF1 was
examined. Specifically, whole cell protein extracts were collected from primary granulosa cell cultures 30 or 60 min after treatment with cAMP, IGF1, or a combination of cAMP and IGF1. Protein extracts were subjected to Western blot analyses using antibodies against phosphorylated Akt or phosphorylated Erk1/2. The individual treatment of granulosa cells with cAMP or IGF1 increased phospho-Akt levels compared with the untreated control (Fig. 2A). There was also a synergistic effect on Akt phosphorylation after cells were co-treated with cAMP and IGF1. Conversely, cAMP but not IGF1 stimulated Erk1/2 phosphorylation at both time points (Fig. 2B). Furthermore, co-treatment of granulosa cells with cAMP and IGF1 did not increase Erk1/2 phosphorylation compared with cAMP treatment alone.

**Areg, Ereg, Btc, and Il6 mRNA abundance is regulated by both Akt and Erk1/2 signaling**

The Western blot analyses suggested that Akt signaling may mediate the synergistic effect of IGF1 on cAMP-dependent regulation of granulosa cell mRNA abundance. Thus, to define which signaling pathways regulated granulosa cell transcript levels, cells were pretreated with the PI3K inhibitor LY294002 or the Mek1/2 inhibitor U0126. Cells were subsequently treated with cAMP, IGF1, or a combination of cAMP and IGF1 for 60 min or 8 h and protein or total RNA collected for Western blot or qPCR analyses respectively. As expected, LY294002 specifically blocked Akt but not Erk1/2 phosphorylation and U0126 specifically blocked Erk1/2 but not Akt phosphorylation in the absence or presence of cAMP, IGF1, or cAMP and IGF1 treatment (data not shown). Pretreatment of cells with each inhibitor revealed that cAMP and cAMP/IGF1 regulation of Ereg and Il6 mRNA abundance was dependent on both Akt and Erk1/2 phosphorylation (Figs 3 and 4). Furthermore, the synergistic effect of IGF1 on cAMP-dependent Ereg and Il6 expression was lost in the presence of both inhibitors. Conversely, cAMP-dependent regulation of Areg and Btc in the absence or presence of IGF1 was mediated by Erk1/2 but not Akt (Figs 3 and 4). Interestingly, inhibition of Akt phosphorylation not only resulted in a loss of the IGF1 synergistic effect on Areg and Btc mRNA abundance but also increased cAMP-dependent expression of both transcripts (Fig. 3).

**NF-κB transcription factors are differentially regulated by the combined treatment of cAMP and IGF1 in primary murine granulosa cell cultures**

Both forkhead box proteins, class O (FOXO) and nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) transcription factors are downstream targets of Akt signaling. In order to assess how these transcription factors are regulated by cAMP and/or IGF1 treatment of murine granulosa cells, Western blot analyses for FOXO1, NF-κB p65, and NF-κB p100/52 were carried out. IGF1 and the combined treatment of IGF1 and cAMP decreased phosphorylated but not total NF-κB p65 approximately twofold (Fig. 5A). At the same time, cAMP, IGF1, and the combined treatment of cAMP and IGF1 increased precursor NF-κB p100 levels twofold (Fig. 5B). Likewise, the levels of transcriptionally active NF-κB p52 were increased modestly by cAMP and IGF1 alone while the combined treatment of cAMP and IGF1 increased NF-κB p52 almost fourfold (Fig. 5B). In contrast to the NF-κB transcription factors, FOXO1 phosphorylation was only modestly increased by

![Figure 2](image_url)
cAMP treatment. There was also no effect of IGF1 alone or in combination with cAMP on FOXO1 phosphorylation in our primary granulosa cell cultures (data not shown).

Discussion

We have long known that IGF1 is an important intraovarian factor that is associated with follicular health and stimulates granulosa cell proliferation and steroidogenesis. Brogan et al. (2010) recently showed that protein levels of IGFBP3, IGFBP4, and IGFBP5 fluctuate upon human chorionic gonadotropin stimulation of ovulation in rhesus macaques. Likewise, Nuttinck et al. (2004) demonstrated cumulus expression of IGF ligand and binding proteins during in vitro maturation of bovine cumulus oocyte complexes. Finally, IGF1-null mice lack antral follicle development and the ability to ovulate after exogenous gonadotropin stimulation (Baker et al. 1996). While these studies indicate a role for IGF signaling during oocyte maturation, cumulus expansion, and ovulation, IGF1-dependent mechanisms that contribute to the ovolatory response have not been reported. In the current study, we have demonstrated that IGF1 is important for amplification of Areg, Ereg, Btc, and Il6 mRNA abundance. Furthermore, our data indicate that these actions of IGF1 on ovolatory response gene expression are mediated, in part, by Akt phosphorylation and may be directly or indirectly regulated by NF-κB transcription factors.

