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Intra-amniální zánět u spontánního předčasného porodu se zachovalým vakem blan – klinické a experimentální aspekty

Intra-amniotic inflammation in women with preterm labor with intact membranes – clinical and experimental aspects

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Abstract of the thesis

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The thesis will be defended .....

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1. SOUHRN

Předčasný porod se zachovalým vakem blan (PTL) představuje přibližně 40 % všech předčasných porodů. PTL je často komplikován intraamniálním zánětem (IAI), který je charakterizován zvýšenou koncentrací zánětlivých mediátorů v plodové vodě. Na základě přítomnosti mikrobiální invaze do amniální dutiny (MIAC) se rozlišují dva klinické fenotypy IAI: i) intraamniální infekce, kdy jsou v plodové vodě přítomny mikroorganismy, a ii) sterilní IAI bez přítomnosti mikrobů v plodové vodě. Oba fenotypy IAI jsou spojeny s horšími neonatálními výsledky, což podtrhuje jejich klinickou závažnost.

Oba fenotypy IAI vykazují kromě přítomnosti MIAC další odlišnosti v charakteristikách intraamniálního zánětu. Těmito rozdíly se zabývá klinická část disertace. Prvním specifickým cílem klinické části bylo stanovení koncentrací interleukinu (IL) - 6 v cervikální tekutině žen s PTL komplikovaným intraamniální infekcí a sterilním IAI. Druhým specifickým cílem bylo stanovení koncentrací IgGFc-binding proteinu (FcgammaBP) v amniální a cervikální tekutině žen s PTL komplikovaným intraamniální infekcí a sterilním IAI.

Oba specifické cíle klinické části této disertace byly zkoumány na stejné kohortě 79 žen s PTL. Přítomnost obou fenotypů IAI byla spojena se zvýšenými hladinami IL-6 v cervikální tekutině. Mezi jednotlivými fenotypy IAI však v koncentracích IL-6 v cervikální tekutině nebyly rozdíly. Koncentrace FcgammaBP v plodové vodě byla zvýšena u obou fenotypů IAI, výrazněji v případě intraamniální infekce. Hladiny FcgammaBP v cervikální tekutině nebyly ovlivněny přítomností žádného z fenotypů IAI.

Animální modely IAI představují jedinečný nástroj k výzkumu předčasného porodu. Umožňují studovat aspekty předčasného porodu, které nelze vyhodnotit v klinických studiích u lidí. Experimentálním cílem této práce bylo vyvinout model IAI u potkana pomocí intraamniální aplikace induktorů zánětu pod ultrazvukovou kontrolou.

Prvním specifickým cílem experimentální části bylo vypracovat systematický přehled literatury zaměřený na metody intraamniální aplikaci induktorů infekce a zánětu za účelem vytvoření zánětem indukovaného modelu předčasného porodu u hlodavců. Druhým specifickým cílem bylo posoudit účinek ultrazvukem navigované intraamniální aplikace lipopolysacharidu (LPS) na hladinu IL-6 v plodové vodě u potkanů. A třetím specifickým cílem bylo vytvořit protokol pro ultrazvukem navigovaného intraamniálního podání induktorů zánětu u potkana.

Systematický přehled literatury ukázal, že se intraamniální podávání induktorů k modelování intraamniální infekce či zánětu u hlodavců používá. Ultrazvukem navigované intraamniální podání bylo popsáno pouze u myši, ale ne u potkana. Naše studie prováděná na sedmi samicích ukázala, že intraamniální aplikace navigovaná ultrazvukem je proveditelná u potkana. Podání 10 μg *E. coli* LPS sérotypu O55: B5 intraamniálně vedlo k rozvoji IAI a nebylo spojeno s předčasným porodem a ani s vyšší úmrtností plodů. Ultrazvukem navigovaná intraamniální aplikace induktorů zánětu u potkana byla podrobně popsána v protokolu, který podporuje proveditelnost a reprodukovatelnost této techniky pro budoucí výzkum.

2. SUMMARY

Preterm labor with intact membranes (PTL) is responsible for approximately 40% of all preterm deliveries. PTL is frequently complicated by intra-amniotic inflammation (IAI), characterized by the elevation of inflammatory mediators in the amniotic fluid. Based on the presence or absence of microbial invasion of the amniotic cavity (MIAC), two different clinical phenotypes of IAI are distinguished—i) intra-amniotic infection, when microorganisms are present in the amniotic fluid, and ii) sterile IAI, when there are no microorganisms in the amniotic fluid. The clinical severity of both phenotypes of IAI is underlined by their association with adverse neonatal outcomes.

In addition to the presence or absence of MIAC, there are also differences between the phenotypes of IAI in terms of their intra-amniotic inflammatory status characteristics. The clinical part of this thesis has addressed these differences in women with PTL. The first specific aim of this clinical study was to determine the concentration of interleukin (IL)-6 in the cervical fluid of women with PTL complicated by intra-amniotic infection and sterile IAI. The second specific aim was to determine the concentration of IgGFc-binding protein (FcgammaBP) in the amniotic and cervical fluids of women with PTL complicated by intra-amniotic infection and sterile IAI.

Both specific aims of the clinical part of this thesis were investigated in the same study population, consisting of 79 women with PTL. The presence of both phenotypes of IAI was associated with a higher concentration of IL-6 in the cervical fluid than the absence of IAI. However, there were no differences in the concentration of IL-6 in the cervical fluid between the phenotypes of IAI. The concentration of FcgammaBP in amniotic fluid was elevated in the presence of both phenotypes of intra-amniotic inflammation, being more pronounced in the presence of intra-amniotic infection. The concentration of FcgammaBP in the cervical fluid was not altered by the presence of either phenotype of IAI.

Animal models of IAI represent a unique tool in the research of preterm delivery, enabling the study of aspects that cannot be evaluated in human clinical studies. Therefore, the objective of this thesis was to develop a rat model of IAI established by ultrasound-guided intra-amniotic administration of an inflammatory agent. The first specific aim of the experimental part of this thesis was to perform a systematic review of literature on methods of intra-amniotic administration of infectious and/or inflammatory agents to develop a rodent model of inflammation-driven preterm delivery. The second specific aim was to assess the effect of ultrasound-guided intra-amniotic administration of lipopolysaccharide (LPS) on the concentration of IL-6 in the amniotic fluid of rats. The third specific aim was to define a detailed protocol for ultrasound-guided intra-amniotic administration of an agent in a rat.

A systematic review of the literature revealed that intra-amniotic administration of triggering agents was used to model intra-amniotic infection/inflammation in rodents. Intra-amniotic administration under ultrasound guidance has been described in mice, but not in rats. Our experiments performed on seven rat dams showed that ultrasound-guided intra-amniotic administration of an agent was feasible in rats. Administration of 10 μg of Escherichia coli LPS serotype O55:B5 per gestational sac resulted in the development of IAI and did not induce labor or fetal mortality. The processes of ultrasound-guided intra-amniotic administration of an agent in a rat were summarized as a protocol to offer detailed guidelines supporting the feasibility and reproducibility of this technique for future research.

3. INTRODUCTION

3.1 PRETERM DELIVERY

Preterm delivery is defined by the World Health Organization (WHO) as delivery occurring before 37 weeks of gestation [1,2]. It is a heterogeneous entity resulting from various maternal and/or fetal disorders and is a leading cause of infant morbidity and mortality [3].

The estimated global rate of preterm delivery is approximately 11%, with almost 15 million babies born preterm annually [3,4]. In European countries and other developed countries, the rate of preterm delivery is between 6% and 9% [3,4]. It is estimated, that more than 1 million children under 5 years of age die due to preterm delivery and associated complications per year [5]. In addition, those who survive are at a greater risk of a range of short- and long-term morbidities. In general, 75% of all perinatal deaths and more than 50% of all postnatal morbidities are related to preterm delivery [4].

