Possible therapeutic effect of magnesium sulfate in pre-eclampsia by the down-regulation of placental tumor necrosis factor-alpha secretion

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ABSTRACT. Objective. To examine the effect of magnesium sulfate (MgSO4) on tumor necrosis factor-alpha (TNF-α) secretion by preeclamptic placentas. Study design. Cotyledons of six, term, normotensive and ten, pre-eclamptic placentas were dually perfused for six hours (6h), with MgSO4 (6-7 mg %) in the maternal reservoir [normotensive (n = 3); pre-eclamptic (n = 5)], and with control medium (without MgSO4) [normotensive (n = 3); pre-eclamptic (n = 5)]. Perfusate samples from the maternal and the fetal circulations were collected every 30 min throughout the 6h of perfusion, and examined for TNF-α levels using ELISA. Statistical significance was determined using a 2-way analysis of variance. Results. Pre-eclamptic placentas perfused with control medium (without MgSO4) secreted higher levels of TNF-α into the fetal and the maternal circulations (1.60 ± 0.59 pg/mL/g of cotyledon and 14.28 ± 2.69 pg/mL/g of cotyledon, respectively), as compared to the fetal and maternal circulations of normotensive placentas (0.25 ± 0.09 pg/mL/g of cotyledon and 6.73 ± 1.11 pg/mL/g of cotyledon, respectively) (p < 0.01). Addition of MgSO4 to normotensive placentas did not affect TNF-α levels in the fetal or maternal circulations. However, exposure of pre-eclamptic placentas to MgSO4 significantly decreased TNF-α levels in both the fetal (0.89 ± 0.09 pg/mL/g of cotyledon versus 1.60 ± 0.59 pg/mL/g of cotyledon; p < 0.05) and the maternal circulations (4.74 ± 2.78 pg/mL/g of cotyledon versus 14.28 ± 2.69 pg/mL/g of cotyledon; p < 0.01). Conclusion. Down-regulation of placental TNF-α secretion by MgSO4 in pre-eclampsia might indicate a possible therapeutic effect for this agent in reducing maternal, endothelial dysfunction and in improving neonatal outcome in pre-eclampsia, by reducing TNF-α levels in maternal and fetal circulations.

Keywords: magnesium sulfate, placental perfusion, pre-eclampsia, tumor necrosis factor-alpha

Pre-eclampsia is a syndrome characterized by the onset of hypertension and proteinuria after 20 weeks of gestation. Additional signs and symptoms that can occur include visual disturbances, headache, epigastric pain, thrombocytopenia, and abnormal liver function [1]. These clinical manifestations result from mild to severe microangiopathy of target organs, including the brain, liver, kidney, and placenta [2]. Pre-eclampsia affects 3-5% of pregnancies worldwide, and is considered to be a leading cause of maternal and fetal morbidity and mortality. However, the etiology of this disease remains undefined [3]. The current theory concerning the development of pre-eclampsia proposes that pre-eclampsia is a two-stage disease. A combination of immunological, environmental and genetic factors results in impaired trophoblast invasion and defective placentaion. This may lead to a reduction in uteroplacental perfusion, resulting in placental ischemia/hypoxia. Ischemic conditions in the placenta, during the late stages of gestation, initiate induced-release of several angiogenic factors including pro-inflammatory cytokines into the maternal circulation. Consequently, systemic endothelial dysfunction and the clinical manifestations of pre-eclampsia are seen [4, 5]. Pro-inflammatory cytokines are thought to link placental ischemia with cardiovascular and renal dysfunction. The placenta is an integral component of this inflammatory response as it actively produces a variety of cytokines and immunomodulatory hormones [6, 7]. Blood pressure regulatory systems, such as the renin-angiotensin system (RAS) and the sympathetic nervous system, interact with pro-inflammatory cytokines, which affect angiogenic and endothelium-derived factors regulating endothelial function [6, 8].
Tumor necrosis factor-alpha (TNF-α) is a well known member of the TNF superfamily that is involved in numerous cellular processes. TNF-α activities, mediated through two distinct receptors TNFR1 and TNFR2, include regulation of cytokines expression, immune receptors, proteases, growth factors and cell cycle genes, which, in turn, regulate inflammation, survival, apoptosis, cell migration, proliferation and differentiation [9]. A number of groups have reported that circulating levels TNF-α are increased in women with pre-eclampsia [10, 11], suggesting its possible involvement in the pathogenesis of this disorder. However, there is a controversy about the expression of placental TNF-α levels in pre-eclampsia [12-14].

