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Research Article

Multi-Drug Resistant Pathogenic Bacteria Caused Intrauterine Infection in Albino Rat Mediated Preterm Labour

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Abstract

Background and Objective: Preterm birth is one of the important causes of neonatal mortality and morbidity in developing and developed countries. It is induced by various factors, including bacterial infections. However, bacterial multidrug resistance leads to intrauterine inflammation, which is difficult to be cured. Therefore, we hope to find out the pathogenic mechanism of bacteria to alleviate the development of gynaecological inflammation. **Materials and Methods:** Live and heat-killed multi-drug resistant pathogenic bacteria (*Escherichia coli* ATCC 25586, *Streptococcus pyogenes* ATCC 19615 and *Fusobacterium nucleatum* ATCC 25586) were inoculated to Albino rat. Cytokine level in experimental and control animal was analyzed to determine the inflammation in the brain tissues. Then preterm delivery was analyzed in experimental animals and compared with control. **Results:** The preterm delivery varied between 20% and 75% in experimental animals. The amount of IL-1 β increased in the brain of live *S. pyogenes* treated animals on day 1 of birth and it was high than other animals infected with *E. coli* and *F. nucleatum* ($p < 0.001$). This trend was also observed on IL-6 and tumour necrosis factor in the experimental animals treated with bacteria. Among these three bacterial strains, the live cells of *S. pyogenes* showed 75% preterm delivery. **Conclusions:** It is concluded that multi-drug resistant pathogenic bacteria-induced pre-term delivery in Albino rat and preterm labour was mainly associated with intrauterine inflammation and brain injury induced by pathogenic bacteria.

Key words: Pathogenic bacteria, uterine infection, Pre-term labour, elevated cytokine, brain injury

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Preterm birth is defined as delivery before 37 weeks of gestation and it is a serious obstetric and health problem. It is one of the important causes of neonatal mortality and morbidity in developing and developed countries. Preterm birth is the result of either medically indicated interventions or spontaneous developments. Extrauterine or intrauterine infections, placental abruption, hormonal disruptions, multiple gestations and other factors involved in spontaneous preterm labour¹. However, a large number of preterm cases were reported without any cause or called 'idiopathic'. It was estimated that about 40% of preterm births are associated with intrauterine infection². Bacterial pathogens caused inflammation within the gestational tissue and were involved in preterm births. The amniotic fluid of women infected with pathogenic bacteria showed more microbial colonies and inflammatory cytokines were also high than term labours³. The systemic administration of various bacteria or bacterial products to pregnant animals induced preterm delivery and labour⁴. Also, periodontal disease, pneumonia and pyelonephritis have been linked with premature parturition⁵⁻⁷.

Patients with intrauterine inflammation or intra-amniotic infection and subclinical intrauterine infections are associated with preterm delivery⁸. Animal models have been proven in developing an observation of the pathophysiology and physiology of parturition. In animal models, especially mice various inflammatory stimuli have been generated to analyze preterm delivery. Rabbits and other non-human primates have also been used to determine preterm delivery^{9,10}. Clinical studies revealed that multiple drug-resistant bacteria from the intrauterine region are associated with spontaneous preterm birth mainly associated with prelabour rupture of membranes¹¹. Animal model studies revealed that inoculation with the bacterial toxin, or live bacteria induce preterm birth. It was found that ascending vaginal bacterial infection is the general route by which pathogenic bacteria gain access into the uterine cavity of women¹². The bacterial species isolated from the placenta and fetal membranes were similar to the bacteria commonly found in the lower genital tract. Investigation of preterm birth using the animal model provides the mechanisms that regulate inflammation, infection and preterm parturition. Ascending vaginal infections in animal models help give data on preterm delivery based on infection and in evaluating new approaches that could be generally clinically translatable. In recent years, ascending vaginal infections were experimentally analyzed using murine models of various bacteria including, *Ureaplasma urealyticum*, *Escherichia coli* and *Streptococcus*¹³⁻¹⁵.