Studies in the mouse and rat showed that IGF1 increases the responsiveness of granulosa cells to FSH due to increased expression of the FSH receptor (Zhou et al. 1997, Minegishi et al. 2000). However, in our study, the cooperative increases in Areg, Ereg, Btc, and Il6 mRNAs by cAMP and IGF1 were rapid (i.e. within 2 h) and independent of gonadotropin receptor signaling suggesting cross talk between downstream targets of cAMP and IGF1 signaling. Interestingly, Fan et al. (2008) recently showed that loss of phosphatase and tensin homolog (PTEN), which is a potent negative regulator of the PI3K/Akt pathway in granulosa cells, results in increased number of ovulated follicles and pups per litter. Likewise, we demonstrated that the cooperative increases in granulosa cell gene expression were regulated, in part, by activation of the Akt signaling pathway (Figs 2 and 3). These data are consistent with the synergetic increase in the extracellular matrix factor cartilage link protein (Crt1) by FSH and IGF1 in granulosa cells (Sun et al. 2003). Taken together, these data provide evidence that activation of Akt phosphorylation by both gonadotropins and IGF1 is an important mechanism for regulating the expression of genes associated with the ovolatory response.

Downstream targets of Akt signaling include several classes of transcriptional regulators including the FOXO
and NF-κB family of transcription factors (Huang & Tindall 2007, Gingery et al. 2008, Oeckinghaus et al. 2011). Studies by Richards et al. (2002b) previously showed that FOXO1 is expressed in granulosa cells during follicular growth and that phosphorylation of FOXO1 is reduced upon in vivo FSH and LH stimulation. However, at the time points analyzed in this study, IGF1 and the combined treatment of cAMP and IGF1 had little effect on FOXO1 phosphorylation. Conversely, changes in the balance of NF-κB transcription factors were detected (Fig. 5). Specifically, the phosphorylation of p65 was decreased by IGF1 and cAMP/IGF1 while the protein levels of p52 were increased by cAMP/IGF1. NF-κB p65 is a downstream transcription factor of the canonical NF-κB pathway that is typically activated by TNFα. Conversely, NF-κB p52 is processed from the p100 precursor upon activation of the noncanonical NF-κB pathway (Hayden & Ghosh 2008, Oeckinghaus et al. 2011). Interestingly, in our granulosa cell culture, the changes in NF-κB transcription factor phosphorylation/ expression and Akt phosphorylation were temporally similar, suggesting that Akt signaling may interact with both the canonical and the noncanonical NF-κB pathways to alter the balance of these transcription factors in granulosa cells.

NF-κB transcription factors can exert stimulatory or repressive effects on gene expression. While we have not conclusively demonstrated that Areg, Ereg, Btc, or Il6 gene expression is regulated by NF-κB p65 or p52, several studies have demonstrated that Il6 gene expression is positively regulated by p65 (Kang et al. 1996, Chen et al. 2008, Ndlovu et al. 2009). Conversely, our data suggest that decreased phosphorylation of p65 and/or increased processing of p52 may directly or indirectly contribute to the synergistic effects of cAMP and IGF1 on Il6 mRNA abundance. Our data, for the first time, also suggests that NF-κB transcription factors may directly or indirectly regulate the expression of EGF-like factors. Like Il6, increases in Ereg expression were correlated with decreased p65 phosphorylation and

