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Does high molecular weight-hyaluronic acid prevent hormone-induced preterm labor in rats?

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Abstract. – OBJECTIVE: The aim of our study was to test if oral high hyaluronic acid (HMW-HA) administration was effective in contrasting induced preterm birth (PTB) in female Wistar rats.

MATERIALS AND METHODS: A total of 24 pregnant rats were pretreated with placebo or low (2.5 mg/day)/high dose (5 mg/day) of HMW-HA (day 15) and then induced to delivery with mifepristone plus prostaglandin E2 (PGE2) (3 mg/100 μL + 0.5 mg/animal) on the 19th day of pregnancy. The delivery time was recorded and the messenger RNA (mRNA) levels of pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)1β, IL-6] were detected in the uterine tissues by real-time polymerase chain reaction (real PCR). Immunohistochemistry was performed alongside.

RESULTS: Oral HMW-HA was well absorbed in the body and was able to significantly delay the timing of delivery and decrease mRNA synthesis of pro-inflammatory cytokines.

CONCLUSIONS: HMW-HA, by acting in the management of PTB, may represent a new approach to protecting physiological pregnancy.

Key Words:
Hyaluronic acid (HA), High-molecular-weight hyaluronic acid (HMW-HA), Pregnancy, Preterm delivery, Rats.

Introduction

Hyaluronic acid (HA) belongs to the family of glycosaminoglycans (GAGs) and is a component of the extracellular matrix (ECM) that is diffused in the epithelial, connective, and nervous tissues[1]. Molecular weight can differ based on the number of repeating disaccharides in HA molecule[2], and each molecular weight activates different molecular pathways. Therefore, HA can act in multiple processes displaying diverse functions[3]. In general, low-molecular-weight hyaluronic acid (LMW-HA) displays pro-inflammatory and pro-angiogenic properties, playing a pivotal role in wound healing processes[4]. Conversely, high-molecular-weight hyaluronic acid (HMW-HA) is a lubricating and an immunosuppressor agent that binds to fibrinogen and modulates inflammatory cytokines and migration of stem cells[5,6]. HMW-HA also has a fundamental role in female reproductive biology, from folliculogenesis to birth. It constitutes a viscoelastic matrix that protects the oocyte and maintains the integrity of fetal membranes[7]. Deficiency of HMW-HA in the uterine cervix may expose the fetus to a greater risk of ascending infections, thus also increasing the risk of preterm birth (PTB)[8]. According to World Health Organization (WHO)[9,10], PTB is defined as delivery before 37 completed weeks of gestation. It globally affects 5-18% of the pregnancies and represents the leading cause of mortality in children under 5 years of age. It is associated[9,10] with increased risk of lifelong health complications as neurological, respiratory and gastrointestinal deficits. Prevention of PTB is complicated because 60% of the cases derive from unknown etiology[11]. Under physiological conditions, the cervical extracellular matrix undergoes drastic structural rearrangements toward the end of the pregnancy to fulfill the functional change of the cervix during parturition. Therefore, understand-

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ing the molecular mechanisms behind cervical remodeling represents an important first step in planning strategies to prevent PTB[12]. Considering the importance of immunotolerance for successful pregnancies, the anti-inflammatory activities of HMW-HA are pivotal to maintain a physiological gestation. Indeed, HMW-HA stimulates not only the secretion of anti-inflammatory cytokines, but it also inhibits the expression of pro-inflammatory factors that may induce a general inflammation status responsible for PTB[13]. The aim of this study was to investigate the effects of HMW-HA supplementation in counteracting PTB in a rat model of hormone-induced PTB.

Materials and Methods

In Vivo Model

Wistar albino female rats, weighing approximately 200-220 g, were kept at 22 ± 3°C with the relative humidity of 30-70% and 12/12 hours photoperiod on a standard rodent pellet diet, with tap water available *ad libitum*.

