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LIF and TNF alpha concentrations in embryo culture media are predictive for embryo implantation in IVF

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ABSTRACT

Objective: There is strong evidence that the cytokines leucemia inhibitory factor (LIF) and tumor necrosis factor (TNF) alpha are related to embryo development and implantation. The aim of this study was to determine the levels of LIF and TNF alpha in embryo culture media and to assess its relationship to the outcome of *in-vitro* fertilization and embryo transfer. **Methods:** A total of 99 patients were included in this prospective trial and underwent either IVF or ICSI procedure. A total of 865 oocytes were collected. Embryos were cultured in sequential media until day 5. A standardized morphology evaluation of all embryos, including a detailed pronuclear scoring, was performed daily during this period followed by the replacement of one or two selected embryos. Collected embryo culture fluids of days 3 and 5 were analysed for LIF and TNF alpha on days 3 and 5. **Results:** Mean TNF alpha concentration in culture media on day 3 was 0.54 and 0.37 pg/mL on day 5 and was significantly lower in women conceiving than in not conceiving (0.43 pg/mL versus 0.59 pg/mL on day 3). Mean LIF concentration on day 3 was 31.5 pg/mL and 35.5 pg/mL on day 5 and was significantly higher in women conceiving (56.2 pg/mL versus 22.2 pg/mL on day 3). **Conclusions:** The results indicate that LIF could have a function in early embryogenesis and as a factor required for embryo implantation. High TNF alpha concentrations seem to be predictive of implantation failure.

1. Introduction

Cytokines are proteins of a mass of 8 to 30 kDa which are secreted by the widest range of cells. Their fundamental function is the effect and change of properties of the widest range of cells and tissues. Cytokins bind to their specific receptor in cell membranes and in this way induce a cascade of signal paths which result in the actual effect of the cytokin on a cell or a tissue. The tumor necrosis factor (TNF) is a pleiotropic cytokine which is found ubiquitously in the human body. First described by Old in 1985, TNF alpha is identified as a cytotoxic protein following endotoxin treatment in rabbit serum[1]. This factor was responsible for haemorrhagic necrosis in the tumour tissue. However, the effective spectrum of TNF alpha is much more varied than originally expected. Thus, TNF alpha is a mediator in apoptosis processes and plays an important role in infection and immune reactions. In the pathogenesis of many diseases, TNF alpha has a crucial function: among other things with sepsis, multiple sclerosis, osteoporosis, instances of cancer and diabetes[2]. A whole number of human cell types produce cytokin: this

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includes primarily monocytes and macrophages but also NK cells, T-lymphocytes and mast cells. Some studies in the past few years have stressed the meaning of TNF alpha in reproductive medicine. It appears to play an important role in pregnancy. Thus, TNF alpha protein, TNF alpha-RNA and also its receptors TNF-R1 and TNF-R2 are expressed during the pregnancy in different tissues such as ovaries[3], endometrium[4], placenta[5] and in the foetus itself[6]. TNF alpha seems to have a significant function in the differentiation and growth processes in a normally progressing pregnancy[7]. Its negative effects are discussed in conjunction with habitual miscarriages and IVF/ICSI failures[8-10].

The leukemia inhibitory factor (LIF) is part of the interleukin-6 family and like its receptor LIF-R is expressed by different cells: this includes, among other things, activated T-cells, monocytes, astrocytes, osteoblasts, keratinocytes, myoblasts, mast cells, fibroblasts and hepatocytes[11]. This explains the participation of LIF in many processes in the human body such as haematopoiesis, bone metabolism, inflammatory reactions, differentiation and proliferation processes[11]. In the past 20 years, many researchers have worked with the LIF in the area of reproductive medicine. In 1988, Smith *et al*. identified LIF as the first protein which had an effect on the differentiation of embryonic cells of mice[12]. Many studies analysed the effects of LIF on blastocyste development and implantation. Thus, *LIF* mRNA was expressed in mouse pre-implantation embryos during development from the fertilized egg to the blastocyst[13]. Cheng *et al*. recorded a lower blastocyste formation rate and a significant decrease in the implantation rate following the addition of a LIF-antisense nucleotide[14]. Embryos in a culture medium enriched with LIF also demonstrated a higher rate of blastocystes in the mouse model[15]. In arrested embryos, a lower expression of LIF was noted[16]. Several studies in the past 15 years have shown that LIF plays an important role in the development and implantation of embryos[17]. Its interactions with other cytokines of the reproductive tract are still primarily unclarified and require other studies.