One-third of preterm deliveries represent iatrogenic preterm delivery, when the delivery is medically indicated due to maternal and/or fetal complications, such as preeclampsia and fetal growth restriction [6-8]. The remaining two-thirds of preterm deliveries represent spontaneous preterm deliveries [6,8,9].

Based on the evolving clinical presentation, two basic phenotypes of spontaneous preterm delivery can be distinguished:

i) Preterm labor with intact membranes (PTL) is defined as regular uterine contractions accompanied by cervical ripening and accounts for 40%–45% of all preterm deliveries.

ii) Preterm prelabor rupture of membranes (PPROM) is characterized by the spontaneous rupture of fetal membranes prior to the onset of uterine contractions and accounts for 30%–35% of all preterm deliveries [10].

3.2 PRETERM LABOR WITH INTACT MEMBRANES

Based on the current knowledge, PTL is a heterogeneous entity attributable to multiple pathological conditions and processes [8,13,14]. Infection and inflammation are the leading causes of PTL, with a prevalence of approximately 30%–40% [30,31]

3.2.1 Microbial invasion of the amniotic cavity

The presence of microorganisms in the amniotic fluid is considered a pathological finding, referred to as microbial invasion of the amniotic cavity (MIAC) [19]. Microorganisms can enter the amniotic cavity by i) ascension from the vagina and the cervix, ii) hematogenous spread through the placenta, iii) retrograde dissemination from the peritoneal cavity through the fallopian tubes, and iv) iatrogenic inoculation during invasive intrauterine procedures [6]. The most common pathway is the ascending route of the lower genital tract.

The frequency of MIAC in women with PTL ranges from 16% to 40%. Low gestational age is typically associated with a higher frequency of MIAC [20,23-26]. The most common bacteria found in the amniotic fluid of women with PTL are genital mycoplasmas, specifically *Ureaplasma* spp. and *Mycoplasma hominis* [19,27].

3.2.2 Intra-amniotic inflammation

Intra-amniotic inflammation (IAI) is characterized by the elevation of many inflammatory cytokines, chemokines, antimicrobial peptides, and lipids in the amniotic fluid. Based on the presence or absence of MIAC, two different clinical phenotypes of IAI are distinguished: i) intra-amniotic infection, when microorganisms are present in the amniotic fluid; and ii) sterile IAI, when the amniotic fluid is free of microorganisms.

Intra-amniotic infection complicates approximately 11% of PTL pregnancies [13]. The microorganisms present in the amniotic fluid activate the innate immune system through the engagement of pattern recognition receptors (PRRs) [13]. Toll-like receptors (TLRs) are essential PRRs that can recognize specific components of microorganisms called pathogen-associated molecular patterns (PAMPs) [28]. The presence of intra-amniotic infection in PTL is associated with a shorter latency period and a lower gestational age at delivery than the absence of IAI in PTL [31,32].

Sterile IAI complicates 26% of pregnancies with PTL. Thus, sterile IAI is more common than intra-amniotic infection [33]. Despite intensive research, the development of sterile IAI in PTL is not completely clear [33,34]. In the absence of bacteria in the amniotic fluid, the following conditions might lead to sterile IAI: i) damage of fetal membranes, leading to the release of endogenous molecules (damage-associated molecular patterns [DAMPs]), called alarmins, into the amniotic fluid, resulting in a subsequent inflammatory response through the PRR system [33-38]; ii) microbial colonization or infection of the choriodecidual space, which stimulates the fetal membranes to produce inflammatory mediators that are released from the fetal membranes into the amniotic fluid [39,40]; or iii) a combination of these two processes. Sterile IAI has a similar rate of adverse pregnancy and neonatal outcomes to intra-amniotic infection [33]. This fact highlights the clinical seriousness of sterile IAI in PTL pregnancies.

3.2.3 Diagnostic approaches

The diagnosis of intra-amniotic infection and sterile IAI is based on the examination of the amniotic fluid collected by transabdominal amniocentesis [41,42].

Cultivation of the amniotic fluid was considered the "gold standard" for the diagnosis of MIAC for decades. However, many microbes involved in MIAC are difficult or impossible to cultivate. Therefore, the adoption of non-cultivation, PCR-based techniques to diagnose MIAC is necessary [25,27,43].

Currently, the evaluation of IL-6 levels in the amniotic fluid is considered the gold standard for the diagnosis of IAI [44]. A IL-6 level of 2600 pg/mL in the amniotic fluid, when measured by enzyme-linked immunoassay (ELISA), has been broadly accepted as a cut-off value for IAI [23,45,46]. However, the use of ELISA in clinical medicine is very limited because it takes hours to obtain results, and the results are not rapidly available for clinical management. Another alternative is an automated electrochemiluminescence immunoassay method developed for use in large-volume clinical biochemistry laboratories and measuring IL-6 concentrations in body fluids in less than 20 min. The cut-off value of 3000 pg/mL for IAI using this method with the immune-analyzer Cobas e602, which is a part of the Cobas 8000 platform (Roche Diagnostics, Basel, Switzerland), was determined by our team [47].

Despite the fact that amniocentesis in women with PTL is associated with a low complication rate [48,49], some clinicians might be reluctant to broadly apply amniocentesis in PTL management due to the invasive nature of this procedure. Thus, the non-invasive testing may be an alternative strategy. The potential directions of non-invasive testing for intra-amniotic

inflammatory complications include the evaluation of maternal blood, vaginal fluid, and cervical fluid.

The inflammatory biomarkers in the maternal plasma cannot be considered an exclusive reflection of the inflammatory status of the amniotic cavity as they are non-specific [50-52]. The concentration of cytokines in the vaginal fluid might be determined by the local vaginal microbiome rather than by the intra-amniotic environment. Therefore, neither vaginal fluid component serves as an appropriate source of potential biomarkers [53].

The cervical fluid, collected from the cervix of women with PTL, is a unique mixture of cervical and uterine origin. Given the close anatomical proximity between the cervix and fetal membranes, the composition of cervical fluid might reflect the microbial and inflammatory status of intra-amniotic and choriodecidual space [54]. The presence of MIAC was related to the elevation of IL-6 [55,56], IL-8 [55,57,58], IL-18 [59], and MCP-1 [60] in the cervical fluid, whereas IAI was associated with elevated IL-6 [55], and IL-8 [55] in the cervical fluid.

The main difference between the phenotypes of IAI is the presence or absence of MIAC. Nevertheless, distinct characteristics of the inflammatory response are also expressed between these phenotypes. Levels of amniotic fluid IL-6 are significantly higher in intra-amniotic infections than in sterile IAI [33]. In addition, women with intra-amniotic infection had higher concentrations of several inflammatory-related proteins than those with sterile IAI [61]. These data demonstrate a much stronger inflammatory response in the amniotic fluid of women with intra-amniotic infection than in that of women with sterile IAI. With respect to the fact that the confirmation/exclusion of MIAC in common clinical practice requires more than 24 hours, additional markers enabling early differentiation between intra-amniotic infection and sterile IAI might be of value.

The inflammatory response, characterized by elevated white blood cells count and levels of glucose and IL-6 in the amniotic fluid, differs between intra-amniotic infection and sterile IAI in PTL [33,61]. However, there is a lack of knowledge on whether the concentrations of other inflammatory mediators in amniotic and other body fluids vary between women with PTL and presence of intra-amniotic infection and those with PTL and sterile IAI. Therefore, the clinical part of this thesis has focused on the differences in concentrations of two thoroughly selected inflammatory mediators in the amniotic fluid and the cervical fluid between women with PTL and intra-amniotic infection and those with PTL and sterile IAI.

3.3 ANIMAL MODELS OF PRETERM DELIVERY

Currently, there is no ideal animal model for simulating all pathways of human preterm delivery. Thus, various animal species have been used in research, all of them with specific ability to mimic processes in clinical cases of human preterm delivery and with important differences in terms of reproductive biology [62].