In Vivo Experiments

At the beginning of the experiment, vaginal smears were taken from n=24 rats to determine estrous cycles. Following estrous cycle determination, female rats were mated with male rats in a mating cage. The following day, vaginal smears were taken from the female rats and a sperm search were performed under a microscope. If the smear proved positive, the female rats were separated and regarded as first-day pregnant animals. Then the pregnant rats (total n=24) were divided in 4 groups of 6 animals each and treated as follows: Group 1: physiologic solution (control group); Group 2: mifepristone + prostaglandin E2 treatment (preterm birth, PTB group); Group 3: 2.5 mg HMW-HA per day + mifepristone + prostaglandin E2 treatment (low dose HA + PTB group); Group 4: 5 mg HMW-HA per day + mifepristone + prostaglandin E2 treatment (high dose HA + PTB group).

Preterm Birth Model

Preterm birth was induced with a combination of 3 mg/100 μL mifepristone (catalogue number: 459982500, Agros Organics, Hampton, VA, USA) and 0.5 mg/animal prostaglandin E2 (PGE2, catalogue number:2268-5, BioVision, Milpitas, CA, USA) on day 19 of pregnancy. Mifepristone was suspended in olive oil and was given as intraperitoneal injection at 9:00 am and PGE2 was intravaginally applied at 4:00 pm, according to a published protocol[14]. The time to delivery was recorded and defined as the number of hours from the time of mifepristone injection to delivery of the first pup.

HMW-HA Treatment

HMW-HA (molecular weight 1,000-1,500 kDa) (HyaSource® Vita, TS-Biotech, Linqu, Shandong, P.R.C.) was administered by oral gavage to animals at the doses of 2.5 and 5 mg per day as it is equivalent to 100 and 200 mg per day in women (60 kg body weight), which are applied according to the Kimura et al[15]. The reason for the treatments starting from the 15th day was because it correlates to the occurrence of preterm birth that is on the 27-35th weeks. The aim was to see the delaying effect of HA supplementation (i.e. after entering the 3rd Trimester) on the risk of a preterm birth.

Histological Examinations

Following the delivery, rats were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg). Uterine and cervical tissues were removed and divided in two (Figure 1). Left pieces were fixed in buffered 10% formaldehyde for three days and then blocked until histological analysis. Right pieces were kept at -80ºC for real time PCR. After routine histological procedures, 5 μm sections were taken with a rotary microtome (catalogue number: RM 2255, Bannockburn, IL, USA). For histomorphological evaluations, sections were stained with Hematoxylin and Eosin (H&E) (catalogue number: H9627, Saint Louis, MO, USA) and Masson's trichrome (MT) (catalogue number: HT15, Saint Louis, MO, USA) for connective tissue differences.

Immunohistochemical Examination

Sections were mounted on poly-L-lysine-coated slides. The streptavidin-biotin-peroxidase assay was carried out using the primary antibodies against IL1β (1/100 dilution) (catalogue number: sc-7884; Heidelberg, Germany), IL-6 (1/100 dilution) (catalogue number: sc-7884; Heidelberg, Germany) and TNF-α (1/100 dilution) (catalogue number: NB600-587; Colorado, USA). After deparaffinization, sections were treated with trypsin (catalogue number: TA-125-TR, Lab Vision Corporaiton, Fremont, CA, USA) for 15 minutes and endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxide in PBS

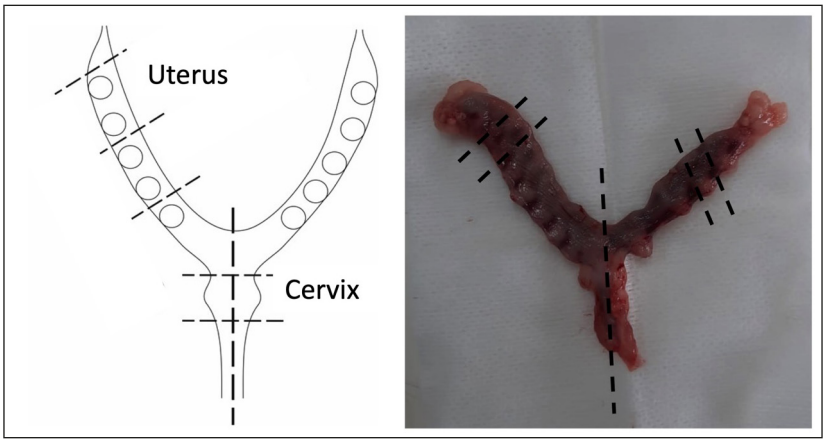

Figure 1. Postpartum uterus tissue.