It was the goal of this perspective study to measure the concentration in the TNF alpha and the LIF for the cytokine considered significant for the early implantation phase in the culture medium and to study a possible correlation with embryo morphology or the success of the pregnancy.

2. Materials and methods

2.1. Patients

Ninety-nine infertile couples were consecutively included in this prospective trial. In 49 cases, conventional IVF was performed and, in 50 cases, ICSI procedure was applied. Indications for assisted reproduction were tubal factor, endometriosis, male factor or idiopathic sterility. In cases with male subfertility, ICSI was performed.

2.2. Ovarian stimulation and sperm preparation

Ovarian stimulation was induced by FSH-application on day 3 of the cycle after pituitary down-regulation by 0.2 mg intranasal nafarelin 2-4 times a day 6-8 days prior to the expected period. When the mean diameter of the dominant follicle reached 18 mm, 10 000 IU of HCG was administered on the same evening. Oocyte recovery was performed by transvaginal route 36-38 h after an HCG injection. Collected oocytes were incubated in culture media (IVF 'in-house-media', SIVF).

All semen samples were evaluated according to the guidelines of the World Health Organisation (WHO) for volume, total sperm count, concentration, leukocytes, motility, vitality and morphology. Ejaculate samples were prepared by a PureSperm (Nidacon, Sweden) cushion centrifugation and a swim-up procedure. After several centrifugation and washing steps the semen pellet was incubated in culture media for 30-60 min at 37 °C for the swim-up of motile sperm.

2.3. IVF and ICSI fertilization

After oocyte collection, the cumulus-oocyte complexes were identified under the microscope in the follicular fluid. IVF and ICSI procedures were carried out using standard protocols as described previously[18,19]. The oocytes were incubated in culture media overnight. A fertilization check was performed after 16-18 h later (appearance of two pronuclei).

2.4. Pronuclear scoring

Each zygote was placed in a microdroplet of 10-15 μL culture media (IVF 'in-house-media', SIVF) into a Petri dish (5×5 microdroplet rows). In this 'single cell culture' zygotes were scored on an inverted microscope at magnification of ×400. Zygotes were individually checked on day 1 for the following criteria: number, size and alignment of pronuclei, halo-effect, alignment and number of nucleoli, appearance of vacuoles in the ooplasma and granulation of ooplasma[20]. With the use of an injection pipette, the oocyte could be turned around and so the exact juxtaposition of pronuclei could be evaluated. The zygote score was calculated as a cumulative score of all items mentioned above.

2.5. Embryo culture and embryo development scoring

After scoring, zygotes were transferred into fresh culture media followed by further cultivation to the blastocyst stage (day 5). If an embryo had not yet developed to the blastocyst stage on day 5, another 24 h of culture followed before the embryo transfer (ET) was performed. When there were no

additional embryos for ET available, embryos which had not developed to blastocysts had to be transferred on day 3 or day 4. The following criteria were included in the embryo scoring system: fusion of the pronuclei, cleavage stage, fragmentation, compactation of the embryo within 72 h, cavitation within 96 h, expansion of the blastocyst, inner cell mass and trophoectoderm development. Twenty-four hours after insemination the embryo should have developed from its pronucleus stage by fusion of the 2 pronuclei and the first cleavage to the 2-cell stage. Further cleavage stages were noted in the embryo scoring on days 2 and 3. On days 4, 5 and 6 of the culture the compacting process, cavitation and expansion of the embryo was evaluated. From day 2 to day 6 every embryo was given a grade 1-3 according to its development regarding cleavage stage, fragmentation and inner cell mass development.

2.6. TNF alpha- and LIF- measurement

Culture medium of each embryo was collected per patient on day 3 and day 5 of culture. TNF alpha and LIF concentrations were measured using an enzyme-linked immunoabsorbent assay (Quantikine®, R&D Systems, Minneapolis, USA).

2.7. Embryo transfer and pregnancy assessment

In this trial, the day of embryo transfer was chosen for each patient individually. Transfer of embryos at the blastocyst stage was preferred, although, in some cases embryos were transferred on day 2, 3 or 4 depending on their morphological development. A single embryo transfer was performed in 66% of cases. Two weeks after embryo transfer a single serum β-HCG measurement was performed. The sonographic appearance of a gestational sac in utero was defined as a clinical pregnancy.