Cerebral palsy, preterm birth and intrauterine inflammation are very much associated with preterm birth and described in recent years^{16,17}. Mice model analysis revealed that lipopolysaccharide at low concentrations did not induce preterm delivery. Preterm delivery is mainly associated with less virulence pathogenic bacteria from the genus *Ureaplasma* and has been isolated from the amniotic cavity of individuals with preterm chorioamnionitis¹⁸. Administration of lipopolysaccharide to pregnant mice through significantly induced cytokines level and preterm labour¹⁹. Also, inoculation of live *Escherichia coli* through intracervical or intrauterine region induced elevated levels of cytokines and induced preterm labour²⁰. In a study, rhesus monkeys have been used as a model organism to analyze bacteria-induced preterm delivery. Findings have been showing a positive correlation between B *Streptococcus* infection and preterm labour. This has been mediated by prostaglandin and cytokine production²¹. Production of elevated levels of cytokines in the immune system at the time of maternal infection is highly harmful to infants, mainly damage the brain tissues of the newborn²². Inflammation and intrauterine infection constitute potential risk factors for adverse neurological disorders in preterm infants. The recent finding revealed that female individuals delivered preterm had increased numbers of *Gardnerella*, *Shigella*, *Escherichia*, *Streptococcus*, *Dorea*, *Megasphaera*, *Gemella*, *Sneathia* and *Atopobium* as well as decreased *Lactobacilli* species in vagina²³. Genital tract infection is very much associated with preterm birth and about 25-40% of preterm birth was associated with genital tract infection. However, these ranges may vary based on the methods of analysis²⁴. In bacteria, specific growth-inhibiting peptides have been associated with the regulation of various cellular processes. These include multidrug tolerance, pathogenesis, biofilm formation, cell differentiation, stress response and quorum sensing²⁵. Bacterial vaginosis was associated with preterm premature rupture of membranes, chorioamnionitis, spontaneous abortion and amniotic fluid infection. These results from the changes of specific vaginal microbial flora, bacteria from the genus *Lactobacillus*, with other anaerobes such as *Mycoplasma hominis*, *Mobiluncus* spp., *Bacteroides* spp. and *Gardnerella vaginalis*²⁶. The sexually transmitted diseases in lower genital infections due to *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis* populations were highly associated with preterm birth²⁷.

The main objective of the study is to analyze the intrauterine inflammation-associated brain injury and preterm labour in Albino rats.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Obstetrics, Central Hospital Affiliated to Shandong First Medical University Lab, China from May, 2020-July, 2021.

Experimental animal: Albino rats were reared and treated following the guidelines for the care of laboratory experimental animals. The experimental animals were maintained in an animal house with good aeration and approved by the ethical committee of Central Hospital affiliated to Shandong First Medical University, Shandong Province, China. The temperature of the animal house was $24 \pm 2^\circ\text{C}$ and 12 hrs light and dark photoperiod was maintained throughout the experiment. The experimental animal was acclimatized in the animal house before mating. All experimental animals were allowed free access to water and food. Female rats between 8 and 14 weeks (250-350 gm) were allowed to mate with potent males. All-female animals were subjected to vaginal plug analysis to confirm mating. Analysis was conducted 17th day of a 21-22 day of Albino rat's pregnancy.