The animal species used to model preterm delivery associated with inflammation/infection are rat, mouse, rabbit, sheep, and rhesus monkey. Currently, rodents (rats and mice) are the most frequently used animals because they are easy to house and treat. In addition, they are relatively inexpensive [63]. Numerous infectious and inflammatory agents have been used to create models for different animals, including the following: i) inactivated and live microorganisms [64-67], ii) PAMPs [68-71], iii) DAMPs: HMGB-1 [72], and iv) cytokines and immune proteins: IL-1 and TNF-α [73]. Animal models of preterm delivery associated with inflammation and infection can be classified as systemic or localized [63].

The systemic models are induced by intraperitoneal or intravenous administration of triggering agents. Because intraperitoneal or intravenous administration is relatively easy to accomplish in most species, systemic models are widely used to model preterm delivery associated with inflammation and infection. Intraperitoneal administration of LPS to mice causes systemic activation of the immune system, with a dramatic increase in maternal serum cytokines and a high rate of preterm delivery among animals. A large proportion of fetal deaths have been observed in this model, with some pups consequently reabsorbed [68,74].

Localized models, which include intrauterine, intra-cervical, and intra-amniotic administration of agents, are a better representation of the regional nature of human preterm delivery associated with inflammation and infection [63]. The intra-amniotic administration of triggering agents constitutes a direct method of IAI induction. This represents a clinically relevant issue as both forms of IAI are frequently observed in human cases of preterm delivery [33,34]. Given the specific anatomy of the rodent uterus with multiple small gestational sacs, intra-amniotic administration may be technically challenging.

Developing an animal model of IAI is an issue addressed in this thesis. In our view, intra-amniotic administration of a triggering agent is an optimal route for the induction of precisely defined IAI. Rodents are the most available, easy to house, and treat animal models [63]. As a low volume of the amniotic fluid in mice limits the availability of a sufficient amount for analysis [75], we consider rats as a better option to create a rodent animal model of IAI. Intra-amniotic administration can be performed via laparotomy or under ultrasound guidance [76]. However, laparotomy might represent a stress stimulus with an endocrine response that influences the function of many organs [66]. Therefore, a rat model with ultrasound-guided intra-amniotic administration of a triggering agent was chosen in this study.

4. OBJECTIVES OF THE THESIS

The objectives of this thesis were divided into two basic components. The first part, based on clinical studies in pregnant women with PTL, addresses the differences between the two phenotypes of IAI. The second part, which was experimental, focuses on the establishment of an animal model of IAI as a unique and irreplaceable tool in the research of preterm delivery.

4.1 CLINICAL OBJECTIVES

The clinical objective of this thesis was to determine the levels of selected inflammatory mediators in the amniotic fluid and the cervical fluid of women with PTL with respect to the presence of both phenotypes of IAI—intra-amniotic infection and sterile IAI.

There were two specific aims of the clinical part:

I-A. To determine the concentration of IL-6 in the cervical fluid of women with PTL complicated by intra-amniotic infection and sterile IAI

I-B. To determine the concentration of IgGFc-binding protein (FcgammaBP) in the amniotic fluid and the cervical fluid of women with PTL complicated by intra-amniotic infection and sterile IAI

4.2 EXPERIMENTAL OBJECTIVES

The experimental objective of this thesis was to develop a rat model of IAI established by ultrasound-guided intra-amniotic administration of an inflammatory agent.

There were three specific aims of the experimental part:

II-A. To perform a systematic review of available methods of intra-amniotic administration of infectious and/or inflammatory agents to create a rodent model of inflammation-driven preterm delivery

II-B. To assess the effect of ultrasound-guided intra-amniotic administration of LPS on the concentration of IL-6 in the amniotic fluid in rats

II-C. To develop a step-by-step protocol for ultrasound-guided intra-amniotic administration of an agent in a rat to support the reproducibility and feasibility of this approach

5. SET OF PATIENTS, METHODS AND STATISTICAL ANALYSIS

5.1 CLINICAL OBJECTIVES

The specific aims of the clinical part of the thesis were derived from the same cohort of patients. Most of the methodology was identical for both aims, and the statistical analysis was based on the same approach. Therefore, the clinical objectives have been described together, with an emphasis on items specific to a particular aim.

5.1.1 Set of patients

This retrospective cohort included pregnant women who were admitted to the Department of Obstetrics and Gynecology at the University Hospital Hradec Kralove in the Czech Republic between March 2017 and May 2020.

The inclusion criteria were as follows: 1) singleton pregnancy, 2) maternal age ≥ 18 years, 3) gestational age between 22+0 and 36+6 weeks, 4) PTL, and 5) the performance of transabdominal amniocentesis at the time of admission to determine IAI.

The exclusion criteria were as follows: 1) pregnancy-related and other medical complications such as fetal growth restriction, gestational or pre-gestational diabetes, gestational or chronic hypertension, and preeclampsia; 2) structural or chromosomal fetal abnormalities; 3) signs of fetal hypoxia; and 4) significant vaginal bleeding.

Gestational age was determined by first-trimester fetal biometry. PTL was diagnosed as the presence of regular uterine contractions (at least two contractions every 10 minutes) and cervical length, measured using transvaginal ultrasound, shorter than 15 mm or within the 15–30 mm range with a positive PartoSure test result [77].

5.1.2 Methods

Paired cervical fluid and amniotic fluid samples were collected at the time of admission from all women included in this study. Each cervical fluid sample was obtained by placing a Dacron polyester swab in the cervical canal for 20 seconds to achieve saturation. Once collected, the polyester swab was inserted into a polypropylene tube containing 1.5 mL of phosphate-buffered saline; the tube was then shaken for 20 min. On removal of the polyester swab, the tube was centrifuged at 300 × g for 15 min at room temperature. The supernatant was divided into aliquots and stored at −80°C until further analysis.

Ultrasonography-guided transabdominal amniocentesis was performed after cervical fluid sampling. Approximately 2–3 mL of the amniotic fluid was aspirated, and the amniotic fluid was immediately divided into polypropylene tubes. The amniotic fluid samples were used for the following: i) the assessment of amniotic fluid IL-6; ii) PCR analysis of *Ureaplasma* spp., *Mycoplasma hominis*, and *Chlamydia trachomatis*; iii) sequencing of the 16S rRNA gene; iv) aerobic and anaerobic cultivation; v) stored at −80°C until further analysis.

The concentrations of IL-6 in the amniotic fluid (fresh samples) and of IL-6 in the cervical fluid (samples with one freezing/thawing cycle) were assessed using an automated electrochemiluminescence immunoassay method. IL-6 concentrations were measured using an immuno-analyzer Cobas e602, which is part of the Cobas 8000 platform [78]. The concentrations of FcgammaBP (samples with one freezing/thawing cycle) were assessed in the amniotic fluid and the cervical fluid using an ELISA.

5.1.3 Clinical definitions

MIAC was determined based on a positive PCR result for *Ureaplasma* spp., M. hominis, C. trachomatis, or a combination of these species or positivity for the 16S rRNA gene, findings of aerobic/anaerobic culture of the amniotic fluid, or a combination of these parameters. IAI was defined as an IL-6 concentration of ≥3000 pg/mL in the amniotic fluid [81]. Intra-amniotic infection was defined by both MIAC and IAI. Sterile IAI was defined as the presence of IAI without concomitant MIAC. Negative amniotic fluid was defined as the absence of MIAC and IAI.

5.1.4 Statistical analyses

The normality of the data was tested using the Anderson-Darling test. The women's demographic and clinical characteristics were compared using a nonparametric Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables and are presented as median values (interquartile range [IQR]) and as number (%), respectively.