2.8. Statistical analysis

Statistical analysis of data was performed by using SPSS 13.0. Results were expressed as mean and standard deviation. The student's t-test was used to compare differences between groups. A $P<0.05$ was considered significant.

3. Results

3.1. Pregnancy data

The data of 99 women were analysed in this prospective trail. In 49 cases, conventional IVF was performed, in 50 cases ICSI procedure was applied. A total of 856 oocytes were collected with a mean number of 8.82±4.8 oocytes per patient. A total of 438 oocytes (mean number of 4.56±3.14 per patient) showed positive signs of fertilization 16-18 h after insemination. In 14 oocytes abnormal fertilization was noted. The mean fertilization rate calculated was (62.7±22.0)%.

In 60.6% of cases embryo transfer (ET) took place on day 5 of culture (blastocyst stage), in 22.5% on day 3, in 11.2% on day 4, in 4.5% on day 2 and in 1.2% on day 6. A total of 59 women had only one embryo transferred (single embryo transfer), 28 women had a transfer of two embryos (double embryo transfer) and 2 women had three embryos transferred. In 10 cases no embryo transfer was performed. The single embryo transfer rate was 66%. Pregnancy rate per ET counted 24.7 % (22 clinical pregnancies).

3.2. Embryo scoring

Within 16-18 h after fertilization the pre-embryo reached the pronuclear stage (presence of 2 pronuclei). Embryos were assessed in a detailed Embryo Scoring System between day 2 and day 5 or 6 of culture. The cleavage stage was noted on day 3, 4 and 5. A total of 52% of embryos on day 4 developed to a compacted embryo and 1.6% to the blastocyst stage. On day 5, 44% of embryos developed to blastocysts.

3.3. TNF alpha

Mean TNF alpha concentrations were significantly lower in the culture medium on day 5 than on day 3 (Table 1). The TNF alpha concentrations were significantly lower in culture media of pregnant women than in non-pregnant women on day 3 and 5 (Table 2). There was no significant difference in TNF alpha concentrations after SET or DET (double embryo transfer), but concentrations were significantly lower in pregnant women in SET as well as in DET cases (Tables 3 and 4).

Embryos which developed to the stage of a full expanded blastocyst showed a defined inner cell mass and high developed trophoectoderm on day 5. TNF alpha concentrations on day 5 showed no significant correlation with the development of the inner cell mass, although lower concentrations were measured in culture media of embryos with a well-developed inner cell mass (0.35±0.9 pg/mL vs. 0.38±0.1 pg/mL, n. s.). In culture media of embryos with highly developed trophoectoderm significant lower TNF alpha concentrations were found than in that of embryos with poorly developed trophoectoderm (0.33±0.1 pg/mL vs. 0.38±0.1 pg/mL, $P<0.05$).

3.4. LIF

Mean LIF concentrations were significantly higher in the culture medium on day 5 than on day 3 (Table I). Significant higher LIF concentrations were measured in the group of women who conceived after ET, than in women who did not conceive (Table 5). These results also applied in cases of

SET and DET (Tables 6 and 7). There was no significant difference in concentrations comparing SET-cycles and DET-cycles.

In culture media of embryos with highly developed trophoectoderm significant higher LIF concentrations were found than in that of embryos with poorly developed trophoectoderm (42.3±14.9 pg/mL vs. 35.5±16.1 pg/mL, $P<0.05$). The quality of the inner cell mass in blastocysts on day 5 showed no significant correlation with the LIF concentration.

Table 1

Mean concentrations of TNF alpha and LIF (pg/mL) in the IVF embryo culture medium on day 3 and day 5.

Item	Day 3	Day 5	P
TNF alpha	0.54±0.07	0.37±0.05	<0.05
LIF	31.5±11.6	35.5±10.7	<0.05

Table 2

Mean concentrations of TNF alpha (pg/mL) on day 3 and 5 in pregnant and not pregnant women.

TNF alpha	Pregnant	Not pregnant	P
Day 3	0.43±0.08	0.59±0.05	<0.05
Day 5	0.26±0.05	0.42±0.07	<0.05

Table 3

Mean concentrations of TNF alpha (pg/mL) in single embryo transfer (SET) cycles on day 3 and 5 in pregnant and not pregnant women.

