Urine neopterin concentrations as a marker for successful blastocyst implantation after assisted reproductive technologies

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Dietmar Fuchs graduated in chemistry in 1980 from the University of Innsbruck, Austria. After employment at the Institute of Medical Chemistry and Biochemistry, he became Reader in Medical Chemistry (1986), and Associate Professor and co-director of the Ludwig Boltzmann Institute for AIDS-Research at the Medical Faculty. He was Honorary Visiting Fellow (Aston University, Birmingham, UK) and Honorary Senior Research Fellow (University of Birmingham) from 1994–1998. His scientific interests lie in the fields of Clinical Biochemistry and Clinical Immunology focusing on the metabolism of neopterin and tryptophan. He has published 730 articles in peer-reviewed scientific journals and received several national and international awards.

Abstract Successful blastocyst implantation requires intricately orchestrated adaptation processes involving maternal and fetal mediators. The pivotal role of distinct immune response pathways in early pregnancy is widely acknowledged. Pro-inflammatory cytokines, e.g. interferon-γ (IFN-γ), are the primary inducers of tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) and of neopterin biosynthesis by GTP-cyclohydrolase I. IDO activity has been proposed to be of high clinical relevance in the context of pregnancy. To date, insights arising from clinical studies on IDO activity and neopterin concentration during the very early days of pregnancy are still few. Early morning urinary neopterin concentrations in 61 women undergoing assisted reproduction treatment (72 cycles in total) were examined, upon exclusion of infections, daily over a period of 2 weeks after embryo transfer. Twenty of the study participants (28%) became successfully pregnant, and four women experienced abortion. Neopterin concentrations significantly increased after blastocyst transfer when implantation was successful (chi-squared = 23.291, P < 0.01; Friedman test), opposed to non-significant changes of neopterin in women with unsuccessful treatment (chi-squared = 8.203). The steady increase of neopterin concentrations upon blastocyst transfer indicates that heightened production of neopterin in very early phases of pregnancy may serve as an early predictor of successfully progressing pregnancies in humans.

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Introduction

Successful onset and maintenance of pregnancy meets a potential quandary, as the implanting blastocyst is semiallogeneic. Hence, the maternal immune system has to recognize the developing child as ‘self’ and/or to build immune tolerance in order to allow invasion of such semiallogeneic tissue instead of rejecting it. Well-orchestrated adaptation processes of the maternal immune system to the fetus at local and systemic levels have been proposed, which are capable of ensuring immunotolerance and successful pregnancy. These processes range from innate responses such as natural killer cells and dendritic cells to adaptive responses such as increasing numbers of regulatory T cells. (Aluvihare et al., 2004; Clark, 2003; Tafuriet al., 1995; Zhou and Mellor, 1998).

Among several candidate immunoregulators, T-helper type 1 (Th1)-type cytokine interferon-γ (IFN-γ) is intensely discussed to elicit pathways which are involved in tolerance induction (Fuchs et al., 1989; Liu and Janeway, 1990; Schroehnsnadel and Fuchs, 2006; Wood and Sawitzki, 2006). Pro-inflammatory stimuli like IFN-γ are the primary inducers of tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) and of neopterin biosynthesis by GTP-cyclohydrolase in human monocyte-derived macrophages and dendritic cells (Figure 1) (Huber et al., 1984; Weiss et al., 1999; Wirleitner et al., 2002). During murine pregnancy, activation of IDO was demonstrated as a necessary requirement for maternal tolerance induction (Munn et al., 1998), whereby it is still hotly debated why IDO knockout mice do not show an impaired reproductive fitness. During human pregnancy, the maternal decidua and the fetal syncytiotrophoblast, which invades the uterine, express IDO and this expression at the fetomaternal interface may prevent immunological rejection of the fetus by suppression of T cell responsiveness (Kamimura et al., 1991; Mellor and Munn, 2001; Munn et al., 1998).

Earlier studies show that tryptophan degradation and neopterin formation are increased in pregnant women, indicating activation of IDO under immunological control mechanisms. Human pregnancy was found to be associated with increasing serum and urine neopterin concentrations correlated with the gestational age and rather quickly returning to normal after birth (Bichler et al., 1983; Schroehnsnadel et al., 1996; Schroehnsnadel et al., 2003). It is assumed that immune recognition and response is induced in early pregnancy. However, insights supporting the role of neopterin and tryptophan metabolism during early pregnancy – before a gestational age of 12 weeks – are still sparse.