Kruskal-Wallis and Mann-Whitney U tests were used for the analyses, as appropriate, and the results are presented as median values (IQR). Spearman partial correlation was performed to adjust the results for gestational age at sampling. Spearman correlation was used to assess the relationship between the concentrations of the evaluated inflammatory mediators in the amniotic fluid or the cervical fluid and gestational age at sampling. Receiver operating characteristic (ROC) curves were constructed to assess the predictive values of selected inflammatory mediators in the amniotic fluid and the cervical fluid for the presence of intra-amniotic infection.

All *p*-values were obtained using two-tailed tests. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using the IBM SPSS Statistics for Mac OS version 27.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 9 for Mac OS X (GraphPad Software, San Diego, CA, USA).

5.2 EXPERIMENTAL OBJECTIVES

5.2.1 Specific aim II-A

We searched for studies that employ intra-amniotic administration of infectious or inflammatory agents to establish a rodent model of inflammation-driven preterm delivery. The search was conducted in two electronic databases (PubMed and Scopus) on February 2, 2019.

Studies that used a rodent model of inflammation-driven preterm delivery initiated by intra-amniotic administration of infectious or inflammatory agents were considered eligible. Primary experimental case-control studies were included. Type of outcome for inclusion: preterm delivery, intra-amniotic infection, sterile IAI, intra-uterine (histological chorioamnionitis) inflammation, and inflammatory complications.

Studies were excluded if any of the following applied: 1) human or in vitro studies, 2) animal models other than rodents, 3) models of preterm delivery other than inflammation-driven preterm delivery, 4) routes of administration of infectious or inflammatory agents other than administration into the gestational sac, and 6) rodent models to study other conditions or diseases without relation to preterm delivery.

The following data were extracted from each article included in this review: author, year of publication, study methodology, information about the study animals and their type (number, species, strain), information about the timing of intervention, description of technique of intra-

amniotic administration, information about the infectious or inflammatory agent used, and outcomes of the study.

For quality assessment of identified studies, the checklist of the CAMARADES group was adjusted [82].

5.2.2 Specific aim II-B

All procedures were performed in accordance with the Act on the Protection of Animals against Cruelty, Act No. 246/1992 Coll., with the approval of the Czech Ministry of Education Youth and Sports (No. 41058/2016-MZE-17214).

Pregnant Wistar rats were purchased from Velaz laboratory (Prague, Czech Republic). On embryonic day 18, intra-amniotic administration of 10 μg of E. coli LPS (serotype O55:B5) in 100 μL of phosphate-buffered saline (PBS) was performed under ultrasound guidance using 27 G needle. Controls were injected with 100 μL PBS alone. Each accessible gestational sac was injected and its localization was recorded.

On embryonic day 19 and 24 hours after ultrasound-guided intra-amniotic administration, the uterine horns were exposed using an abdominal incision and removed from the abdominal cavity. Using a sterile 30 G needle, the amniotic fluid was harvested from all sacs and stored in polypropylene tubes at -70°C until analysis. After the procedure, the animals were sacrificed.

The concentrations of IL-6 in the amniotic fluid samples were assessed using the Rat IL-6 Quantikine ELISA Kit.

The normality of the data was tested using the Anderson-Darling test. Because the IL-6 levels in the amniotic fluid were not normally distributed, non-parametric Mann-Whitney U tests were used for the analyses, as appropriate. All p-values were obtained using two-tailed tests. All statistical analyses were performed using GraphPad Prism version 9 for Mac OS X. Differences were considered significant at $p < 0.05$.

5.2.3 Specific aim II-C

All steps of the entire process of ultrasound-guided intra-amniotic administration of an agent performed on the experimental animals used in Specific aim II-B were recorded and summarized in the protocol.

6. RESULTS

6.1 CLINICAL OBJECTIVES

6.1.1 Clinical characteristics of study population

A total of 79 women with singleton pregnancies with PTL were included in the study. IAI was found in 42% (33/79) of the women; intra-amniotic infection and sterile IAI were observed in 15% (12/79) and 27% (21/79) of the women, respectively.

6.1.2 Specific aim I-A

Differences in the concentrations of IL-6 in the cervical fluid were found among the subgroups of women with PTL in crude analysis (intra-amniotic infection: median, 587 pg/mL; IQR, 166 – 2427; sterile IAI: median 590 pg/mL; IQR, 245 – 1495; with negative amniotic fluid, 149 pg/mL; IQR, 30 – 569; $p = 0.004$), as well as after the adjustment for gestational age at sampling ($p = 0.002$).

Women with intra-amniotic infection and sterile IAI had higher concentrations of IL-6 than those with negative amniotic fluid ($p = 0.01$, adj. $p = 0.004$; $p = 0.005$, adj. $p = 0.003$; respectively). No differences in the concentrations of IL-6 in the cervical fluid were found between women with intra-amniotic infection and sterile IAI ($p = 0.81$).

6.1.3 Specific aim I-B

Differences in the concentrations of amniotic fluid FcgammaBP were identified among the subgroups of women with intra-amniotic infection, sterile IAI, and negative amniotic fluid (infection: median 139.7 ng/mL, IQR 74.2-205.3; sterile: median 54.2 ng/mL, IQR: 44.8-127.0; negative: median 19.7 ng/mL, IQR: 15.9-23.6) in the crude analysis and after the adjustment for gestational age at sampling (both $p < 0.0001$).

Women with intra-amniotic infection had higher amniotic fluid FcgammaBP concentrations than did women with sterile IAI (adj. $p = 0.02$) and with negative amniotic fluid (adj. $p < 0.0001$). Women with sterile IAI had higher amniotic fluid FcgammaBP concentrations than those with negative amniotic fluid (adj. $p < 0.0001$). The amniotic fluid FcgammaBP cutoff value of 120 ng/mL was found to be optimal in the prediction of intra-amniotic infection with area under the receiver operating characteristic curve of 86% ($p < 0.0001$).

No difference in cervical fluid FcgammaBP concentrations was found among the subgroups (intra-amniotic infection: median 341.1 ng/mL, IQR 95.2-614.8; sterile IAI: median 341.2 ng/mL, IQR 138.1-523.4; and negative amniotic fluid: median 200.9 ng/mL, IQR 56,7-443.8; $p = 0.18$). There was no difference in cervical fluid FcgammaBP concentrations between women with and without intra-amniotic infection (with infection: median 341.1 ng/mL, IQR 95.2-614.8 vs. without infection: median 227.0 ng/mL, IQR 95.7-455.4; $p = 0.45$).

6.2 EXPERIMENTAL OBJECTIVES

6.2.1 Specific aim II-A

In total, 13 studies fulfilled our selection criteria and were included in the review [65,72,83-93]. No other rodent animals than rats and mice were used in the included articles.

Two distinct ways of administration of infectious or inflammatory agents into the gestational sacs were used. Five mice studies used transabdominal ultrasound-guided intra-amniotic administration of the agent [72,89,90,92,93]. Laparotomy with visualization of the uterine horns followed by direct puncture of the gestational sacs was the second identified route of administration of agents. Laparotomy was used in three mice studies [65,83,86] and in all studies with rats [84,85,87,88,91].

Infectious or inflammatory agents used in the studies were classified as follows: (1) live microorganisms, *Ureaplasma parvum*; (2) bacterial products, extracellular membrane vesicles; (3) PAMPs, LPS; and (4) DAMPs, HMGB-1, S100-B, and surfactant protein A.

Ureaplasma parvum was the only live microorganism used in the included studies. [65]. Extracellular membrane vesicles from group B Streptococcus strain A909 were used in one study [86]. LPS was the most common triggering agent. LPS serotype O55:B55 was used in one study [87], while serotype O111:B4 was utilized in seven studies. The fourth group of the triggering agents consisted of DAMPs (HMGB-1 protein and protein S100B) that induced sterile IAI. [72] [93].

6.2.2 Specific aim II-B

In total, four rats were administered LPS and three rats were administered PBS. In total, 19 gestational sacs were injected in the LPS group and 17 gestational sacs were injected in the PBS group.

