CLINICAL STUDY

# Maternal Gram-Negative Bacterial Infection Induced Apoptosis of the Implanting Blastocyst

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**Received 28 September 2006; received in revised form 20 October 2006; accepted 31 October 2006; published online 02 November 2006**

## Abstract

**Objective:** Genital tract infections of gram-negative bacteria lead to a pathogenic condition known as bacterial vaginosis. Endotoxins (lipopolysaccharides: LPS) are the main antigenic components of these bacteria and can trigger the immune system through the secretion of proinflammatory cytokines. Many of the cytokines are known to be involved in the process of embryonic development and implantation of the blastocyst on to a receptive endometrium for successful pregnancy to occur. In earlier studies, in a mouse model it was shown that the normal pattern of expression for IL-1, TNF-α, CSF-1 etc., in the embryos and uterine horns are altered when the animals are exposed to a 'minimum dose' (MD) of LPS. The current study investigates whether LPS can cause apoptosis in the preimplantation stage embryos as an end effect.
**Material and Methods:** 5 μg LPS were injected per mouse (weighing ≈ 20 gr) on day 0.5 of pregnancy. The degree of apoptosis induced by the MD of LPS was assessed by comet assays on individual preimplantation stage embryos.
**Results:** MD of LPS was found to cause cell deaths in more than 88% of the embryos by the time they reach to the stage of implantation.
**Discussion:** We conclude that LPS can cause implantation failure and pregnancy loss due to apoptosis of blastomeres during embryonic development to the blastocysts *in vivo*.

**Keywords:** lipopolysaccharides (LPS), blastocyst, implantation, pregnancy, apoptosis

## Özet

### Maternal Gram-Negatif Bakteriyel Enfeksiyonlar İmplante Olan Blastokistlerin Apoptozisini Artırmaktadır

**Amaç:** Gram-negatif bakteriler, bakteriyel vajinöz olarak adlandırılan genital yol enfeksiyonlarına yol açmaktadır. Endotoksinler (LPS: lipopolisakkaridler), bu bakterilerin ana antijenik komponentlerini oluşturmakta ve proenflamatuvar sitokinlerin sekresyonu aracılığı ile bağışıklık sistemini aktive etmektedir. Bu sitokinlerin birçoğu sağlıklı bir gebelik için embriyonik gelişim ve blastokistin reseptif endometriyuma implantasyonu aşamasında rol sahibidirler. Önceki bazı çalışmalarda fare modelinde embriyolar ve uterin korunuda IL-1, TNF-α, CSF-1, vb. sitokinlerin ekspresyon paterninin ortama "minimum doz" (MD) lipopolisakkaridlerin eklenmesi ile değiştiği saptanmıştır. Bu çalışmada, lipopolisakkaridlerin bir sonuç olarak preimplantasyon aşamasındaki embriyolarda apoptozise yol açıp açmadıkları incelenmiştir.
**Materyal ve Metot:** Her fareye (yaklaşık 20 gr ağırlığında) 5μg lipopolisakkarid enjeksiyonu gebeliğin ilk 12 saati içinde yapılmıştır. Minimum doz lipopolisakkarid ile indüklenen apoptozisin derecesi her bir preimplantasyon aşamasındaki embriyo için ayrı ayrı değerlendirilmiştir.
**Sonuçlar:** Minimum doz lipopolisakkarid enjeksiyonu implantasyon aşamasına gelmiş embriyoların >%88'inde hücre ölümüne yol açmıştır.
**Tartışma:** Lipopolisakkaridler, *in vivo* blastokistlerde embriyonik gelişim sırasında blastomer apoptozisi yolu ile implantasyon başarısızlığı ve gebelik kaybına yol açmaktadır

**Anahtar sözcükler:** lipopolisakkarid, blastokist, implantasyon, gebelik, apoptozis

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## Introduction

*Escherichia coli, Klebsiella, Salmonella, Serratia, Pseudomonas* are some of the common Gram-negative bacteria which populate the genital tract of women and cause infections (1,2). Endotoxins are the most injurious and fatal component of the Gram-negative bacterial cell wall (3). Although antibiotics for killing the bacteria are available, there is no effective way to stop the toxic effects of the endotoxins once it has entered the bloodstream. When endotoxins or lipopolysaccharides (LPS) enter the bloodstream, the body's defense system releases inflammatory substances and causes fever to help fight the infection (4). If the body's defense system is overwhelmed by the multiplying bacteria endotoxin, then the immune responses "overreact" causing tissue destruction, a condition called endotoxic shock.