The aim of the present study was to analyse urinary concentrations of neopterin in women undergoing assisted reproductive technologies to obtain information about changes of the immune system during the first 2 weeks of pregnancy. Such insights would then allow the identification of early markers/predictors for successful blastocyst implantation after treatment.

Materials and methods

Patients

Urinary neopterin concentrations from the very early phase of pregnancy were determined in 61 women (average age 33 years, range 24–41 years) after day-5 (blastocyst stage) transfer in vitro fertilization (IVF, n = 13) or intracytoplasmic sperm injection (ICSI, n = 59). Women were asked to collect early morning urine specimens daily over 14 days, starting the day after the IVF or ICSI, and kept in the freezer at −20°C until measured. In total, 72 fertilization attempts were followed. For three individuals, urine collections were available during a longer period of pregnancy. During the study period, women did not show any evidence of systemic illness or fever.

The study was in accordance with the Helsinki declaration and all individuals included in the study gave informed consent that the results of urinary neopterin measurements were solely used for scientific purposes in a blinded manner.

Neopterin measurement

Determination of neopterin was performed using HPLC on reversed-phase C18 with Sörensen potassium phosphate buffer (0.015 mol/l, pH = 6.4) as eluent (flow rate = 1.0 ml/min) (Schroecksnadel et al., 2006a, b) as follows: 100 μl urine were mixed with 1000 μl elution buffer containing 2 g/l EDTA to dissolve eventual urinary sediments. Neopterin was detected by measurement of its natural fluorescence (excitation wavelength 353 nm, emission wavelength 438 nm). Physiological differences in urine densities were taken into account by calculating the ratio of neopterin versus creatinine concentrations, which were measured in parallel to neopterin in the same chromatographic run by detection of its UV absorption at 235 nm wavelength. The quotients neopterin/creatinine were expressed in μmol neopterin/mol creatinine.

![Figure 1](natural killer (NK) cells and T-cells, stimulated within the innate and adaptive T-helper type 1-type immune response, release the pro-inflammatory cytokine interferon-γ (IFN-γ). In turn, IFN-γ stimulates enzymes GTP-cyclohydrolase (GCH), which underlies the accumulation of neopterin in human macrophages (MP) and dendritic cells (DC), and indoleamine 2,3-dioxygenase (IDO) which initiates the degradation of tryptophan via the kynurenine pathway in several cells of various species. In turn, the production of neopterin and kynurenine derivatives as well as the depletion of tryptophan during immune response could contribute to immunosuppression via the induction of T-cell tolerance and apoptosis.)
Statistical analysis

Not all the study participants completed collection of urine specimens throughout the whole observation period of 2 weeks. There was an increasing number of missing specimens at the later days of the follow-up; during days 11–14, drop-out number of participants was from two to five in the pregnant group and from eight to 16 in the non-pregnant group. Consequently, group comparisons were only performed for the first 10 days in which the number of missing specimens was zero in the pregnant group and four or more in the non-pregnant group (Figure 2).

Because not all the data sets showed normal distribution, non-parametric statistics were applied for data analyses. For comparison of grouped data, Friedman test and Wilcoxon signed ranks test were used. In order to compare serial measurements between pregnant and non-pregnant women, urinary neopterin concentrations were plotted versus time (not shown). Thereby it was concluded that the variable changed during the observation period in a rather linear way and, thus, the slopes of regression lines fitted to the data of each individual by least square estimation were compared between the two groups by non-parametric Mann–Whitney U-test (Matthews et al., 1990).

Statistical Package for the Social Sciences version 14 (SPSS, Chicago, IL, USA) was used. P-values below 0.05 were considered to indicate significant differences.

Results

Among the 72 attempts of assisted reproduction treatment, 20 were successful (28%) and resulted in a viable pregnancy, five after IVF and 15 after ICSI. Four women had a multiple pregnancy (three twins, one triplets) and four experienced abortion at a later time during follow-up. On day 1, neopterin concentrations in women who later became pregnant (mean ± SEM 160 ± 13.3 μmol/mol creatinine) were slightly lower than in women who did not (mean ± SEM 182 ± 10.0 μmol/mol creatinine), this difference was however not significant. A significant increase of neopterin concentrations was observed in the group of pregnant women (chi-squared = 23.291, P < 0.01; Friedman test) but not in the non-pregnant group (chi-squared = 8.203, not significant; Friedman test). When percentage changes were compared after setting baseline values of individual women as 100%, the percentage change of concentrations was highest on day 9 with 121 ± 9.1% in pregnant women versus 105 ± 6.2% in non-pregnant women (Figure 2). Likewise, when compared with baseline, the increase of neopterin concentrations became significant from day 4 onwards in the pregnant women (U = 2.083, P < 0.05, paired rank test). In women with twin or triplet pregnancies, neopterin concentrations were only slightly higher as compared with single pregnancies (details not shown).