at room temperature for 10 min. Then, primary antibodies were applied for 2 h at 4°C temperature and washed in PBS. After washing, the secondary antibodies (catalogue number: 85-9043, Invitrogen Corporation, Carlsbad, CA, USA) were applied for 30 min, followed by washings in PBS. The peroxidase activity was visualized with diaminobenzidine (DAB) (catalogue number: 11718096001, Roche, Merck, Darmstadt, Germany). Slides were counterstained with Mayer's hematoxylin, dehydrated, cleared, and analyzed on a light microscope[14].

Scoring of Immunohistochemistry

For quantitative measurements, the percentage of immunopositive cells were determined as average of measurements in 3 different random fields by Image J software (available at: https://imagej.nih.gov/ij/download.html). Immunostaining intensity was categorized accordingly to the following scores: 0 (no staining), 1 (weak, but detectable staining), 2 (moderate staining), and 3 (intense staining). The H-score values were derived for each specimen by calculating the sum of the percentage of cells with the formula: H-score= $\sum$Pi (i+1). Where i (1→4) is the intensity of staining with respective values of 0, 1, 2, or 3 (absent, weak, moderate, or strong, respectively) and Pi is the percentage of stained cells for each intensity, varying from 0% to 100%. For each slide of tissue, 5 different fields were randomly selected and evaluated microscopically at 200X magnification. The H-score evaluation was performed by at least 2 independent experienced histologists, blinded to the source of the samples taken from different random fields of the same sections, and the average score was utilized.

Expression of Cytokine Messenger RNA in Tissues

RNA from tissues was extracted with FFPE RNeasy mini kit (Qiagen, Milan, Italy) according to the indication of the manufacturer; 1 µg of total RNA was used for complementary DNA (cDNA) synthesis, and 1 µg of total cDNA was used for each real-time reaction; analyses were performed in triplicate for each sample as previously described. Total cDNA levels were standardized by normalization to the Glyceraldehyde-3-phosphate dehydrogenase (GADPH) control and presented as the fold increase (ratio of the experimental gene value/GADPH gene value) to the control sample. GAPDH and 18S ribosomal RNA was used to normalize the polymerase chain reactions (PCRs) with comparable results. Primers for housekeeping genes and primers for TNF-α, IL1β, IL-6 were used (Table I). All primer sets have an annealing temperature of 60°C and were checked for primers efficiency over 90% on cDNA standard curve.

Statistical Analysis

Statistical analysis were performed using the SPSS software for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA). Data are presented

Table I. Real-time PCR primer list.

Primer	
TNF-α	ATGGGCTCCCTCTCATCAGT GCTTGGTGGTTTGCTACGAC
IL1β	TGGCAACTGTCCCTGAACTC AGGGCTTGGAAGCAATCCTTA
IL-6	CACTTCACAAGTCGGAGGCT TCTGACAGTGCATCATCGCT

as mean ± SEM. Kruskal-Wallis nonparametric test and post hoc Mann-Whitney U test were used to compare the groups. Statistical significance was set at $p<0.05$. All *in vitro* experiments were performed at least 3 times unless otherwise stated. Graphs were performed with GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

The delivery time of the first fetus was noted as the duration in hours from the time of mifepristone and PGE2 administration. The gestation period in these rodents normally lasts 22 days, with variations between 21 and 23 (rarely up to 26)[14,15]. In our study, the average gestation period in the Group 1 was 22.8 days. When the delivery times were compared after mifepristone administration, a significant decrease was observed in Group 2 (26.3 hours) compared to Group 1 (91.3 hours). While no significant difference was observed in Group 3 (30.0 hours) compared to Group 2, a significant increase in delivery time was observed in Group 4 (59.1 hours) compared to Group 2 (Figure 2).