Pathogenic bacteria and inoculum preparation: The pathogenic bacterial strains, *Escherichia coli* (ATCC 25586), *Streptococcus pyogenes* (ATCC 19615) and *Fusobacterium nucleatum* (ATCC 25586) were cultured in a Nutrient broth medium. The culture was incubated for 18 hrs and at $37 \pm 2^\circ\text{C}$. Both heat-killed bacteria and live bacteria were used to analyze the induced preterm delivery in rats. For the preparation of heat-killed bacteria, the bacterial strains were cultured in a Nutrient broth medium and centrifuged at $5000 \times g$ for 10 min. The precipitated bacteria were washed with Phosphate-Buffered Saline (PBS) and maintained the final concentration as 1.5×10^{12} bacteria mL^{-1} . It was serially diluted and plated on a Nutrient agar medium. After 24 hrs incubation, the developed colony on the agar plates were counted using an automated colony counter. Then the bacteria were heat-killed by boiling the PBS supplemented culture for 5 min. The heat-killed bacteria was again plated on a solid nutrient agar medium and incubated for 18-24 hrs. Also, the motility of this organism was tested after viability analysis. For the experiments with live bacteria, the bacterial strains (0.1 gm each) were continuously cultured and grown in a minimal volume of sterile medium (10 mL), further surgeries were performed. The undiluted bacteria were stored at room temperature during a surgical operation. The bacterial

strains were quantified by a plating method and pre- and post-bacterial numbers were determined.

Pre-term labour analysis: Albino rats were anaesthetized with 10-15 mg kg^{-1} b. wt. of 2.5% (v/v) tert-amyl alcohol and 2.5% (w/v) tribromoethyl alcohol mixture in phosphate-buffered saline. In the lower abdomen, a 2.0 cm midline incision was made carefully then 100 μL of PBS suspended bacteria, killed bacteria or sterile PBS were injected into the centre of the right uterine horn very close at the place between two adjacent fetuses. Further, the abdominal incision was aseptically closed using vicryl sutures through the peritoneum of the rat and staples at the skin of the animal. Experimental procedures end within 20-25 min. The number of experimental animals was freed into the cages and allowed back to normal condition. Then, the number of dead pups or new-born was observed. The fetus that appeared in the lower vagina or the cage within 48 hrs of surgery was considered as preterm delivery. All experimental animals were monitored continuously for a week to observe the time of delivery and the health of delivered pups.

Role of progesterone in delaying preterm delivery: In the case of intrauterine injections, generally, the right uterine horn was recognized and progesterone was injected between fetuses. To determine the role of additional progesterone supplementation, progesterone was injected before the supplementation of bacteria (killed or alive) at various concentrations (0-2.5 mg progesterone per rat). After 2 hrs of injection, these animals underwent anaesthesia and intrauterine administration of heat-killed bacterial strains (*E. coli*, *S. pyogenes* and *F. nucleatum*, 10^9 CFU/mL). These experimental mice were monitored at a specific time or they were sacrificed after 16 hrs of surgical procedure for the collection of serum. Progesterone content was determined by High-Performance Liquid Chromatography²⁸.

Cytokines analysis: Cytokines were determined from the brain tissue. Albino rats were anaesthetized after 18 hrs of intrauterine infection with the selected bacterial pathogens. To the control, only PBS was injected. After 18 hrs of treatment with live bacteria, the brain was dissected out from the sacrificed animal and stored at -20°C for the determination of cytokines activity. The tissue sample (0.1 gm) was lysed with lysis buffer (2.0 mL) and total protein content was initially estimated. The amount of Albino rat IL-1 β , IL-6 and tumour necrosis factor- α were determined.

RESULTS

Intrauterine infection and preterm delivery: The experimental animal was infected with live *E. coli*, *S. pyogenes* and *F. nucleatum* at 1.5×10^{12} CFU/mL and heat-killed bacteria. Both live and heat-killed bacteria-induced preterm labour. Multidrug-resistant bacteria such as *E. coli* (ATCC 25586), *S. pyogenes* (ATCC 19615) and *F. nucleatum* (ATCC 25586) were associated with intrauterine inflammation in experimental animals and induced preterm delivery. The rate of preterm delivery varied between 20% (*F. nucleatum* killed shown in Table 1) and 75% (*S. pyogenes* Live shown in Table 1).