On comparing the slopes of regression lines fitted to urinary neopterin values plotted versus time there was a stronger increase of urinary neopterin in pregnant (median slope 1.413, 95% CI –0.576 to 4.308) than in non-pregnant women (median slope –1.275, 95% CI –2.897 to 0.886; U = 258, P < 0.021).

In two individuals, neopterin analyses were possible throughout the first 70 and 80 days respectively of pregnancy. Further, samples were obtained from one woman throughout the entire pregnancy and during puerperium. In the first two cases, this study observed that neopterin concentrations continued to steadily increase with time reaching concentrations of approximately 350 and 225 μmol/mol creatinine, and in the third women neopterin concentrations around 400 μmol/mol creatinine were reached at the end of pregnancy, including a few peaks around day 60 of pregnancy (details not shown). In one female post-partum measurements were available, and Caesarean section was performed as was requested by the mother, because of an intrauterine death during her previous pregnancy. After Caesarean section was performed, neopterin concentrations abruptly declined but again two prominent peaks were observed within the following month.

Discussion

Despite the intensive research performed over previous years, immunotolerance in pregnancy is still far from being fully understood, and it has been estimated that the implantation of more than 50% of conceptions in humans is unsuccessful (Clark, 2003). A better understanding of the mechanisms involved in maternal pregnancy maintenance

![Figure 2](attachment:figure2.png)

**Figure 2** Urinary neopterin concentrations (μmol/mol creatinine; mean ± SEM) in women after successful (n = 20, filled circles; chi-squared = 23.291, P < 0.01; Friedman test) and non-successful (n = 52, open circles; chi-squared = 8.203) treatment. Percentage changes were compared after setting baseline values of individual women at 100%.
would provide the basis for developing suitable therapeutic and predictive tools for this condition.

In the present study, an increase of urinary neopterin concentrations was observed in women as early as 4 days after treatment in successfully progressing pregnancies. An increase of neopterin concentrations was seen preferentially in early pregnancy but not in women who did not become pregnant. It indicates pro-inflammatory pathways caused by pregnancy which most probably involve immune system activation and production of IFN-γ. No other explanation (e.g., infections) was evident, which could explain the raise of neopterin concentrations in the later days. Thus, the increase of neopterin would be in line with a role of IFN-γ in tolerance induction, as was previously suggested several times (Fuchs et al., 1989; Liu and Janeway, 1990; Wood and Sawitzki, 2006). Thereby the activation of IDO could play an important role, although it may represent just one of the major players induced by the cytokine and being able to induce specific immunotolerance (Wood and Sawitzki, 2006). Notably, only humans and mice produce larger amounts of neopterin because their monocyte-derived cells like macrophages or dendritic cells are deficient for the enzyme pyruvyl tetrahydropterin synthase, which in other cells and cells of other species including macrophages converts 7,8-dihydronopterin triphosphate to 5,6,7,8-tetrahydrobiopterin, the cofactor of amino acid hydroxylases including nitric oxide synthases. So monitoring of neopterin concentrations cannot be applied as a urine pregnancy test in mouse biology.

In vitro and in vivo, tryptophan degradation by IDO is strongly induced by pro-inflammatory cytokines such as IFN-γ in a broad variety of cells in humans and other species (Byrne et al., 1986; Werner-Felmayer et al., 1989; Wirleitner et al., 2002; Yoshida et al., 1981). Interestingly, in the endometrium and decidua, IFN-γ is largely derived from decidual natural killer (dNK) cells. This cell population is increasingly acknowledged to control trophoblast invasion and vascular remodelling through their professional ability to secrete an array of angiogenesis-regulating molecules, chemokines and cytokines, such as vascular endothelial growth factor, placental growth factor and interleukin-8 (Hanna et al., 2006). Hence, the up-regulation of neopterin ensuring subsequent successful blastocyst implantation might be an additional task of this unique decidual cell population. IFN-γ strongly induces GTP-cyclohydrolase I and gives rise to the secretion of neopterin in human and primate monocyte-derived macrophages and dendritic cells (Figure 1) (Huber et al., 1984; Munn et al., 1998; Murr et al., 2002). As dNK cells and dendritic cells have been postulated to promote pregnancy maintenance by forming an immunological synapse (Karimi et al., 2008), increasing neopterin production associated with successful pregnancy production may be mutually ensured within this synapse via dNK cell- and dendritic cell-dependent pathways.