Examination of uterine and cervical tissue sections showed intact layers with normal histological structure, no histological differences were observed in tissue layers (H&E) (Figure 3-4). Masson's trichrome staining revealed a different pattern of collagen packing, and fibrils were observed as irregular and increased in Group 2 and Group 3 with respect to Group 1 and Group 4 (Figure 3-4 black arrows).

With immunohistochemical analyses of uterine and cervical tissues following delivery, we observed staining of TNF-α (Figure 5) and IL1β (Figure 6) in Group 2 and Group 3. H-score analyses evidenced that TNF-α and IL1β positive cells were significantly increased in Group 2 and Group 3 with respect to Group 1 and Group 4, ($p<0.05$) in which we detected no staining. We observed staining of IL-6 in Group 1 and Group 4 (Figure 7).

The mRNA expression for TNF-α, IL1β and IL-6 was also investigated by real time PCR in uterus tissue. As reported in Figure 8, TNF-α and IL1β were upregulated in Group 2 respect to all other groups. In detail, TNF-α mRNA was upregulated and expressed 2.1-fold in Group 2 group *vs.* Group 1 (Figure 8a); IL1β was significantly upregulated and expressed 3.4-fold in Group 2 group *vs.* Group 1 (**$p<0.01$) (Figure 8b). The HMW-HA treatment at low dose (Group 3) significantly reversed the upregulation of IL1β mRNA caused by PTB induction (Group 2) ($^{\#}p<0.05$) (Figure 8b); the HMW-HA treatment at high dose (Group 4) significantly reversed the upregulation of both TNF-α and IL1β mRNA observed in the case of Group 2 ($^{\#}p<0.05$ and $^{\#\#}p<0.01$) (Figure 8a-b). IL-6 mRNA was significantly down-regulated in Group 2 (0.3-fold) and Group 3 (0.2-fold) with respect to the Group 1 (**$p<0.01$). The HMW-HA treatment at high dose (Group 4) partially reversed this effect, but without reaching statistical significance.

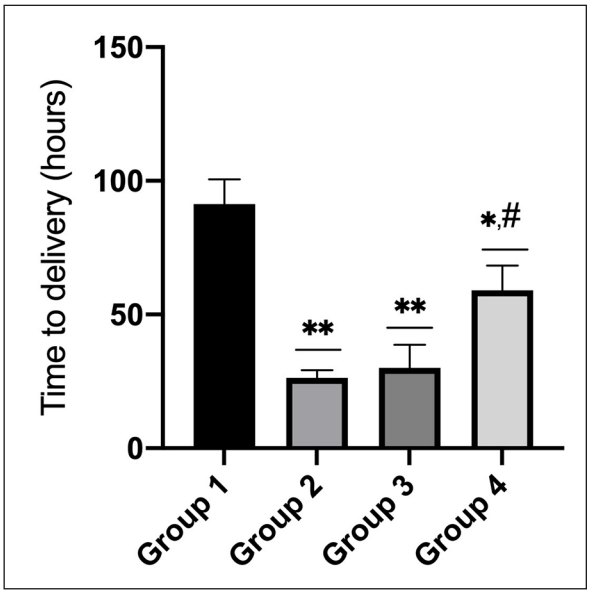

Figure 2. Delivery times in each group after mifepristone administration. Group 2 and Group 3 *vs.* Group 1 **$p<0.01$; Group 4 *vs.* Group 1 *$p<0.05$; Group 4 *vs.* Group 2 $^{\#}p<0.05$ performed with Mann-Whitney U Test. Kruskal Wallis Test. All group $p=0.001$.

Discussion

Our results suggest that oral HMW-HA (molecular weight 1.000-1.500 kDa) is well absorbed and distributed in the cervical and uterine tissues when administered in a rodent model. Our data also indicates that HMW-HA significantly counteracts the effects of mifepristone and PGE2 on PTB induction in Wistar albino female rats by delaying the delivery time and reversing the upregulation of pro-inflammatory cytokines in uterine tissues. In our experiments, we used a hormone-induced model of PTB according to what was reported by our previous study and Gálik