Pathogenic bacteria mediated cytokine level: To analyze brain inflammation, the brain samples were collected from the experimental rat and control animals. On day 1 of birth,

cytokines level enhanced in the treated rats than control. The amount of IL-1 β increased in the brain from 0.18 ± 0.05 - 0.43 ± 0.03 compare control with *S. pyogenes* treated animal and this increased amount was high than other animals treated with *E. coli* (0.39 ± 0.05 ng mg⁻¹) and *F. nucleatum* (0.26 ± 0.04 ng mg⁻¹) (Fig. 1a. $p < 0.001$). This trend was also observed on IL-6 which increased from 5.066 ± 0.04 - 21.5 ± 0.06 compare control with *S. pyogenes* treated animal (Fig. 1b) and tumour necrosis factor- α which increased from 0.043 ± 0.03 - 0.15 ± 0.07 compare control with *S. pyogenes* treated animal (Fig. 1c) in the experimental animals treated with pathogenic bacteria.

Serum progesterone level in experimental animals: In this study, serum progesterone level was observed for 16 hrs of treatment in pregnant Albino rats treated with bacterial inoculums. We can find the control group result was 40.38 ± 3.22 in 4 hrs and 33.9 ± 2.78 in 16 hrs. So, in the control

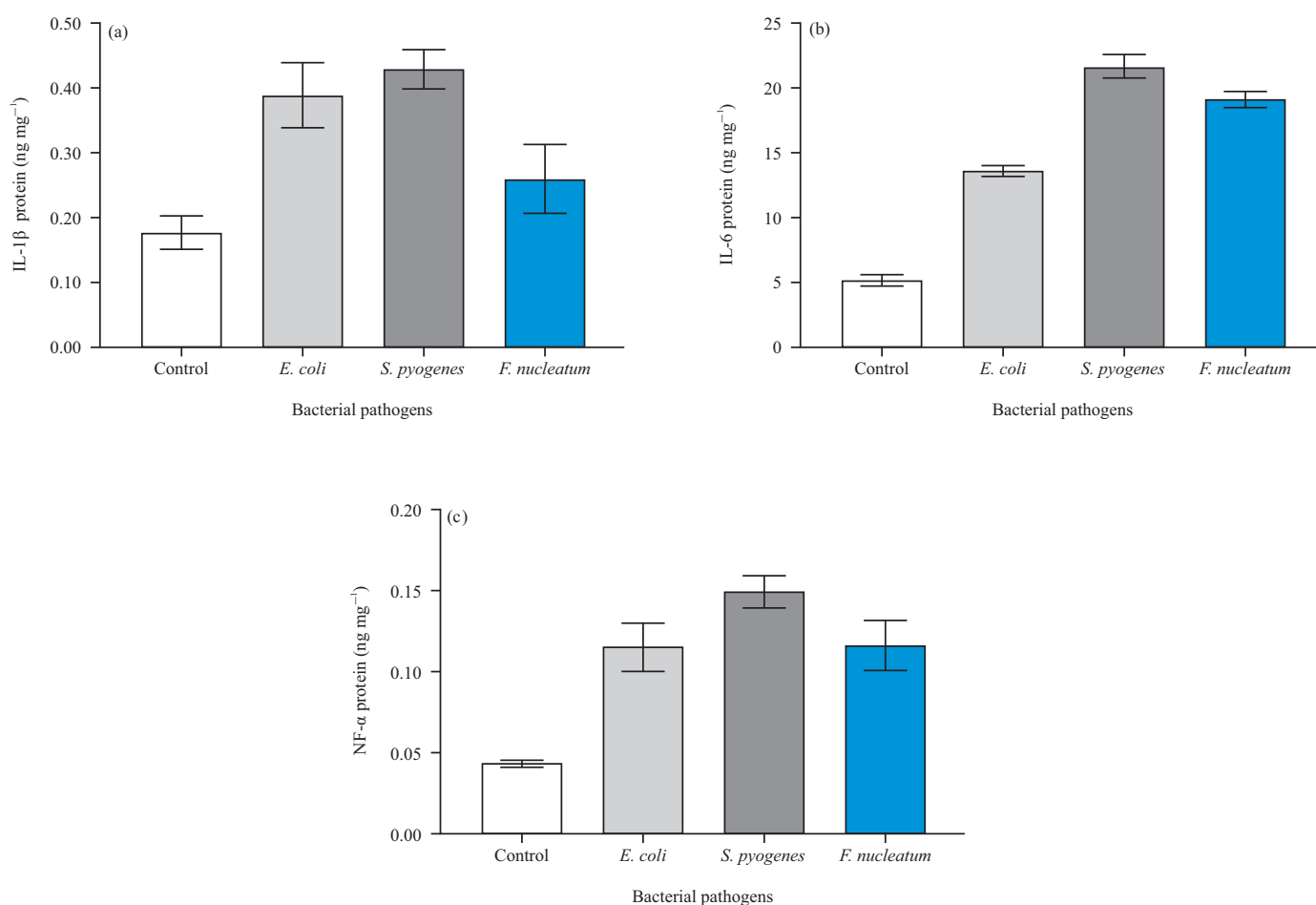


Fig. 1(a-c): Cytokine activity in the brain tissue of Albino rats, (a) IL-1 β , (b) IL6 and (c) TNF- α

Tissue homogenate was prepared from the brain tissue of the experimental animal and the amount of cytokine was expressed as nanogram/mg protein, Error bar: standard deviation ($p < 0.001$)

Table 1: Influence of pathogenic bacteria causing intrauterine infection associated with preterm delivery

Bacteria	Treatments	Number of bacteria	Experimental animals used	Preterm delivery (%)
<i>Escherichia coli</i>	Heat Killed	$1-3 \times 10^9$	11	60
	Live	$1-2 \times 10^9$	11	55
	Control	-	10	0
<i>Streptococcus pyogenes</i>	Heat Killed	$2-4 \times 10^9$	11	60
	Live	$2-4 \times 10^9$	11	75
	Control	-	10	0
<i>Fusobacterium nucleatum</i>	Heat Killed	$1-3 \times 10^9$	11	20
	Live	$1-3 \times 10^9$	11	25
	Control	-	10	0

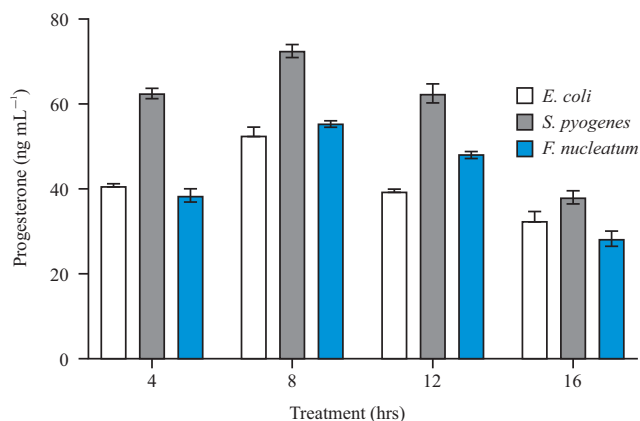


Fig. 2: Progesterone content in the experimental rat inoculated with heat-killed bacterial pathogens
 Triplicate experiment was performed and an error bar indicated standard deviation