In humans with inflammatory conditions, a close association exists between neopterin concentrations and the kynurenine-to-tryptophan ratio, which is a suitable index of tryptophan degradation (Schroecksnadel et al., 2006a, b). It is generally assumed that pro-inflammatory cytokines like IFN-γ might induce IDO in parallel to neopterin formation, and it thus could be important for the induction of tolerance in the early phase of pregnancy (Kamimura et al., 1991; Mellor and Munn, 2001; Zhou and Mellor, 1998). A weak correlation between the serum kynurenine to tryptophan ratio, an estimate of IDO activity, and IFN-γ concentrations was described recently in first-trimester gestation (Barrientos et al., 2009). However, because treatment was performed in women who had not previously become pregnant naturally, results of this study may not adequately reflect a normal pregnancy. Certainly, one cannot exclude that neopterin and tryptophan metabolism may have behaved differently in this particular selection of women who were included in this study.

Accumulating data suggest that alterations in the balance of Th1 to Th2 type cells and cytokines are important in immune regulation during pregnancy (Clark et al., 2005). Whereas investigations on the cellular level often were inconclusive (Palfi et al., 1999), this study’s results are able to extend the ongoing discussion that an initial pro-inflammatory response, as is indicated by increasing neopterin concentrations, might be required for pregnancy development and/or blastocyst differentiation, as no such response was evident in women who did not become pregnant with assisted reproduction treatment. Also, this initial immune response may be involved in creating a microenvironment which prevents fetal survival. It is reasonable to speculate that, e.g., the IDO expression which was found to be required for induction of tolerance is initially cytokine induced. Unfortunately, such data on IDO activity and tryptophan degradation were not available in this study since ethical constraints excluded daily blood withdrawal. Still in accordance with findings from murine pregnancy, IDO could be related to this study’s findings on higher neopterin production rates in pregnant versus non-pregnant women after treatment, but notably, there are several other pathways which are considered to be related to tolerance induction and at last some of which are induced by pro-inflammatory stimuli such as IFN-γ. These pathways seem to reflect the down-regulatory and anti-inflammatory activities which are directed to dampen an immune response once activated (Fuchs et al., 1989; Wood and Sawitzki, 2006). Thus, the balance between pro- and anti-inflammatory cytokine pathways may be important for the fate of the fetus.

Similar pathways seem to exist with regard to the development of immunodeficiency in chronic diseases which are associated with inflammation and immune activation such as chronic infections or malignancy (Fuchs et al., 1989; Murr et al., 2002). In these patients, increased neopterin concentrations have been described earlier to parallel disease progression and to indicate a higher risk of death which is most probably associated with immune system failure (Fuchs et al., 1989; Schroecksnadel and Fuchs, 2006; Wood and Sawitzki, 2006).

Neopterin concentrations were also documented throughout longer periods of gestation in two women and throughout the entire gestational length in one woman and steadily increasing neopterin concentrations until delivery were found. The steady increase of neopterin throughout the first 3 months of pregnancy was interrupted by a few spikes, which were seen in all three cases and most impressive around days 55–70. They were unrelated to clinically overt infectious episodes. However, serological analyses were not available. Thus, it remains to be identified
in continuing studies whether these spikes are related to specific developmental phases during pregnancy.

In conclusion, this study describes an early increase of urinary neopterin concentrations in women who underwent successful assisted reproduction treatment compared with women without pregnancy. Assisted reproduction treatment may not represent an adequate model to extrapolate the findings to normal human pregnancy, but results should provide a further basis for a discussion on the role of pro-inflammatory stimuli in the induction of immunotolerance, which is required for successful nidation. Mean values of neopterin concentrations differed significantly between women with successful or unsuccessful interventions, and future research is needed to confirm: (i) whether neopterin monitoring allows early detection of pregnancy development in women after assisted reproduction treatment; and (ii) how neopterin concentrations and IDO activity might be up-regulated to allow successful blastocyst implantation.

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References


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