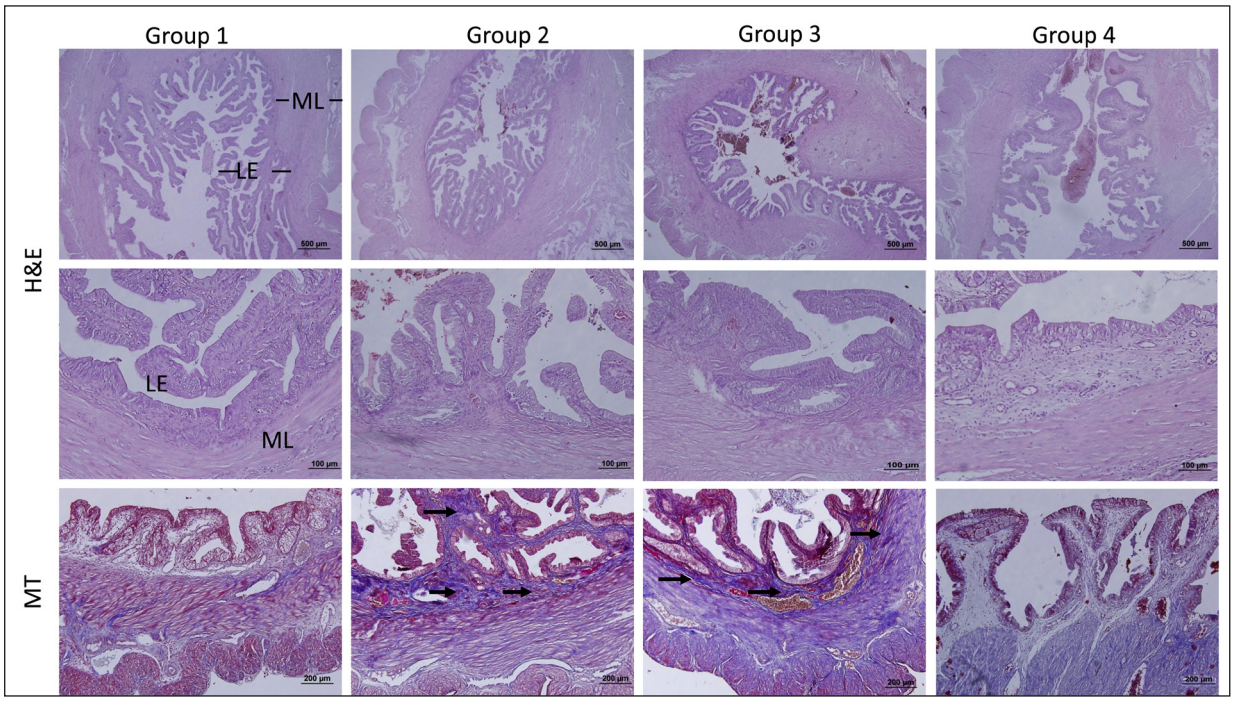

Figure 3. Transverse sections of histologic Hematoxylin-Eosin (H&E) and Masson's Trichrome (MT) staining of uterus. H&E and MT staining were performed on formalin-fixed and paraffin-embedded sections of rats' uterus. LE: Luminal epithelium, ML: Muscular layer. Black arrows show irregular and increased different pattern of fibrils. Magnification: 4×, 10× and 20×. Scale bar = 500, 100 and 200 µm.