TNF alpha SET	Pregnant	Not pregnant	P
Day 3	0.43±0.05	0.60±0.07	<0.05
Day 5	0.26±0.01	0.44±0.05	<0.05

Table 4

Mean concentrations of TNF alpha (pg/mL) in double embryo transfer (DET) cycles on day 3 and 5 in pregnant and not pregnant women.

TNF alpha DET	Pregnant	Not pregnant	P
Day 3	0.44±0.04	0.58±0.06	<0.05
Day 5	0.24±0.01	0.40±0.07	<0.05

Table 5

Mean concentrations of LIF (pg/mL) on day 5 in pregnant and not pregnant women.

LIF	Pregnant	Not pregnant	P
Day 3	56.2±13.7	22.2±9.3	<0.05
Day 5	59.7±14.9	25.9±10.1	<0.05

Table 6

Mean concentrations of LIF (pg/mL) in single embryo transfer (SET) cycles on day 3 and 5 in pregnant and not pregnant women.

LIF in SET	Pregnant	Not pregnant	P
Day 3	55.4±13.2	21.4±9.4	<0.05
Day 5	62.8±14.1	26.6±10.3	<0.05

Table 7

Mean concentrations of LIF (pg/mL) in double embryo transfer (DET) cycles on day 3 and 5 in pregnant and not pregnant women.

LIF in DET	Pregnant	Not pregnant	P
Day 3	57.9±13.3	22.3±9.1	<0.05
Day 5	52.5±12.9	25.0±9.8	<0.05

4. Discussion

It is well known that both TNF alpha and LIF play a key role in human reproduction. Nevertheless, there is controversy about whether TNF alpha has a favourable or a rather negative impact in embryonic development, implantation and pregnancy. Hunt *et al*. found that both TNF alpha and its receptors are expressed in the endometrium and ovary[4].

Here the TNF alpha seems to increase the permeability of the endometrium but also the proliferation of the endometrial epithelium is inhibited through the effects of TNF alpha[21]. A possible factor here is induced apoptosis processes in the epithelial cells of the endometrium. TNF alpha as well as its receptors were proven to be present in the ovary, in the endometrium, in the placenta and in the decidua and in the foetus itself [3-5,22]. It is probable that TNF alpha plays an important role in the progression of a normal pregnancy. However, many authors also ascribe negative properties to cytokine in terms of the pregnancy and embryonic development. A study by Silver *et al*. reported massage instances of haemorrhages and miscarriages following administration of a high bolus of TNF alpha[23].

LIF, on the other hand, has been expressed in numerous cells of the human body. Due to the variety of its actions, a precise differentiation of its effective mechanisms appears to be difficult. LIF can also be proven in most tissues of the reproductive tract in mammals and in humans specifically, including in the endometrium, oocytes and blastocystes. LIF was also formed depending on the menstruation cycle. It has its highest concentration during implantation. It seems possible that LIF is a critical parameter for the success of nidation in the uterus. Dunglison *et al*., among others, studied the effect of exogenetically added LIF to the culture medium[15]. Here, by adding LIF to the culture medium there was an increase in the blastocyst formation rate and an improved quality of the blastocysts.

Cytokines are not only discussed as part of embryonic development and implantation but also in conjunction with female infertility. Female indications which require assisted fertilization are numerous (tubular blockages, endometriosis and others), with causes that are elsewhere in women with habitual miscarriages and IVF/ICSI failures. While coagulation disorders and infections were discussed, another clarification attempt could also be dysregulation on the cytokine level.

In a study by Laird *et al*., both uteral fluid as well as tissue biopsies of the endometrium were obtained in three different groups of women: fertile women, idiopathically infertile women and women with frequent miscarriages[24]. There was a higher LIF level in the aspirated uteral fluid and the endometrial biopsies of fertile women. Hambartsoumian *et al*. were able give further credence to Laird *et al*[25]. The results in infertile women showed a significant dysregulation

of LIF production during the proliferation phase and the secretion phase. Unlike fertile patients, infertile women showed no LIF increase in the secretion phase. The dysregulation of the endometrial LIF production can thereby be a clarification for the occurrence of multiple implantation failures following assisted fertilization and idiopathic infertility. The assumption of many work groups that LIF has a positive effect on the early development and implantation of embryos has a high degree of probability. The use of LIF perhaps seems possible in the near future in assisted reproduction following development of recombinant LIF.