Magnesium sulfate (MgSO₄) is the drug of choice for the treatment of severe pre-eclampsia [15], prevention of eclampsia [16] and prevention of recurrent eclamptic seizures [17]. Previously, we have shown that MgSO₄ might selectively vasoconstrict the vasogenic and effect of angiotensin II (Ang-II) and endothelin-1 (ET-1) on placental vasculature [18]. Although the mechanism of action of MgSO₄ as an anticonvulsant agent in pre-eclampsia/eclampsia is not clearly understood, some possible mechanisms including vasodilatation of cerebral vasculature, inhibition of platelet aggregation, protection of endothelial cells from damage by free radicals, prevention of calcium ion entry into ischemic cells, decreasing the release of acetylcholine at motor end plates within the neuromuscular junction, and as a competitive antagonist to the glutamate N-methyl-D-aspartate receptor (which is epi-leptogenic), have been proposed [19].

Limited data have suggested that MgSO₄ may have neuroprotective effects on preterm neonates [20-22]. Although possible mechanisms by which MgSO₄ might be neuroprotective, such as blocking of glutamate receptors [23], have been suggested, the specific mechanism of this potential effect remains unclear. Inflammatory cytokines, such as TNF-α, interleukin (IL)-6 and IL-1β, have been linked to increased risk of neuronal morbidities including cerebral palsy (CP) [24-26]. Recently, our group reported that MgSO₄ may differentially affect the capacity of the fetal and the maternal compartments of normotensive human placenta to secrete the inflammatory cytokines TNF-α and IL-6, in presence of Ang-II [27]. Therefore, one of the potential mechanisms for the neuroprotective effects MgSO₄ might be by affecting the expression levels of these cytokines.

The current study was performed to compare the capacity of ex vivo-perfused, pre-eclamptic and normotensive placentas to produce TNF-α, and to examine the possible effect of MgSO₄ on the capacity of these placentas to produce TNF-α.

**METHODS AND MATERIALS**

**Study population**

After obtaining local institutional approval (no. 4188 and 4543), six placentas from term (37-40 weeks), normotensive pregnancies and ten placentas from term, pre-eclamptic pregnancies were collected immediately after vaginal or caesarean deliveries. Pre-eclampsia was defined as a new onset of hypertension and proteinuria after 20 weeks of gestation. Hypertension was defined as either a systolic blood pressure greater than 140 mmHg or diastolic blood pressure greater than 90 mmHg on two occasions at least four hours apart. Proteinuria was defined as either more than 300 mg per 24 hours, or 2+ or greater on urine dipstick. Women with pre-existing complications such as chronic hypertension, diabetes mellitus, autoimmune and renal diseases were excluded from the study. Women with intrauterine fetal death or women with preterm (less than 37 weeks of gestation) delivery were also excluded.

**Placental perfusion**

Three normotensive placentas and five pre-eclamptic placentas were perfused for six hours (h) with medium alone (control medium); and another three normotensive placentas and five pre-eclamptic placentas were perfused for 6h with medium containing MgSO₄ (6-7 mg%) in the maternal reservoir (maternal administration of standard doses of MgSO₄ for pre-eclampsia results in plasma levels of 4.8-7 mg%). The perfusion experiments were performed using the method previously described by Holcberg et al. [28] with certain modifications.