Figure 4 Inhibition of Erk1/2 phosphorylation alters the mRNA abundance of Areg, Ereg, Btc, and Il6. Short-term granulosa cells were serum starved, pretreated with 0.1% DMSO or 10 μM U0126 for 30 min, and subsequently left untreated (white bars) or treated with cAMP, IGF1, a combination of cAMP and IGF1 for 8 h (black bars). QPCR reactions, data normalizations, and statistical analyses of Areg, Ereg, Btc, Il6, and Actb were carried out as described in Fig. 3. Significant fold changes in mRNA abundance between treated and untreated cells are indicated by different letters (*P<0.05). For each treatment, statistically significant differences in mRNA abundance between DMSO- and U0126-treated cells are indicated with asterisks (*P<0.05, ***P<0.001).
increased p52 processing (Fig. 5). While previous studies have demonstrated Sp1-dependent regulation of the Ereg promoter (Sekiguchi et al. 2002), our data suggests a possible novel mechanism regulating Ereg gene transcription. In contrast to Il6 and Ereg, treatment with the PI3K/Akt inhibitor LY294002 also suggested that the altered balance of NF-κB transcription factors by IGF1 may have a suppressive effect on Areg and Btc expression. These data not only indicate a potential role for NF-κB on Areg and Btc transcription but also suggest novel, differential regulation of the EGF-like factors, which may be mediated by the NF-κB transcription factors.

Thus, taken together, we have demonstrated that IGF1 amplifies cAMP-dependent increases in the mRNA abundance of EGF-like factors and Il6, which have well-described roles in mediating the events associated with ovulation. Our data indicate that IGF1 may enhance Areg, Ereg, Btc, and Il6 expression above an important threshold level or sustain the expression of these ovulatory response genes in order to optimally initiate meiotic resumption and/or cumulus expansion. The synergistic effect of cAMP and IGF1 on granulosa cell gene expression also suggests that any disruptions in this synergy (e.g. due to metabolic disturbance) may contribute to the development of anovulatory infertility.

Materials and Methods

Animals

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska–Lincoln. CF-1 female mice were purchased from Charles River Laboratory (Wilmington, MA, USA) and had ad libitum access to food and water before killing.

Granulosa cell collection and culture

Ovaries were isolated from 24-day-old CF-1 mice and incubated for 15 min at 37 °C in collection medium (1× Leibovitz (Sigma–Aldrich), 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), and 1% penicillin/streptomycin (Invitrogen)) containing 6 mM EGTA (Sigma–Aldrich). Ovaries were subsequently incubated for 15 min at 37 °C in collection medium containing 0.5 M sucrose (Sigma–Aldrich). Ovarian follicles were punctured with a 27-gauge needle in collection medium and cells were applied to 40 μm filters (BD Falcon, Franklin Lakes, NJ, USA) to remove cumulus–oocyte complexes. Cells were cultured on tissue culture plates pretreated with 0.25% human fibronectin (Sigma–Aldrich) in complete medium (DMEM/F12 (Sigma–Aldrich), 10% heat-inactivated FBS (HyClone), and 1% penicillin/streptomycin (Invitrogen)) for 72 h. Cells were subsequently maintained in serum-free medium (DMEM/F12 (Sigma–Aldrich) and 1% penicillin/streptomycin (Invitrogen)) for 24 h. Serum-starved cells were incubated in the absence or presence of 1 mM 8-bromoadenosine 3′,5′-cAMP (8-br-cAMP; Sigma–Aldrich), 100 ng/ml IGF1 (Cell Signaling Technology, Danvers, MA, USA), or a combination of 8-br-cAMP and IGF1 for 0.5, 1, 2, 4, or 8 h. After treatment, cells were collected in TRI reagent (Ambion, Inc., Austin, TX, USA; 2, 4, and 8 h) or protein extraction buffer (described below; 0.5 and 1 h). In a subset of experiments, granulosa cell cultures were pretreated with vehicle control (0.1% dimethyl sulfoxide (DMSO)), 50 μM LY294002 (Cell Signaling Technology), or 10 μM U0126 (Cell Signaling Technology) for 30 min before 8-br-cAMP, IGF1, or 8-br-cAMP plus IGF1 treatment for 1 or 8 h.

RNA extraction and RT

RNA was extracted and purified from granulosa cells using the Ambion Ribopure Kit according to the manufacturer’s directions. Total RNA (~1 μg) from each sample was combined with 5 units of RQ1 RNase–free DNase (Promega) and incubated at 37 °C for 30 min to eliminate genomic DNA contaminants. The RNA was subsequently combined with 400 units of Moloney murine leukemia virus reverse transcriptase (Promega), 500 μM dNTPs (Promega), and 100 ng of random primers (Roche Applied Science) and incubated at 37 °C for 2 h.