Twenty-four hours after administration, all animals remained alive and had not delivered. All fetuses, except one, were alive. From the rats administered LPS, a sufficient volume of the amniotic fluid was obtained from 16 gestational sacs with LPS and from 33 without LPS for the analysis. From the rats administered PBS, sampling was successful from nine gestational sacs with PBS and 32 sacs without PBS.

Differences in the concentration of IL-6 in the amniotic fluid were found among the subgroups of gestational sacs (with LPS: median 538 pg/mL; IQR 192.6–843.2 pg/mL; without LPS: median 36 pg/mL, IQR 35.6–52 pg/mL; with PBS: median 35.6 pg/mL, IQR 35.6–44.5 pg/mL; without PBS: median 35.6 pg/mL, IQR 35.6–35.8 pg/mL; $p \leq 0.0001$).

The concentration of IL-6 in the amniotic fluid from gestational sacs with LPS were higher than that in the amniotic fluid from gestational sacs with PBS ($p < 0.0001$) and those without LPS ($p < 0.0001$) and without PBS ($p < 0.0001$). No differences in the concentration of IL-6 in the amniotic fluid were identified between gestational sacs with PBS and those gestational sacs without LPS ($p = 0.63$) and without PBS ($p = 0.36$). The concentration of IL-6 in the amniotic fluid from gestational sacs without LPS from rats administered LPS was higher than that in the amniotic fluid from gestational sacs without PBS from rats administered PBS ($p = 0.04$).

6.2.3 Specific aim II-C

The bottle warmer was turned on in advance to warm up the ultrasound gel. The temperature of the warmer was set to 37°C. The oxygen tank and the level of isoflurane in the anesthesia vaporizer was checked. The hose switch was opened to allow anesthetic gases to flow into the induction chamber. The heating of the Vevo Imagine Station heating pad was turned on. The infrared lamp was turned on. Five pieces of tape, approximately 10 cm in length, were prepared and used to attach the animal and rectal probe to the heating pad during the procedure. Hair removal cream and a few pieces of cotton swabs and gauze pads were prepared and used to remove fur.

A Vevo 3100 ultrasound machine was turned on. The MX250S transducer (15–30 MHz) was connected to an ultrasound machine.

The oxygen tank was turned on, and the flow rate was set to 2 L/min. Isoflurane was initiated at a concentration of 5%. The rat was removed from the cage and placed in an induction chamber. After anesthesia was initiated, the isoflurane concentration was lowered to 1.5%. The hose switch connecting the anesthesia vaporizer and nasal mask on the heating pad at the ultrasound station was open. The rat was carefully removed from the induction chamber and moved to the heating pad of the imaging station. The animal was placed in the prone position on the heating pad, and the rat's snout was placed on the heating pad to the nasal mask. After stabilization, the animal was placed in the supine position (with the face and torso facing up).

The electrode gel was applied between the rat's paws and the electrodes of the heating pad. The rat's paws were fixed to the electrodes of the heating pad using previously prepared tapes. The rectal probe was inserted into the rectum of the rat to measure body temperature.

Depilatory cream was applied to the rat's abdomen for 3 min. Then, the fur and cream were removed using pieces of wet gauze.

The lower part of the rat's abdomen was covered with the ultrasound gel. The maternal bladder was identified and used as a midline reference point for localizing and mapping gestational sacs with pups. First, the right part of the abdomen of the animal was scanned from the bladder to the thorax. Then, the left part of the abdomen of the animal was scanned from the bladder to the thorax. The positions of the pups and placentas were recorded.

The MX250S transducer was replaced by an MX400 transducer (20–46 MHz). The MX400 transducer was placed in the transducer holder. The syringe with the substance was placed in the syringe holder. The target gestational sac was visualized on the ultrasound image. The syringe holder was moved toward the animal body surface inside the gel layer with a 27 G needle perpendicular to the skin until the needle tip could be visualized. When the tip of the needle was visible on the ultrasound image, puncture was performed. The agent was injected when the tip of the needle was inside the gestational sac. Successful application was confirmed by visualization of the fluid jet. The needle was then slowly pulled out. The procedure was repeated to inject all accessible gestational sacs in the animal. A new sharp needle was used for each new administration to ensure the successful passage of the needle through the skin.

The abdomen was completely dried before the animal was moved into the cage. The rectal probe was removed, and the paws were released. The isoflurane vaporizer was turned off. The animal was placed in a cage, where it was kept under a heat lamp and watched until recovery from anesthesia.

7. DISCUSSION

7.1 CLINICAL OBJECTIVES

7.1.1 Specific aim I-A

The principal findings of the specific aim I-A, evaluated in women with PTL, were as follows: i) intra-amniotic infection and sterile IAI were found in 15% and 27% of the women, respectively; ii) cervical fluid IL-6 concentration was positively correlated with amniotic fluid IL-6 concentration; iii) women with intra-amniotic infection and sterile IAI had a higher concentration of IL-6 in the cervical fluid than those without IAI; and iv) no differences in the concentration of IL-6 in the cervical fluid were found between women with intra-amniotic infection and those with sterile IAI.

In this study, we confirmed the results reported in previous studies [55,56] that in PTL pregnancies, IAI is associated with higher cervical fluid concentrations of IL-6 than in those without IAI. To extend the knowledge of this field, women with PTL were further divided into three subgroups: intra-amniotic infection, sterile IAI, and without IAI. As expected, women with both clinical phenotypes of IAI had higher concentrations of IL-6 in the cervical fluid than those without IAI. However, no difference in the cervical fluid IL-6 concentration was found between women with intra-amniotic infection and sterile IAI. These observations show that an inflammatory and/or infectious environment in the cervical compartment is present in both clinical phenotypes of IAI. Given the tight anatomical proximity between the cervix and fetal membranes, we hypothesize that the composition of the cervical fluid might reflect the microbial and inflammatory status of the choriodecidual space. This hypothesis is driven by the fact that the presence of bacteria in the chorioamnion is associated with an elevation of IL-6 concentration in the cervical fluid [40]. In addition, the presence of microorganisms in the chorioamnion is also related to higher concentrations of IL-6 in the amniotic fluid, irrespective of the presence or absence of microorganisms in the amniotic fluid [39,40]. These facts collectively suggest that presence of microorganisms in the chorioamniotic membranes closely related to the elevation of the concentration of IL-6 in both cervical and amniotic fluids.

Therefore, the elevation of IL-6 concentration in the cervical fluid in women with PTL with sterile IAI can be explained by the possible presence of microorganisms in the chorioamnion and/or inflammation in the choriodecidual space. This observation supports the hypothesis that these conditions represent one of the mechanisms playing pivotal roles, apart or in combination with the release of alarmins from necrotic cells or cells undergoing cellular stress, on the development of a sterile intra-amniotic environment in women with PTL.

7.1.2 Specific aim I-B

The principal findings of the specific aim I-B, evaluated in women with PTL, were as follows: i) FcgammaBP was identified as a constituent of amniotic and cervical fluids, ii) the concentration of FcgammaBP in amniotic fluid was elevated in the presence of both phenotypes of IAI, being higher in the presence of intra-amniotic infection, iii) FcgammaBP in the amniotic fluid might be a marker of intra-amniotic infection in women with PTL, and iv) the concentration of FcgammaBP in the cervical fluid was not altered by the presence of either phenotype of IAI.

FcgammaBP is a relatively unknown protein, with limited reports in relation to conditions such as bowel inflammatory disease, autoimmune disease, or thyroid gland tumors [94-96], however, it also represents one of the proteins identified in the amniotic fluid using proteomics [97-100].