Within 15-20 minutes of delivery, a fetal artery and corresponding vein from an intact cotyledon (lateral cotyledons containing part of the membranes) were cannulated. Following successful establishment of the fetal circulation, the placenta was mounted in a perfusion chamber, and the maternal circulation was simulated by placing four catheters into the intervillous space of the lobe, corresponding to the isolated perfused cotyledon. Maternal perfusate that returned from the intervillous space was continuously drained by a maternal venous catheter, placed at the lowest level on the maternal decidual surface to avoid significant pooling of perfusate. Perfusion medium consisted of two liters (L) of M-199 cell culture medium [M-199 media (Sigma Chemicals Co., St. Louis, USA)], enriched with bovine serum albumin (1 gr/L), glucose (1 gr/L) (Sigma), heparin (10 IU/mL) (Bect Kama, Israel) and Gentamycin (40 μg/mL) (Teva, Petah Tekva, Israel). The pH of the medium was adjusted to 7.4 with bicarbonate (Sigma). The two reservoirs, containing the perfusion medium for the maternal and the fetal circuits, were placed into heated water baths at 37°C, and were equilibrated with a pre-humidified gas mixture of 95% O₂ and 5% CO₂ in the maternal reservoir and 95% N₂ and 5% CO₂ in the fetal reservoir. A perfusion pressure of 20-40 mmHg, giving a flow rate of 6-8 mL/min in the fetal circulation and 10-12 mL/min in the maternal circulation, was established. The venous return could be recycled into the respective reservoir, giving a closed circuit perfusion. Per fusate samples from the fetal and the maternal circulations were collected in each experiment every 30 minutes until the end of the perfusion, and stored at - 70°C until examined for TNF-α levels by enzyme linked immuno-assay (ELISA). The perfused cotyledon in each placenta was weighed at the end of the perfusion. ELISA results were normalized for gram of perfused cotyledon.
in order to minimize the error that may result from variations in perfused cotyledon weights from different placentas.

In order to minimize the possible effect of labor on placental release of cytokines, each normotensive or pre-eclamptic placent al cotyledon, either after vaginal or caesarean delivery, was perfused for 30 minutes with lactated Ringer’s [Hartman solution (Teva Medical, Ashdod, Israel)] followed by 30 minutes of perfusion with medium enriched with albumin (1 gr/L), glucose (1 g/L) and heparin (10 IU/mL). Subsequently, the perfusion medium in the fetal and maternal compartments was exchanged with fresh, enriched medium.

Validation of placental integrity for each experiment was established throughout the experimental period by ensuring that the rate of perfusate input in both the maternal and fetal circuits equaled the rate of output, and that histological examination of the cotyledon at the end of each experiment revealed no significant morphological changes.

**Examination of collected samples by ELISA**

TNF-α levels in the perfusate samples were measured by an enzyme-linked immunosorbent assay (ELISA), with mouse monoclonal anti-human TNF-α antibodies (first antibodies) (Biosource, Nivelles, Belgium), and mouse monoclonal anti-human TNF-α biotin-conjugated antibodies (second antibodies) (Biosource); the sensitivity of the kit was < 16 pg/mL, and the standard curve range was 4–20,000 pg/mL of recombinant human TNF-α (PEPROTEIN Inc., Rocky Hill, NJ, USA). The first antibodies were incubated overnight in 96-well ELISA plates at 4°C, followed by washing and addition of blocking buffer, consisting of 10% fetal calf serum (Beit HaEmek, Israel) in phosphate-buffered saline (PBS) (Beit HaEmek, Israel) for 2 h at 37°C. Thereafter, blocking buffer was removed, and samples or recombinant TNF-α were added for 1 h incubation at 37°C. After washing, the second antibodies were added and plates were incubated for an additional 1 h at 37°C. After incubation, plates were washed, and Streptavidin HRP was added for 30 minutes (min) at 37°C. After washing, tetramethyl benzidine (TMB) (DakoCytomation Inc., CA, USA) was added for 15 min and the reaction was stopped by adding 2N H2SO4. Optical absorbance was read using an ELISA reader (Model 550) (Biorad, CA, USA) at 450 nm.