In previous studies, we have shown that a very low dose of LPS 250 μg/kg body weight termed as the 'minimum dose' (MD) of LPS is enough for causing pregnancy loss, in a mouse model (5). LPS is also known to cause changes in the embryonic and uterine expressions of some of the proinflammatory cytokines like IL-1, TNF-α, CSF-1 etc., (5-7,9). However, it is not known how such alterations in their expressions render the embryos inefficient for implantation. Some of these cytokines are known for their ability to induce cell death. We were, therefore, interested in detecting the extent of apoptosis induced by this MD of LPS during the preimplantation period of pregnancy. The presence of a very high degree of apoptosis in the blastomeres of the embryos approaching implantation indicates a possible reason for the observed loss of implantation or pregnancy.

## Materials and Methods

Park strain mice used for this study were maintained at our animal care facility as described in a previous study (5). The detection of the minimum dose of LPS "MD" used in this study is described earlier (5). LPS was injected to pregnant females on day 0.5 of pregnancy, in all the experiments. The morphology of the recovered blastocysts were examined and evaluated by visual examination under the microscope (8).

**Superovulation and embryo recovery**

Park strain mice were maintained, superovulated, and mated as described earlier (5). Female mice were killed by cervical dislocation on day 4.375 of the preimplantation period of pregnancy. The embryos were recovered on each day of pregnancy by flushing the excised oviducts and uterine horns with sterile PBS in sterile endotoxin free petri-dishes (8). The recovered embryos were examined under a microscope (Leica DM IL, Leica Microsystems, Germany) and their morphology was studied using 20x and 40x objectives. Morphologically unusual and degraded embryos with degenerating blastomeres and signs of growth arrests were considered as developmentally compromised and abnormal.

**Detection of DNA damage in individual embryos by comet assay**

Detection of DNA damage in individual embryos was carried out with a slight modification of the method described by Takahashi (10). The collected embryos were washed in PBS. Thirty embryos in each experimental group were transferred to a 100 μl drop of 1% agarose with low melting temperature in PBS at 37°C. The agarose drop was placed on a 35 mm plastic Petri dish. Using a stereo dissecting microscope to visualize the embryos, the embryos were gently mixed with the agarose, and then captured in a total volume of about 30 μl with a micropipette. The embryos were quickly placed as a drop on a microscope glass slide which was initially coated with 1% agarose with high-melting temperature. The slides were placed on ice for 5 min to solidify the agarose. The blastocysts were incubated in lysing buffer (1.25 M NaCl, 100 mM Tris-HCl, pH 10, 50 mM EDTA, 1% Triton X-100 and 10% DMSO) for 3 hours at ambient temperature to denature DNA (and dissociate DNA-associated proteins) and to degrade RNA.

The slides were removed from the lysing buffer and washed with chilled DDW. The slides were placed on a horizontal gel electrophoresis unit (Pharmacia, USA) filled with fresh electrophoresis buffer (10N NaOH, 200 mM EDTA, 0.0004% dimethylsulphoxide [DMSO], pH 13.0) to a level of 25 mm above the slides. The slides were equilibrated in the electrophoresis buffer for 20 min. to allow the unwinding of DNA under alkaline conditions. The electrophoresis was conducted for 25 min. at 25V, 4°C using an electrophoresis compact power supply (BIO-RAD, USA). After electrophoresis, the slides were neutralized by adding neutralization buffer (0.4 M Tris-HCl, pH 8.0) drop-wise for 5 min. at 4°C. The DNA was detected by adding a 50 μl drop of ethidium bromide (1 μg/ml) to the slides for 10 min. The stained slides were washed with chilled double distilled water (DDW) and observed under a fluorescence microscope (Leica DM4000B, Leica Microsystems, Germany) with an excitation filter of 515-560 nm and a barrier filter of 590 nm.