FcgammaBP was discovered more than 30 years ago as a specific site for the fragment of crystallizable (Fc) region of the immunoglobulin (Ig) G antibody in the small intestinal and colonic epithelia [101]. This specific site differed from previously recognized receptors in the Fc region of IgG [101]. The specific site for the Fc region of IgG was later termed FcgammaBP and identified as a protein primarily localized in the mucosal granules of the small intestinal and colonic epithelia that are secreted into the intestinal lumen. Based on the current knowledge, FcgammaBP is considered a protein that provides immunological protection to the intestinal tissue and facilitates the interaction between the intestinal mucus and potentially harmful stimuli (such as microorganisms and alarmins) with the ultimate goal of protecting the mucosal surface [94,101,102]. However, its exact biological function has yet to be fully elucidated.

FcgammaBP has been found in low concentrations in human serum from healthy individuals [95]. However, its serum concentrations were elevated in the presence of autoimmune diseases such as Crohn's disease, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, and progressive systemic sclerosis [95]. The presence of FcgammaBP has been further proven in the amniotic fluid, urine, saliva, and cerebrospinal fluid [97,100]. Liu et al. found the presence of FcgammaBP in the amniotic fluid in the second trimester of uncomplicated pregnancies [97]. In addition, FcgammaBP was shown to be among the most abundant (35/1624) proteins found in the amniotic fluid [97]. Our group described the presence of FcgammaBP in the amniotic fluid in pregnancies complicated by PPROM and PTL [98,99]. The finding of this study that FcgammaBP is a constituent of the amniotic fluid in PTL pregnancies is in line with the abovementioned findings.

Previously, the concentration of FcgammaBP in the amniotic fluid was shown to be higher in women with PPROM with MIAC and acute histological chorioamnionitis than in those without these complications [98]. Interestingly, no differences in the amniotic fluid concentration of FcgammaBP between the presence and absence of the abovementioned complications were identified in women with PTL, where amniotic fluid was obtained from the forewaters at the end of the first stage of labor [99].

In this study, we found an elevated amniotic fluid concentration of FcgammaBP in the presence of both phenotypes of IAI. Interestingly, the concentrations of FcgammaBP in amniotic fluid were higher in the presence of intra-amniotic infection than in the presence of sterile IAI. The results from this study show that both infectious and non-infectious stimuli might trigger the production of FcgammaBP.

In this study, the concentration of FcgammaBP was measured in paired amniotic and cervical fluid samples. Interestingly, the FcgammaBP concentrations were higher in the cervical fluid samples than in the amniotic fluid samples, despite the fact that cervical fluid samples obtained with a swab were diluted in 1.5 mL of the buffer. These observations suggest that epithelial cells and/or immune cells in the endocervical canal are able to produce FcgammaBP. This finding supports the key role of the cervix during pregnancy, which is immunological protection against the ascension of microorganisms from the vagina and the cervix toward the upper genital tract [103-106].

Cervical fluid sampling can be clinically relevant given the non-invasive nature of this procedure. However, only a weak positive correlation between the concentration of FcgammaBP in the amniotic fluid and the cervical fluid was found in PTL. Due to intact membranes in pregnancies with PTL, the protein composition of a cervical fluid sample may reflect the situation in the cervical compartment rather than that in the intra-amniotic cavity. The study shows that FcgammaBP in the cervical fluid is not a useful marker for the diagnosis of intra-amniotic complications in women with PTL.

Confirmation of intra-amniotic infection is a challenge for clinicians. The necessity to rule in or rule out the presence of microorganisms in amniotic fluid makes the diagnosis of intra-amniotic infection time-consuming and more expensive when the techniques used to identify either non-culturable or difficult-to-culture microorganisms are employed. Therefore, from a clinical point of view, there is an urgent need to discover a single marker of intra-amniotic infection that has reliable sensitivity and specificity. In this study, FcgammaBP in the amniotic fluid was identified as a potential marker of intra-amniotic infection in PTL pregnancies.

7.2 EXPERIMENTAL OBJECTIVES

7.2.1 Specific aim II-A

The key findings of specific aim II-A were as follows: 1) intra-amniotic administration of agents to model intra-amniotic inflammation/infection associated with preterm delivery has been used since 2004; 2) the published approaches to administer triggering agents into the gestational sacs include open surgery with direct puncture and transabdominal ultrasound-guided administration; 3) four kinds of triggering agents were used: i) live microorganisms, ii) bacterial products, iii) PAMPs, and iv) DAMPs; 4) LPS was the most commonly used triggering agent; 5) *Ureaplasma parvum* was the only live microorganism used; and 6) HMGB-1, S100B, and surfactant protein A were DAMPs used to model sterile IAI.

The intra-amniotic administration of triggering agents started to be used only recently. Our search (even though there was no time restriction) identified only the studies published after 2004, with majority of studies published in 2018. The possible explanations are the following: i) a change of researchers' view on the importance of intra-amniotic inflammatory response in the research of intra-amniotic inflammation/infection associated with preterm delivery, and ii) better availability of high-frequency ultrasound devices that made the intra-amniotic administration of triggering agents under ultrasound guidance possible.

The majority of the included studies used mini-laparotomy to establish access to the pregnant uterus. Laparotomy is an invasive procedure and has some drawbacks. Pain along with surgical stress can result in a major endocrine response influencing function of many organs [107]. High-frequency ultrasound devices have recently become available for small laboratory animal imaging. Of the included studies, only one research group took advantage of a high-frequency ultrasound device to guide the transabdominal administration into gestational sacs of mice [72,89,90,92,93]. This approach is less invasive than direct puncture of gestational sacs from mini-laparotomy.

The use of transabdominal ultrasound-guided administration has been reported only in mice but not in rats. In our experience, thicker rat skin impedes the needle passage through their abdominal wall. On the contrary, Serriere et al. showed that transabdominal ultrasound-guided aspiration of amniotic fluid was possible even in rats [108].

For animal modeling, intra-amniotic infection can be triggered by live microorganisms, their components, or PAMPs. Except for one study, the included LPS studies used LPS serotype O111:B4. Intra-amniotic administration of LPS O111:B4 to C57BL/6 mice caused preterm delivery in 80%-88% of the cases. The remaining mice delivered at term [89,90,92]. In studies with intraperitoneal and intrauterine administrations, almost all animals delivered preterm [109,110]. It is likely that the intensity of IAI triggered by the intra-amniotic administration of LPS was not strong enough to cause preterm delivery in all animals. However, the exposed pups suffered from severe mortality regardless of preterm or term delivery. The advantage of the intra-amniotic administration of LPS is the absence of signs of systematic involvement and

changes in body temperature in pregnant mice [90]. This scenario mimics a clinical situation in pregnant women.

The intra-amniotic administration of LPS to Sprague–Dawley rats did not cause preterm delivery among animals in five included studies regardless of the dose or serotype of LPS used. This might suggest that Sprague–Dawley rats were not as sensitive to LPS as C57BL/6 mice [84,85,87,88,91].

Genital mycoplasmas are the most frequent microorganisms diagnosed in the amniotic cavity of women with preterm delivery [111,112]. Therefore, their use to model intra-amniotic infection is more clinically relevant than LPS-based studies. Interestingly, in a study by Norman et al., intra-amniotic administration of live *Ureaplasma parvum* did not cause preterm delivery in the CD-1 mouse model [65]. The absence of preterm delivery induction by *Ureaplasma parvum* in this study can be the consequence of bypassing the process of ascending infection due to direct intra-amniotic administration. Other possible explanation is that the serovar of *Ureaplasma parvum* used in this study lacked the capacity to induce preterm delivery. However, exposed pups suffered from mild postnatal inflammation and worsened oxygen-induced lung injury, which demonstrate the significance of intra-amniotic infection [65].

Three kinds of DAMPs were used for the induction of sterile IAI in the included studies; namely, HMGB-1, S100B, and surfactant protein A [72,83,93]. In C57BL/6 mice, intra-amniotic administration of HMGB-1 and S100B caused a similar rate of preterm delivery (approximately 50%) [72]. These findings provide evidence that DAMPs can induce preterm delivery associated with sterile IAI and probably mimic similar situation among humans.