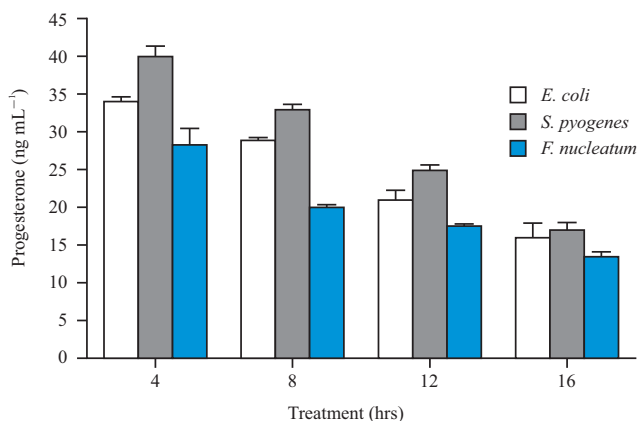


Fig. 3: Progesterone content of experimental animal after ovariectomy
 Triplicate experiment was performed and an error bar indicated standard deviation

rats, progesterone levels did not decrease significantly as shown in Fig. 2. In the experimental groups, pre-treated bacterial inoculums decreased the hormone activity. However, ovariectomy led to a dramatic and rapid decline in serum progesterone level and was statistically significant

($p < 0.001$). Heat killed *E. coli*, *S. pyogenes* and *F. nucleatum* declined progesterone level about 10-15% after 8 hrs. After 4 hrs, progesterone level was 34 ± 1.2 ng mL⁻¹ in *E. coli* (Fig. 3) injected Albino rat and decreased as 16 ± 3.2 ng mL⁻¹ after 16 hrs (Fig. 3). *S. pyogenes* inoculated rat showed 40 ± 2.4 ng mL⁻¹ progesterone after 4 hrs and declined as 17 ± 1.7 ng mL⁻¹ after 16 hrs of post-treatment. In the case of *F. nucleatum* inoculated rats, the amount of progesterone level was 28.3 ± 3.7 ng mL⁻¹ after 4 hrs of post-treatment and this level declined about 50% after 16 of post-treatment (Fig. 3).

DISCUSSION

Intrauterine inflammation is mainly associated with preterm birth and is one of the leading causes of perinatal mortality and morbidity. The analysis in human subjects and animal models revealed bacterial infection mediated preterm birth²⁹. The bacterial pathogens, *E. coli*, *S. pyogenes* and *F. nucleatum* were selected in this study because of their associated lipopolysaccharide toxins and these strains were used for the determination of inflammation-mediated preterm delivery. These pathogenic bacteria involved preterm delivery in Albino rats. Among the strains, the live cells of *S. pyogenes* induced 75% pre-term delivery. Intrauterine infection is caused by the bacterial strains severely associated with inflammation, cerebral palsy and preterm birth¹⁷. Numerous inflammatory and infectious agents, including live and killed *Escherichia coli*, the cell wall of Gram-positive lipoteichoic acid bacteria, group B *Streptococcus* and lipopolysaccharide from Gram-negative bacteria, IL-1 and bacteria such as *Ureaplasma* have been used to analyze preterm delivery in various experimental animals³⁰. PCR method has been used to detect bacteria from the amniotic fluid and bacteria from the genus *Ureaplasma* and *Mycoplasma* associated with preterm labour^{31,32}. *E. coli* colonization in the vagina has been associated with preterm delivery^{33,34}. Pathogenic bacteria caused intrauterine infections and this is involved in preterm labour. Generally, the amniotic fluid is completely sterile,

however, about 1% of pregnant women suffered preterm labour due to bacterial load in the amniotic fluid. Not only because of these pathogenic stages but also, have inflammatory states responsible for preterm birth³⁵. Bacteria such as *Gardnerella vaginalis*, *Mycoplasma hominis* and other groups, including, *Bacteroides*, *peptostreptococcus* have been isolated from the vagina of pregnant women. All of these organisms were less virulent and associated with preterm labour. The bacteria such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were rarely reported in the uterus, however, after membrane rupture, these were associated with fatal infection and chorioamnionitis and other bacterial species such as *Escherichia coli* and *B. Streptococcus* were also associated with preterm labour³⁶.