**Statistical analysis**

Statistical analysis was performed with the SPSS package (SPSS, Chicago, IL, USA). Continuous parameters were summarized as mean ± standard deviation (SD), and examined using Student’s t-test. Categorical parameters were summarized using frequency measures, and statistical analysis was performed using the Fisher’s exact test. Comparisons of TNF-α levels in normotensive versus pre-eclamptic, and in control groups versus MgSO4 groups were performed using two-way analysis of variance (ANOVA), P < 0.05 was considered statistically significant.

**RESULTS**

**Table 1** summarizes the clinical data of the participants. There were no significant statistical differences in maternal age, gravidity and parity, BMI or mode of delivery between the normotensive and pre-eclamptic groups. However, the gestational age at delivery in the pre-eclamptic group was significantly lower as compared with the normotensive group (37.3 ± 2.4 weeks and 40 ± 1.8 weeks, respectively; p < 0.05). Systolic and diastolic blood pressure in the pre-eclamptic group were significantly higher in the pre-eclamptic group (158.6 ± 12.7 versus 103.4 ± 9.9, respectively) as compared to the normotensive group (128.7 ± 8.9 versus 75.8 ± 2.7, respectively; p < 0.001), as expected.

**Pre-eclamptic placentas secrete higher levels of TNF-α than normotensive placentas**

TNF-α levels in the fetal circulation of pre-eclamptic placentas perfused with control medium (without MgSO4) increased with time, reaching significantly higher levels after 330 min of perfusion (1.60 ± 0.59 pg/mL/g of cotyledon), as compared to TNF-α levels in the fetal circulation of normotensive placentas (0.25 ± 0.09 pg/mL/g of cotyledon; p < 0.01) (figure 1A). In the maternal circulation, TNF-α levels also increased with time, reaching

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normotensive</th>
<th>Pre-eclamptic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>- Control group</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>- MgSO4 group</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Maternal age (Y)</td>
<td>25 ± 4.6</td>
<td>25.4 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Gravidity (# pregnancies)</td>
<td>2.3 ± 2</td>
<td>1.5 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Parity (# deliveries)</td>
<td>2.3 ± 2</td>
<td>1.1 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.6 ± 0.7</td>
<td>31.3 ± 4.5</td>
<td>NS</td>
</tr>
<tr>
<td>Mode of delivery (PSd:CSe)</td>
<td>6:0</td>
<td>7.3</td>
<td>NS</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>40 ± 1.8</td>
<td>37.3 ± 2.4</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>128.7 ± 8.9</td>
<td>158.6 ± 12.7</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.8 ± 2.7</td>
<td>103.4 ± 9.9</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Proteinuria (urine dipstick)</td>
<td>0</td>
<td>+3</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

*Plus-minus values are means ± SD.

Statistical analysis between the normotensive and the pre-eclamptic groups was performed using Student’s t-test. Categorical parameters were summarized using frequency measures, and statistical analysis was made using Fisher’s exact test.

*Not significant.

*Vaginal delivery.

*Caesarean delivery.
Effect of magnesium sulfate on placental tumor necrosis-alpha secretion

The levels of TNF-α secreted into the fetal and the maternal circulations of normotensive placentas were not affected by the addition of MgSO₄ to the maternal reservoir of the perfused normotensive placentas (figure 2A and B). However, addition of MgSO₄ to the maternal reservoir of pre-eclamptic placentas resulted in a significant decrease in secreted levels of TNF-α into the fetal and the maternal circulations (figure 3A and B).