7.2.2 Specific aim II-B

The principal findings of the specific aim II-B were as follows: i) ultrasound-guided intra-amniotic administration of an agent was feasible in rats; ii) ultrasound-guided intra-amniotic administration of 10 μg of *E. coli* LPS serotype O55:B5 induced a marked elevation in the concentration of IL-6 in the amniotic fluid of rats; iii); the concentration of IL-6 in the amniotic fluid was elevated in gestational sacs treated with LPS; iv) intra-amniotic administration of 10 μg of *E. coli* LPS serotype O55:B5 did not induce labor within 24 hours; and v) after LPS administration, 95% of fetuses remained alive.

Ultrasound-guided injections of triggering agents have only been recently used to develop a model of inflammation/infection associated with preterm delivery in small laboratory animals [76,113]. This approach has become possible because of the high-frequency ultrasound devices specifically designed for use in small laboratory animals. Owing to their minimal invasiveness, ultrasound-guided injection is advantageous over the classical open surgery approach [76]. Ultrasound-guided intra-amniotic administration of triggering agents to create a model of IAI in mice is currently used by one research group [72,90,92,93]. Serriere *et al.* showed that transabdominal ultrasound-guided aspiration of the amniotic fluid was also possible in rats [108]. However, to the best of our knowledge, our study is the first to use ultrasound-guided intra-amniotic administration of a triggering agent to develop a model of IAI in rats.

LPS, a component of the cell wall of gram-negative bacteria, has been used to model infection and inflammation associated with preterm delivery in animal models for decades [114]. Gayle *et al.* demonstrated a 12-fold and 5-fold increase in IL-6 levels in the amniotic fluid at 6 and 12 hours, respectively, after systemic administration of LPS to rats [115]. In our study, the intra-amniotic injection of 10 μg of *E. coli* LPS serotype O55:B5 per gestational sac under ultrasound guidance triggered a marked elevation in IL-6 levels in the amniotic fluid. Twenty-four hours after administration, the concentration of IL-6 in the amniotic fluid in the

gestational sacs with LPS were 15-fold higher than that in the amniotic fluid in the gestational sacs without LPS. Elevated IL-6 levels were observed only in the gestational sacs with LPS. However, the amniotic fluid from non-injected gestational sacs in rats administered LPS, had slightly higher IL-6 concentrations than that from non-injected gestational sacs in rats administered PBS. This phenomenon could be explained by a potential weak leak of LPS from the injected gestational sacs into the choriodecidual space surrounding non-injected gestational sacs. We can only hypothesize that the presence of LPS leak in the choriodecidual space from injected gestational sacs might have triggered a weak inflammatory response with the release of IL-6 in the amniotic fluid into non-injected sacs.

There is evidence that intra-amniotic administration of LPS 0.1 μg per gestational sac in mice causes a high frequency of preterm delivery (80%) [89,90,92]. In our study, dams were not delivered within 24 h after intra-amniotic administration of LPS despite the development of IAI. This is in line with other rat studies, in which intra-amniotic administration of LPS via small laparotomy did not induce preterm delivery [87,88]. Moreover, intra-amniotic administration of LPS to mice is associated with a high fetal mortality rate, which is not seen in rat studies [87,90-92]. In our study, only 1 of the 19 fetuses from gestational sacs injected with LPS died 24 hours after administration. This intrauterine death could be attributed to IAI induced by LPS; however, needle injury during the intra-amniotic puncture might have also been a possible mechanism. The important attribute of this model is that intra-amniotic administration of LPS did not induce labor and 95% of fetuses remained alive in the inflammatory environment. Due to its endurance and stability, it may represent a more valuable model for research of intra-amniotic inflammatory complications in maternal and fetal compartments than in a similar mouse model.

The assessment of IL-6 concentrations in the amniotic fluid in an animal model represents an approach relevant to human clinical practice. Therefore, we focused on the evaluation of this body fluid in our rat model. To the best of our knowledge, this is the first study to investigate IL-6 levels in the amniotic fluid of rats after intra-amniotic administration of LPS. The possible reason for the absence of other studies could be the fact that the acquisition of a sufficient volume of amniotic fluid for analysis could be a challenge after intra-amniotic injection. In our study, the amniotic fluid volume was reduced in the injected gestational sacs, which impaired amniotic fluid harvesting. The reduction of amniotic fluid volume could have been associated with the development of IAI; however, based on the concurrent occurrence of this event in gestational sacs administered PBS, the leakage of the amniotic fluid through the traumatized membranes was a more probable mechanism.

7.2.3 Specific aim II-C

In specific aim II-C, the protocol of ultrasound-guided intra-amniotic administration of an agent to create a rat animal model of IAI was summarized. The protocol provides complete and consistent instructions on how to develop a rat animal model of IAI by ultrasound-guided intra-amniotic administration of an agent. The ultrasound-guided mini-invasive approach minimizes trauma and stress in animals. In our study, we used LPS administration; however, other agents such as different bacteria and their products or DAMPs can be used in the same way to trigger different phenotypes of IAI in rats. Therefore, this protocol can be a supportive and helpful basis for the establishment of other rat models of IAI.

8. CONCLUSION

8.1 CLINICAL OBJECTIVES

In women with PTL, the presence of both phenotypes of IAI, intra-amniotic infection and sterile IAI, was associated with an elevated concentration of IL-6 in the cervical fluid. However, no differences in the concentration of IL-6 in the cervical fluid were found between the two conditions.

In women with PTL, the concentrations of FcgammaBP in amniotic fluid were elevated in the presence of both phenotypes of IAI, being higher in the presence of intra-amniotic infection. Therefore, FcgammaBP in the amniotic fluid can be considered a potential marker of intra-amniotic infection in women with PTL. The concentration of FcgammaBP of the cervical fluid was not altered by the presence of either phenotype of IAI.

8.2 EXPERIMENTAL OBJECTIVES

A systematic review of the literature demonstrated that intra-amniotic administration of triggering agents is used to model intra-amniotic infection/inflammation in rodents. Intra-amniotic administration under ultrasound guidance has been described in mice, but not in rats.

Our experiments showed that ultrasound-guided intra-amniotic administration of an agent was feasible in rats. The administration of 10 µg of E. coli LPS serotype O55:B5 per gestational sac resulted in the development of IAI and did not induce labor or fetal mortality.

Finally, a step-by-step protocol for ultrasound-guided intra-amniotic administration of an agent in a rat to support the reproducibility and feasibility of this approach was developed.

9. LITERATURE REVIEW

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10. PUBLICATIONS AND LECTURES