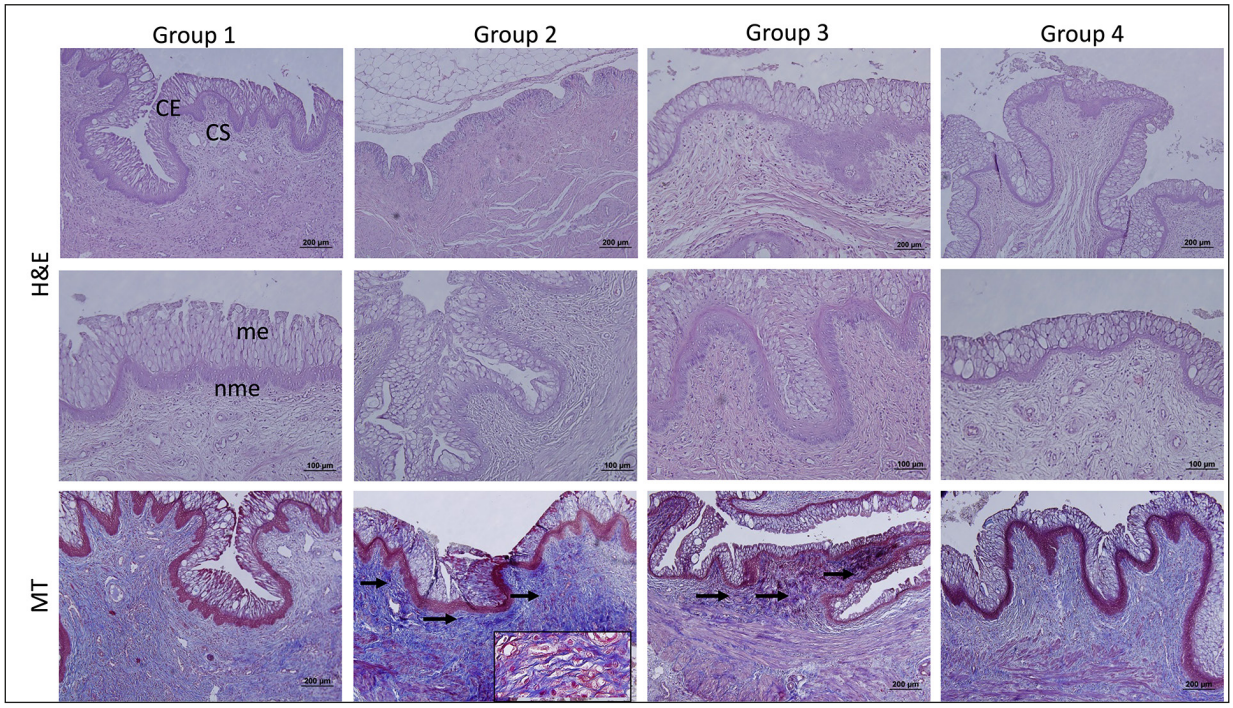

Figure 4. Transverse sections of histologic Hematoxylin-Eosin (H&E) and Masson's Trichrome (MT) staining of cervix. H&E and MT staining were performed on formalin-fixed and paraffin embedded sections of rats' cervix. CE: Cervical epithelia, CS: Cervical stroma, nme: non-mucosal epithelia, me: mucosal epithelia. Black arrows show irregular and increased different pattern of fibrils. Magnification: 10× and 20×. Scale bar = 100 and 200 µm.