Perfusion with MgSO₄ resulted in decreased TNF-α levels in both the fetal and maternal circulations of pre-eclamptic placentas compared to perfusion with the control medium (fetal: 0.89 ± 0.09 pg/mL/g of cotyledon versus 1.6 ± 0.59 pg/mL/g of cotyledon, after 330 min of perfusion; p < 0.05, and maternal: 4.74 ± 2.78 pg/mL/g of cotyledon versus 14.28 ± 2.69 pg/mL/g of cotyledon at the end of perfusion; p < 0.01, respectively) (figure 3A and B).

Moreover, TNF-α levels detected at the end of perfusion in the fetal and maternal circulations of pre-eclamptic placentas exposed to MgSO₄ (0.89 ± 0.09 pg/mL/g of cotyledon and 4.74 ± 2.78 pg/mL/g of cotyledon, respectively), were statistically equal to TNF-α levels in the fetal and maternal circulations of normotensive placentas perfused with control medium (0.43 ± 0.29 pg/mL/g of cotyledon and 6.73 ± 1.11 pg/mL/g of cotyledon, respectively) (figure 4A and B).

In the presence of MgSO₄, no significant differences were detected in TNF-α secretion into the fetal as compared to the maternal circulation of normotensive placentas (table 2). However, pre-eclamptic placentas

**Table 2**

<table>
<thead>
<tr>
<th>Placental group</th>
<th>Fetal circulation*</th>
<th>Maternal circulation*</th>
<th>P-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensive (Control)</td>
<td>0.43 ± 0.29</td>
<td>6.73 ± 1.11</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Pre-eclamptic (Control)</td>
<td>1.74 ± 1.14</td>
<td>14.28 ± 2.69</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Normotensive (MgSO₄)</td>
<td>0.67 ± 0.58</td>
<td>6.5 ± 6.97</td>
<td>NSc</td>
</tr>
<tr>
<td>Pre-eclamptic (MgSO₄)</td>
<td>0.54 ± 0.24</td>
<td>4.74 ± 2.78</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

* Plus-minus values are means ± SD of pg cytokine/mL/g of cotyledon.

**Figure 1**

TNF-α levels in the fetal (A) and the maternal (B) circulations of normotensive (n = 3) and pre-eclamptic (n = 5) placentas after six hours of perfusion with control medium (without the addition of MgSO₄). Results are displayed as mean ± SD.

* p < 0.05 and ** p < 0.01; (2-way ANOVA).

**Figure 2**

Final TNF-α levels in the maternal circulation as compared to fetal circulation of normotensive and pre-eclamptic placentas
DISCUSSION

MgSO₄ is the most frequently used agent for the treatment of pre-eclampsia [15]. Despite its widespread use, reliable data on its mechanism of action in the treatment of this disorder are remarkably few. The reports about the role of the placenta in the increase in serum inflammatory cytokine levels are conflicting. However, an increasing number of studies have suggested that MgSO₄ may act as an anti-inflammatory agent affecting the expression of inflammatory cytokines in pre-eclampsia. Using an ex vivo, placental perfusion system, this is the first report showing over-secretion of TNF-α by pre-eclamptic as compared to normotensive placentas, and that MgSO₄ significantly decreases these higher levels of TNF-α. Our current study suggests that pre-eclamptic placentas secrete higher levels of the inflammatory cytokine TNF-α into both the fetal and maternal circulation, as compared to normotensive placentas. Exposure to MgSO₄ significantly reduces the induced TNF-α secretion into both circulations.

Although, elevated levels of TNF-α and its soluble receptors in serum and placentas of pre-eclamptic women have been recently reported, others have not found changes in placental TNF-α expression [9], suggesting that the increase in serum TNF-α levels seen in pre-eclampsia is not caused by the placenta. Our results show that high levels of TNF-α were detected in the maternal circulation of pre-eclamptic placenta over six hours of perfusion. These findings suggest that the placenta may be responsible for the increase in serum TNF-α levels, thus contributing to the pathophysiology of pre-eclampsia. The discordance between our results and other published data may be related to different techniques used (placental perfusion versus immediate sampling of placental tissue after placental delivery), or due to different assay systems used to measure TNF-α levels. Higher plasma levels of TNF-α were found to be associated with the severity of the pre-eclampsia, suggesting TNF-α as a possible marker of severity [29]. TNF-α is also known as one of the vital circulating factors that mediate endothelial cell activation/dysfunction. It does so through regulation of a wide range of physiological processes in these cells, including prostaglandin production, expression of adhesion molecules and alteration of the balance between oxidant and anti-oxidant molecules [30]. Taking into consideration our current results, the placenta may play a key role in determining the severity of pre-eclampsia in its later stages.