In this study, the concentration of TNF alpha was measured in the culture medium of the embryos. Thus, the goal should be to study whether the embryo expresses and secretes this cytokine itself during its development *in–vitro*. TNF alpha could be proven to be present in all samples. The average concentration of TNF alpha measured higher on day 3 than on day 5 and was significantly lower in pregnant women then in non–pregnant ones on both days. According to our results, the concentration of TNF alpha seems to be associated with the result of the pregnancy following IVF and ICSI. The data correlated here with a study performed in our work group from 2009 in which TNF alpha was proven in the follicle fluid of the dominant follicle in 197 patients which performed a IVF/ICSI program[8]. Also here the concentration of TNF alpha in pregnant women is significantly lower than in non–pregnant women. Both studies give credence to the assumption that high concentrations of TNF alpha have a negative conflict on implantation and the success of pregnancy.

The rate of the single embryo transfers (SET) was relatively high in this study at 66%. More and more reproduction centres also strive to achieve a SET. In young women under 35 years of age, this is a measure to avoid the risk of a multiple pregnancy. Reasons for the transfer of several embryos are implantation failures following a transfer and a strong restriction of female fertility. It was analysed in this study whether the TNF alpha concentration in a SET differs from that of the double embryo transfer (DET). No significant difference could be found. However, significantly lower concentrations were measured in the group of pregnant women both with SET as well as DET.

In the analysis of the possible correlation between embryonal morphology and the TNF alpha concentration it was shown that the TNF alpha neither directly correlated with the degree of maturity of the embryos nor with the number of blastocystes which had developed up to day 5 of the culture. Because, in pregnant women, significantly more blastocystes up to day 5 had developed and the TNF alpha concentration on day 5 was considerably lower in these patients, it could be assumed that, in the culture medium in which more blastocystes developed, there was also a reduced TNF alpha concentration. It cannot be assumed that the concentrations measured in the culture medium originated from the embryo itself because there could be no external effects during breeding through the *in–vitro* culture. The sequential media contained no cytokines which, in turn, would be able to induce the production of TNF alpha or LIF by the embryo.

The cytokine could also be proven to be present in the samples. The average concentration of LIF in the culture medium increased from 31.5 pg/mL on the 3rd day of the culture to 35.5 pg/mL on the 5th day. Physiologically, the embryo is in the stage of the fully expanded blastocystes at the time of implantation. On day 5 of the culture, 44% of the cultivated embryos of our study reached a blastocyste stage. The observed increase of the LIF concentration from the 3rd to the 5th day could reflect a possible increase in LIF expression through the embryo at the time of implantation.

Chen *et al*. studied the expression of LIF and its receptor transcripts (LIF–R) in mouse and human pre–implantation embryos[16]. In total, 30 of 34 human embryos expressed the cytokine LIF. *LIFR* mRNA could be demonstrated in 31 of 34 embryos. The expression of both transcripts showed a decrease in the embryos which were in a 4–cell stage or had idle growth for 24–48 h. Their results demonstrate that LIF and *LIF* mRNA are expressed during the pre–implantation period in human embryos and confirm our measured concentrations of human pre–implantation embryos in the culture medium.

Embryos in the early stage or with arrestation of their growth showed a significantly lower expression of the cytokine[16]. The role of LIF in embryonic development was studied in many work groups in the past few years and resulted in controversial results. In a study by Dunglison *et al*., superfluous human embryos which are provided by pairs according to IVF were bred in a LIF–enriched (1 000 IU/mL) culture medium[15]. The blastocyste formation increased following the use of LIF from 18% to 44% and the quality of the blastocystes increased from 10% to 33%. The addition of LIF in this study had a positive effect on embryonic development.

Embryos with a well–formed trophectoderm had significantly higher LIF concentrations in the culture medium than more poorly formed embryos. Comparison of the concentrations in the group of patients which were designed following transfer with those of the group of women who did not get pregnant showed a significantly higher LIF concentration in the group of pregnant women on day 3 and day 5. There could be a significant relationship between the LIF concentration and the success of the pregnancy. This applied both to women who became pregnant following a SET and following a DET. The culture medium of the embryos, which has resulted in a positive pregnancy result, had significantly more LIF than that of non–pregnant women.

Conflict of interest statement

We declare that we have no conflict of interest.

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