The photographs of the individual embryos were taken by Leica Digital DFC 320R-II camera (Leica Microsystems, Germany). All the steps were conducted in the dark to prevent additional DNA damage. Comet tail length was calculated by measuring the streak of DNA comet tail between the edge of the *zona pellucida* and the end of the visible comet tail through an Image Analysis System (Leica Qwin, Leica Microsystems, Germany). The results of each experiment were analyzed by using one way analysis of variance (ANOVA) with Duncan's multiple range test for comparison of the significance level ($p$) between control and treated values. A $p<0.05$ value was considered as significant difference between the values compared.

## Results

The implantation of blastocyst stage embryos occur on day 4.5 of pregnancy in mouse. This was confirmed by pontamine

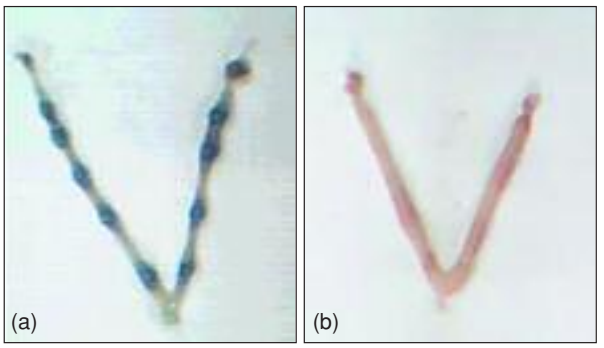


**Figure 1.** Pontamine blue dye test showing implantation sites (day 5.5 of pregnancy) in control and LPS treated animals. (a) Control (b) LPS treated uterine horns.

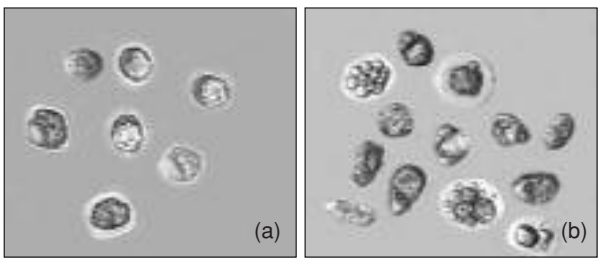


**Figure 2.** Morphology of preimplantation stage blastocysts (day 4.375 of pregnancy) collected from superovulated (a) control (b) LPS treated pregnant females.

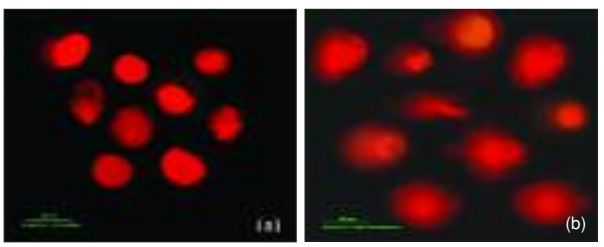


**Figure 3.** Comet assay showing the degree of apoptosis induced in control and LPS treated blastocysts on day 4.375 of pregnancy, collected before implantation. Panel (a) shows normal embryos and (b) shows embryos from LPS treated animals.

blue dye tests as shown in Figure 1a. Injection of 5 µg LPS per mouse on day 0.5 of pregnancy resulted in complete loss of pregnancy as shown by the absence of pontamine blue dye positive sites in the uterine horns on day 5.5 of pregnancy, as shown in Figure 1b.

The blastocyst stage embryos were flushed on day 4.375 of pregnancy from both control or normal and LPS treated animals (Table 1). There were 19.51±1.17% abnormal embryos on day 4.375 of pregnancy in normal animals collected after super-ovulation (Figure 2a). However, only 10% of them were apoptotic with an average comet tail length of 21.6±1.02 µm (Table 2, Figure 3a). The LPS treated animals show severely degenerated embryos 88.96±1.73% (Figure 2b) on day 4.375 of pregnancy, 100% of them were apoptotic, with an average comet tail length of 71±5.93 µm (Figure 3b). This is significantly higher than that of the control group.