10.1 ORIGINAL RESEARCH PAPERS PUBLISHED IN THE JOURNALS WITH IMPACT FACTOR

1. **Stranik J**, Kacerovsky M, Soucek O, Kolackova M, Musilova I, Pliskova L, Bolehovska R, Bostik P, Matulova J, Jacobsson B, Andrys C. IgGFc-binding protein in pregnancies complicated by spontaneous preterm delivery: a retrospective cohort study. *Scientific Reports [IF$_{2019}$ 3.998]*. ***2021 Mar*** *17;11(1):6107. PMID: 33731725.*
2. **Stranik J**, Kacerovsky M, Andrys C, Soucek O, Bolehovska R, Holeckova M, Matulova J, Jacobsson B, Musilova I. Intra-amniotic infection and sterile intra-amniotic inflammation are associated with elevated concentrations of cervical fluid interleukin-6 in women with spontaneous preterm labor with intact membranes. *The Journal of Maternal-Fetal & Neonatal Medicine [IF$_{2019}$ 1.737]*. ***2021 Jan*** *7;1-9. PMID: 33412979.*
3. Soucek O, Kacerovsky M, **Stranik J**, Musilova I, Pliskova L, Bolehovska R, Matulova J, Andrys C. Macrophage inflammatory protein-1α in amniotic and cervical fluids in spontaneous preterm labor with intact membranes with respect to intra-amniotic inflammation. *The Journal of Maternal-Fetal & Neonatal Medicine [IF2019 1.737]*. ***2021 May*** *9;1-9. PMID: 33969779.*
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5. Chalupska M, Kacerovsky M, **Stranik J**, Gregor M, Maly J, Jacobsson B, Musilova I. Intra-Amniotic Infection and Sterile Intra-Amniotic Inflammation in Cervical Insufficiency with Prolapsed Fetal Membranes: Clinical Implications. *Fetal Diagnosis and Therapy [IF$_{2019}$ 2.095]*. ***2020 Dec*** *8;48(1):58-69. PMID: 33291113.*
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7. Kacerovsky M, Romero R, Stepan M, **Stranik J**, Maly J, Pliskova L, Bolehovska R, Palicka V, Zemlickova H, Hornychova H, Spacek J, Jacobsson B, Pacora P, Musilova I. Antibiotic administration reduces the rate of intraamniotic inflammation in preterm prelabor rupture of the membranes. *American Journal of Obstetrics and Gynecology [IF$_{2019}$ 6.502]*. ***2020 Jul****;223(1):114.e1-114.e20. PMID: 32591087.*
8. Musilova I, Kolackova M, Andrys C, Drahosova M, Baranová I, Chmelarova M, **Stranik J**, Jacobsson B, Kacerovsky M. Nicotinamide phosphoribosyltransferase and intra-amniotic inflammation in preterm prelabor rupture of membranes. *The Journal of Maternal-Fetal & Neonatal Medicine [IF$_{2019}$ 1.737]*. ***2019 May*** *15;34(5):736-746. PMID: 31056993.*
9. Musilova I., Spacek R., **Stranik J**., Jacobsson B., Kacerovsky M., Fetal Portal System Flowmetry and Intra-Amniotic Inflammation in Preterm Prelabor Rupture of Membranes. *Fetal Diagnosis and Therapy [IF$_{2019}$ 2.095]*. ***2019 Mar*** *19;46(5):323-332. PMID: 30889602.*

10. Kacerovsky M., Vlkova B., Musilova I., Andrys C., Pliskova L., Zemlickova H., **Stranik J**., Halada P., Jacobsson B., Celec P., Amniotic fluid cell-free DNA in preterm prelabor rupture of membranes. *Prenatal Diagnosis [IF$_{2018}$ 2.434]*. ***2018 Dec*** *;38(13):1086-1095. doi: 10.1002/pd.5366. PMID: 30276834*.
11. Musilova I, Bestvina T, **Stranik J**, Stepan M, Jacobsson B, Kacerovsky M. Transabdominal Amniocentesis Is a Feasible and Safe Procedure in Preterm Prelabor Rupture of Membranes. *Fetal Diagnosis and Therapy [IF$_{2017}$ 1.813]*. ***2017 Feb*** *25;42(4):257-261. PMID: 28237988.*

10.2 OTHER PAPERS PUBLISHED IN THE JOURNALS WITH IMPACT FACTOR

1. **Stranik J**, Kacerovsky M, Vescicik P, Faist T, Jacobsson B, Musilova I. A rodent model of intra-amniotic inflammation/infection, induced by the administration of inflammatory agent in a gestational sac, associated with preterm delivery: a systematic review. *The Journal of Maternal-Fetal & Neonatal Medicine [IF$_{2019}$ 1.737]*. ***2020 Apr*** *29:1-9. PMID: 32349576.*
2. Burckova H, **Stranik J**, Musilova I, Matulova J, Jacobsson B, Kacerovsky M. Intra-amniotic inflammatory complications in preterm prelabor rupture of membranes and long-term neurodevelopmental outcomes of infants: a systematic review. *The Journal of Maternal-Fetal & Neonatal Medicine [IF$_{2019}$ 1.737]*. ***2021 Mar*** *29:1-6. PMID: 33781152.*
3. Musilova I, Elias P, **Stranik J,** Matejkova A, Kacerovsky M. Transvaginal three-dimensional ultrasound of the fetal pelvis to detect anorectal malformation during the second trimester. *Ultrasound in Obstetrics and Gynecology [IF$_{2019}$ 5.571]*. ***2021 Jan*** *27. PMID: 33502057*.

10.3 PAPERS PUBLISHED IN THE JOURNALS WITHOUT IMPACT FACTOR

1. **Stráník J**., Kacerovský M. Předčasný odtok plodové vody v periviabilním období. *Gynekologie a Porodnictví*, **2018**
2. Veščičík P, Kacerovská Musilová I, **Stráník J**, Štěpán M, Kacerovský M. Lactobacillus crispatus dominant vaginal microbita in pregnancy. *Česká Gynekologie.* ***2020*** *;85(1):67-70. PMID: 32414287.*
3. Mls J, **Stráník J**, Kacerovský M. Lactobacillus iners-dominated vaginal microbiota in pregnancy. *Česká Gynekologie.* ***2019*** *;84(6):463-467. PMID: 31948257*.

10.4 LECTURES – INTERNATIONAL

1. Preterm prelabor rupture of membranes complicated by sterile intra-amniotic inflammation, *Salzburg, Austria,* ***2020***.
2. Late preterm prelabor rupture of membranes, *Sahlgrenska University hospital, Gothenburg, Sweden,* ***2019***.
3. Vaginal fluid interleukin-6 as a point-of-care test, *PREBIC meeting, Guangzhou, China,* ***2018***.
4. Rat model of intra-amniotic infection and inflammation, *TriNet meeting, Bratislava, Slovakia****, 2018***.

5. Vaginal fluid interleukin-6 as a point-of-care test, *TriNet meeting, Bodapest, Hungary*, ***2016***.

10.5 LECTURES – CZECH

1. Ultrazvukové vyšetření pánve plodu, *41. celostátní konference Sekce ultrazvukové diagnostiky ČGPS ČLS JEP, Brno,* ***2020***.
2. PPROM: Chlamydia trachomatis v plodové vodě u PPROM, *20. celostátní konference fetální medicíny, Praha* ***2020***.
3. Předčasný porod a císařský řez, *Čechova ultrazvuková konference, Olomouc,* ***2018***.
4. Pozdní PPROM - jak FIRS ovlivňuje neonatální výsledky, *18. celostátní konference fetální medicíny Praha*, ***2018***.
5. PPROM u dvojčat v periviabilním období, *7. konference nemocničních gynekologů, České Budějovice,* ***2017***.
6. PPROM: jak reaguje plod na intra-amniální zánětlivé a infekční komplikace, *17. celostátní konference fetální medicíny, Praha,* ***2017***.
7. Význam ultrazvuku v entitě předčasného porodu, 6. *jihlavská perinatologická konference, Jihlava,* ***2016***.
8. Cerclage – co, kdy, komu a jak?, *Východočeské perinatologické dny, Deštné v Orlických horách*, ***2016***.
9. PPROM: calreticulin v plodové vodě, nadějný marker intraamniální infekce, *16. celostátní konference fetální medicíny, Praha,* ***2016***.
10. Midgut volvulus ve II. trimestru. *36. celostátní konference Sekce ultrazvukové diagnostiky ČGPS ČLS JEP, Brno,* ***2015***.
11. Management předčasného porodu, *Východočeské perinatologické dny*. *Deštné v Orlických horách*, ***2015***
12. Je cervikovaginální tekutina to samé co plodová voda? *15. celostátní konference fetální medicíny, Praha*, ***2015***.
13. Bedside interleukin-6 v plodové vodě u pacientek s PPROM. 4. konference *nemocničních gynekologů, Ostrava,* ***2014***.
14. Bedside interleukin-6 v plodové vodě u pacientek s PPROM. *14. celostátní konference fetální medicíny, Praha,* ***2014***