QPCR analyses

Forward and reverse primers (Table 1) for Areg, Ereg, Btc, Il6, and β-actin (Actb) were designed (Primer Express; Applied Biosystems, Foster City, CA, USA) and synthesized (Integrated DNA Technologies, Coralville, IA, USA). Each set of primers was tested empirically to determine the maximal concentration of primers that could be used to produce specific amplification of the target sequence in the absence of primer dimer amplification. QPCR reactions were carried out using equivalent dilutions of each cDNA sample. Power SYBR Green PCR Master Mix (Applied Biosystems), the empirically determined concentration of each primer, and the 7900HT Fast Real-Time PCR system (Applied Biosystems). The relative abundance of each target mRNA (Areg, Ereg, Btc, and Il6) and the internal control gene Actb in each cDNA sample was determined using serial dilutions of whole ovary cDNA. The relative abundance of Actb between treatments was not statistically different and was therefore an appropriate internal

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
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<tr>
<td>Actb</td>
<td>NM_007393</td>
<td>5'-agatgaccccagacatggtgaga-3'</td>
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<td>5'-tttgctgcttcacaccaagc-3'</td>
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<tr>
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<td>5'-agacgctgcaacacaccaagc-3'</td>
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<td>Il6</td>
<td>NM_031168</td>
<td>5'-agcagccgcttaattacacatgt-3'</td>
<td>5'-tgccatggcaactctttct-3'</td>
</tr>
</tbody>
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control. The relative abundance of each specific gene product was divided by the relative abundance of Actb in each sample to generate a normalized abundance for each gene interrogated. The normalized abundance of each gene in treated cells was compared to untreated control cells and expressed as a fold-change.

**Whole cell protein extracts**

Untreated and treated granulosa cell cultures were collected into modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA) containing phosphatase inhibitors (1 mM Na3VO4 and 1 mM NaF) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics) and sonicated. The cell supernatant was subsequently separated from cellular debris by centrifugation at 12 000 g. Protein concentrations were determined using the Pierce BCA Protein Assay (Rockford, IL, USA) according to the manufacturer’s directions.

**Western blot analyses**

Whole cell extracts were resolved by SDS–PAGE and separated protein transferred to Immobilon PVDF (Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk in TBS-T before incubation with primary antibody (all purchased from Cell Signaling Technology) against phospho-Akt (1:2000), phospho-Erk1/2 (1:2000), phospho-NF-kB p65 (1:1000), NF-kB p100/52 (1:1000), or phospho-FOXO1 (1:2000) followed by HRP-conjugated secondary antibody. Proteins were visualized using West Pico Chemiluminescent Substrate (Pierce) and exposure of blots to X-ray film. Following visualization of phosphorylated Akt, Erk1/2, p65, and FOXO1 as well as total NF-kB p100/52, blots were stripped with Restore Plus Western Blot Stripping Buffer (Pierce), blocked, and incubated with primary antibody against total Akt (1:2000), total Erk1/2 (1:2000), total NF-kB p65 (1:2000), total FOXO1 (1:1000), or β-actin (1:4000) respectively. Total protein for each factor or β-actin was visualized as described and served as a loading control. To quantify the relative amount of protein expressed in each sample, the density of each protein band in each sample was determined using Photoshop. The density of phosphorylated Akt, Erk1/2, p65, FOXO1, and total NF-kB p100/52 in each sample was normalized using the density of the total protein or β-actin in each sample. The normalized abundance of phosphorylated Akt, Erk1/2, p65, FOXO1, and total NF-kB p100/52 in treated cells was subsequently compared with untreated control cells and expressed as a fold-change.

**Statistical analyses**

The qPCR experiments represent n=3–5 replicates for each experimental group (i.e. untreated, treated, and/or inhibitor). Western blot analyses were carried out using two independent samples for each experimental group. All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Specifically, qPCR data were analyzed using one-way ANOVA followed by Tukey’s pairwise comparisons. Differences were considered significant at P<0.05.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This project was supported by State of Nebraska Hatch (NEB-26-198) and Multi-State (NEB-26-181) Research Funds. This study represents a contribution of the University of Nebraska Agricultural Research Division, Lincoln, Nebraska.

**Acknowledgements**

The authors thank Andrea Cupp and Renee McFee for critical reading of this manuscript.

**References**


Received 14 June 2012
First decision 5 July 2012
Accepted 4 September 2012