Our results also suggest that the pre-eclamptic placenta secretes increased levels of TNF-α into the fetal circulation. Since TNF-α, as well as other inflammatory cytokines, have been linked to increased risk of CP [24-26], our data may add support to the leading role of pre-eclampsia in neonatal morbidity. Increased TNF-α secretion into the fetal circulation may result in increased levels of this cytokine in the fetal serum, increasing the risk of neonatal brain damage. The decreased levels of TNF-α secreted into the maternal circulation of pre-eclamptic placentas achieved with MgSO₄, may indicate a possible therapeutic effect in pre-eclampsia. MgSO₄ may act as an anti-inflammatory agent on the feto-placental interface by reducing the

perused with MgSO₄ still secreted significantly higher levels of TNF-α into the maternal circulation (4.74 ± 2.78 pg/mL/g of cotyledon) as compared to the fetal circulation (0.54 ± 0.24 pg/mL/g of cotyledon; p < 0.05).
secretion of the inflammatory cytokine TNF-α. In this manner, MgSO₄ may reduce the inflammatory process in the blood vessels of the pre-eclamptic women. On the other hand, the similar reducing effect of MgSO₄ on the secretion of TNF-α into the fetal circulation suggests a possible neonatal neuroprotective effect in pre-eclampsia. Our results are in agreement with recently reported data regarding the effect of MgSO₄ on inflammatory cytokine production. Makhlof et al. showed that MgSO₄ suppresses endotoxin-stimulated IL-8 production by amniotic and decidual cells in vitro [31]. Moreover, it has been suggested that magnesium deficiency is associated with increased inflammatory responses [32-34].

Previously, we have shown that MgSO₄ and Ang-II may differently affect the capacity of the fetal and the maternal compartments of normotensive human placenta to secrete TNF-α [27]. In the current study, we have shown similar levels of TNF secreted from the maternal side of normotensive perfused placentas in the presence or absence of MgSO₄. However, in the previous study, we compared the effect of MgSO₄ to Ang-II or AngII + MgSO₄ on TNF secretion. In addition, a different protocol was used in the current study (MgSO₄ was added after three hours of perfusion in the previous study; in the current study, MgSO₄ was added from the beginning of the perfusion), because of the sensitivity of the pre-eclamptic placentas used. This could lead to differing interpretations between the two studies.

Nuclear factor kappa B (NFκB) is a nuclear transcription factor that regulates numerous inflammatory mediators. Recently, Rochelson et al. reported that MgSO₄ inhibits endothelial cell activation by reducing the nuclear translocation of NFκB and by protecting its cytoplasmic inhibitor (IkBα) from degradation [35]. These data may provide the missing link to explain the mechanism by which MgSO₄ reduces the inflammatory state in the blood vessels of pre-eclamptic patients.

The fact that TNF-α secretion by the normotensive placenta is not affected MgSO₄ suggests that the effect of MgSO₄ is specific to the pre-eclamptic placenta. This might indicate a different mechanism of regulation of TNF-α secretion in pre-eclampsia as compared to normotensive pregnancies.

In conclusion, MgSO₄ may normalize increased secretion of placental TNF-α in pre-eclampsia. This suggests a possible therapeutic effect of MgSO₄ that acts by reducing TNF-α levels in maternal and fetal circulations, thereby reducing maternal endothelial dysfunction and improving neonatal outcome in pre-eclampsia.

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