## Discussion

Gram-negative bacterial infections of the genital and urinary tracts of pregnant women are known to cause fetal abortions or pregnancy losses (2). Previous studies have shown that various proinflammatory cytokines like TNF, IL-1, IL-6 etc., are involved in such pathogenic conditions (5-7,9). However, all these cytokines may or may not be directly involved in the embryopathy of infected women during pregnancy. Cytokines are usually secreted by both the embryos as well as the uterine endometrium and are also thought to be necessary for normal embryonic growth and development. It was found that a disturbance in the pattern and level of expression of IL-1, TNF-α and CSF-1 occurs, in the embryos and uterine horns of the pregnant animals, which leads to poor development of the embryos to the blastocyst stage, and at the same time renders the uterus non-receptive to the implanting embryos (5-7,9).

The minimum dose "MD" of LPS used in the present study causes 100% pregnancy loss in mice. When the uterine horns were flushed to check if the LPS had an effect on the development of the embryos, we found that about 88.96% of them were morphologically abnormal with severe signs

**Table 1.** Effect of the 'minimum dose' of LPS on development of preimplantation stage embryos collected from superovulated pregnant females

| Days of pregnancy used | No. of animals | Control Animals* | | | LPS Treated Animals* | | |
|---|---|---|---|---|---|---|---|
| | | Total no. of embryos recovered | No. of abnormal embryos recovered | % Abnormal embryos | Total no. of embryos recovered | No. of abnormal embryos recovered | % Abnormal embryos |
| 4.375 | 3 | 59±1.00 | 11.50±0.50 | 19.51±1.17[a] | 45±2.00 | 40±1 | 88.96±1.73[b] |

*Data are expressed as mean ± 1SEM, with all values given in % of abnormal embryos. Means bearing non-similar superscripted alphabets are different from each other at $p \leq 0.05$ (based on Duncan's multiple-range test)

**Table 2.** Effect of the 'minimum dose' of LPS on the degree of apoptosis in preimplantation stage embryos collected from superovulated pregnant animals

| Days of pregnancy used | No. of embryos used | Control Animals* | | LPS Treated Animals* | |
|---|---|---|---|---|---|
| | | % of embryos having tail | Mean tail lenght (μm)* | % of embryos having tail | Mean tail lenght (μm)* |
| 4.375 | 30 | 10 | 21.6±1.02[c] | 100 | 71±5.93[d] |

*Data are expressed as mean ± 1SEM, with all values given in Mean tail length μm. Values bearing non-similar superscripted alphabets differ from other each at $p \leq 0.05$ (based on Duncan's multiple-range test)

of degeneration and loss of formation of a blastocoel cavity. The embryos were tested for apoptosis by comet assay and 100% of the tested embryos exhibited DNA tailing by comet assay. In the normal mice about 19.51% of the embryos were morphologically abnormal. The number of degenerated blastocyst is probably a bit higher than usual because of the fact that these were collected after superovulation. However, only 10% of them were apoptotic as detected by comet assay. This probably indicates that the remaining 9.51% of the morphologically abnormal embryos did not have any apoptotic nuclei in their blastomeres, though they were morphologically abnormal. It is possible that the blastomeres of these embryos were preparing to enter apoptosis, after some time, and therefore could not be detected at this time point (on day 4.375). The normal embryos showed a significantly smaller DNA tail length than the embryos collected from LPS treated animals. The longer tail length indicates a higher degree of apoptosis in the LPS treated embryos as compared to the normal. However, the high degree of apoptosis induced by LPS indicates a possible reason for pregnancy loss. This also supports our earlier observations, and indicates that the disturbances in the embryonic and or uterine secretion of cytokines like TNF-α, CSF-1, IL-1α and IL-1β can probably trigger the apoptotic pathways in the blastomere of the developing embryos. The pathways involved in this process of embryonic pathogenesis is not known and there could also be other possible molecular players or pathways involved. Further studies to decipher the underpinning mechanism are being carried out in our laboratory.

**Acknowledgements**

We thank the All India Council for Technical Education (AICTE), New Delhi, India; The Rockefeller Foundation, New York, USA for financial support to YKJ; and the Council for Scientific and Industrial Research (CSIR), New Delhi for a Junior Research Fellowship and Senior Research Fellowship to VA. We thank Dr. I.K. Patro for the generous gift of LPS for research work.

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