There were previous reports on brain damage due to perinature exposure of bacterial infection to the experimental animals³⁷. The present findings revealed that the bacteria, *E. coli*, *S. pyogenes* and *F. nucleatum* are involved in inflammation. *S. pyogenes* induced more inflammatory responses than *E. coli* and *F. nucleatum*. Inflammation was significantly caused by microbial activity in the uterus of the mother. Inflammation in the placenta has been used to predict brain injury in experimental animals. Previously, a low virulence pathogenic bacteria strain also has been isolated from the placenta of the experimental models³⁸. Urinary tract infection is common among females and about 80-90% urinary tract infection was associated with *Escherichia coli*. The first generation antibiotics showed poor sensitivity on *E. coli* and second or third-generation cephalosporin is recommended to treat these drug-resistant *E. coli* strains³⁹. The mechanism of intrauterine inflammation by the pathogenic bacterial strains and the influence on fetal brain injury was not completely elucidated. However, various animal studies revealed that the inflammation is based on the types of bacteria involved and cytokines generated during inflammation were linked between brain injury and developing brain^{40,41}. This is mainly due to the activity of placental cytokines, cytokines produced in the mother or by the fetus. The cytokine, IL-6 freely cross the placenta and the elevated level indicated fetal inflammatory response syndrome^{42,43}. The cytokine, IL-6 produced by the mother reflected brain injury of offspring⁴⁴.

The experimental animal pre-treated with bacterial inoculums affected the hormone function. A rapid decline in serum progesterone level was observed in ovariectomy animals. Progesterone has anti-inflammatory activities, increasing the link between alterations in progesterone receptor, inflammatory processes and preterm labour⁴⁵. In both preterm labour and term labour, there is strong evidence

of a marked increase in interleukin-6, interleukin-1, Tumour Necrosis Factor (TNF) -alpha and anti-inflammatory interleukin-10, increasing the synthesis of prostaglandin⁴⁶. The tumour necrosis factor and inflammatory cytokines interleukin-1 increased the production of prostaglandin, while both progesterone and IL-10 harm the production of prostaglandin⁴⁷. Elovitz and Mrinalini applied lipopolysaccharide to infect the intrauterine region and associated inflammation in mouse model⁴⁸. Progesterone pre-treatment was mainly associated with inflammatory mediators, suppression of activation of genes associated with contraction, as well as intrauterine inflammation. The pre-treatment with progesterone to the mouse was associated with preserved fetal viability in the mouse and dramatic reduction in preterm labour. These findings showed that the application of progesterone is useful to decrease the risk of preterm delivery in experimental animals. Recently, probiotics, medicinal plants and PDE-4 inhibitors have been suggested to control preterm birth⁴⁹. The impact of progesterone on Toll-like receptors has been analyzed previously. These receptors participated in both the modulation and initiation of the inflammatory response, the regulation of these receptors may modify labour⁵⁰. Hence the administration of progesterone alters labour. In an experiment, mice were pre-treated with progesterone before the intra-uterine environment and the induced effect of LPS in the placenta and cervix receptors were regulated.

CONCLUSION

Intrauterine inoculation of pathogenic bacteria in pregnant Albino rats induced preterm labour. Both live and heat-killed bacteria-induced preterm labour in the experimental animals. Pathogenic bacteria elevated the level of cytokines. The enhanced cytokine level in the brain tissue indicated brain injury. Administration of progesterone showed a protective role and delayed preterm delivery. It is concluded that administration of bacteria-induced pre-term delivery in Albino rats and preterm labour was associated with intrauterine inflammation and brain injury.

SIGNIFICANCE STATEMENT

This study discovered the Borth live and heat-killed bacteria-induced preterm labour in the experimental animals that can be beneficial for intrauterine inoculation of pathogenic bacteria in pregnant Albino rats induced preterm labour. This study will help the researchers to uncover the critical areas of pathogenic bacteria and pre-term delivery,

intrauterine inflammation and brain injury that many researchers were not able to explore. Thus a new theory on medication and treatment may be arrived at.

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