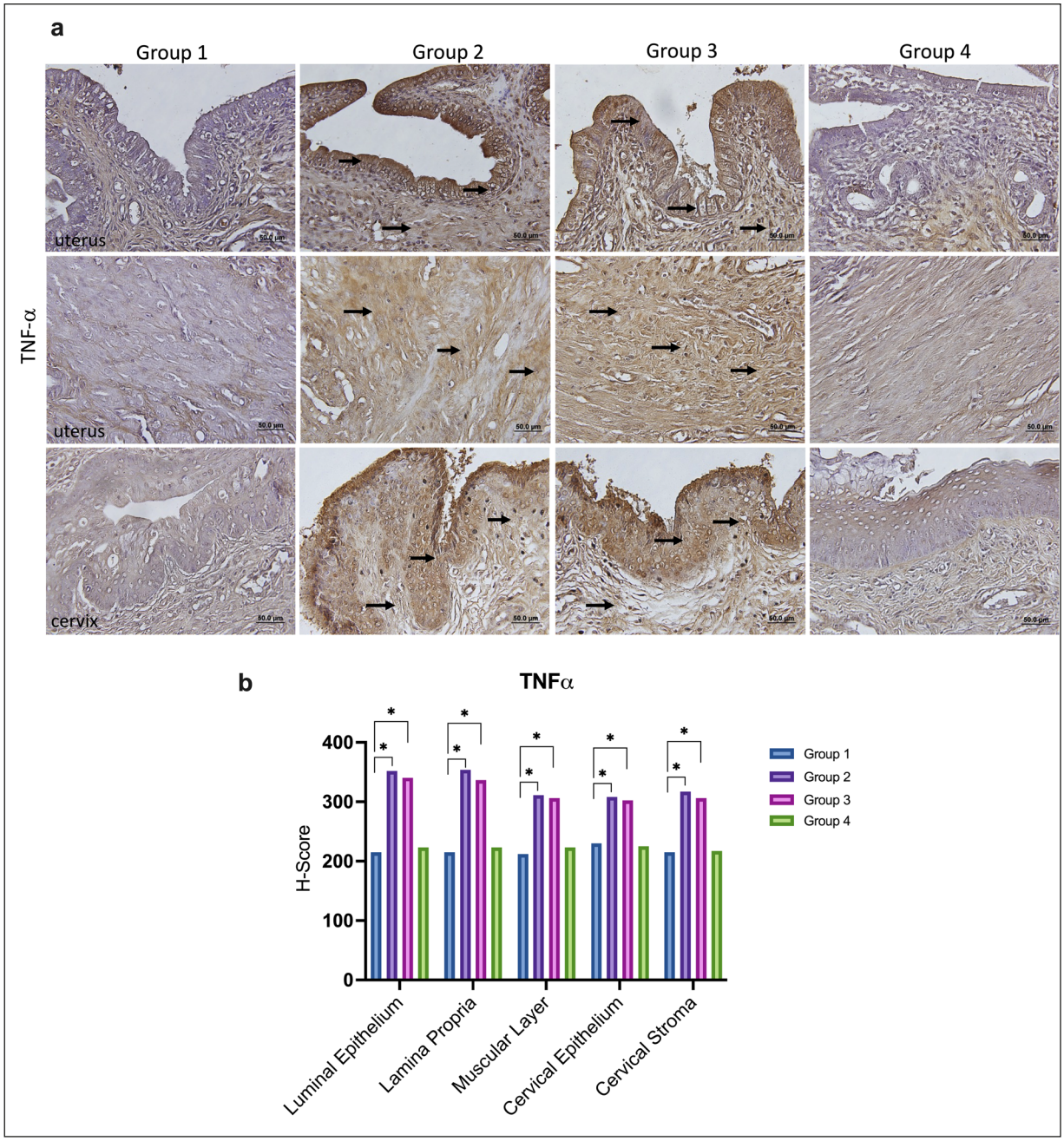

Figure 5. **a**, Immunohistochemical staining of TNF-α in uterus and cervix tissues. Black arrows indicate TNF-α staining. Magnification: 40×. Scale bar = 50 µm. **b**, H-score analyses of TNFα immunoreactivity. The asterisk indicates significant difference with respect to control group (p<0.05).

et al[16]. We used mifepristone, a synthetic steroid that induces PTB faster than lipopolysaccharide (LPS), with complete efficacy within 24 hours[14,16] and antagonizes progesterone receptors due to its great affinity[17,18]. This effect prevented the suppression of oxytocin receptors induced by progesterone and stimulated myometrial contractility and the onset of labor[19]. Intrauterine deaths frequently occur in LPS-induced preterm deliveries[20] and as evidenced by Terrone et al[21], LPS-induced preterm labor occurs within 92 hours with live birth rate around 50%. For these reasons, given the limited number of animals in the groups, we chose a hormone-induced delivery model.

Physiological pregnancy is maintained through a constant balance between maternal/fetal inhibitors and activators to preserve uterine quiescence,

membrane integrity, and cervical competence until labor. During labor, instead, the uterus shifts from a quiescent to a contractile status, and the cervix becomes soft and dilated to allow the passage of the fetus through the birth canal[22]. Proper cervical function is essential for physiological pregnancy, therefore understanding the molecular mechanisms behind cervical remodeling is a key step to prevent PTB[23].

At term birth, hyaluronic acid concentrations decrease because of increased activity of metalloproteinases, enzymes that degrade extracellular matrix and basement membrane components, at the time of birth. The change in hyaluronic acid concentration leads to leukocyte migration and dilation of the cervix[24-27].

In our experiment we used HMWHA because LMWHA is well known to have opposite ef-

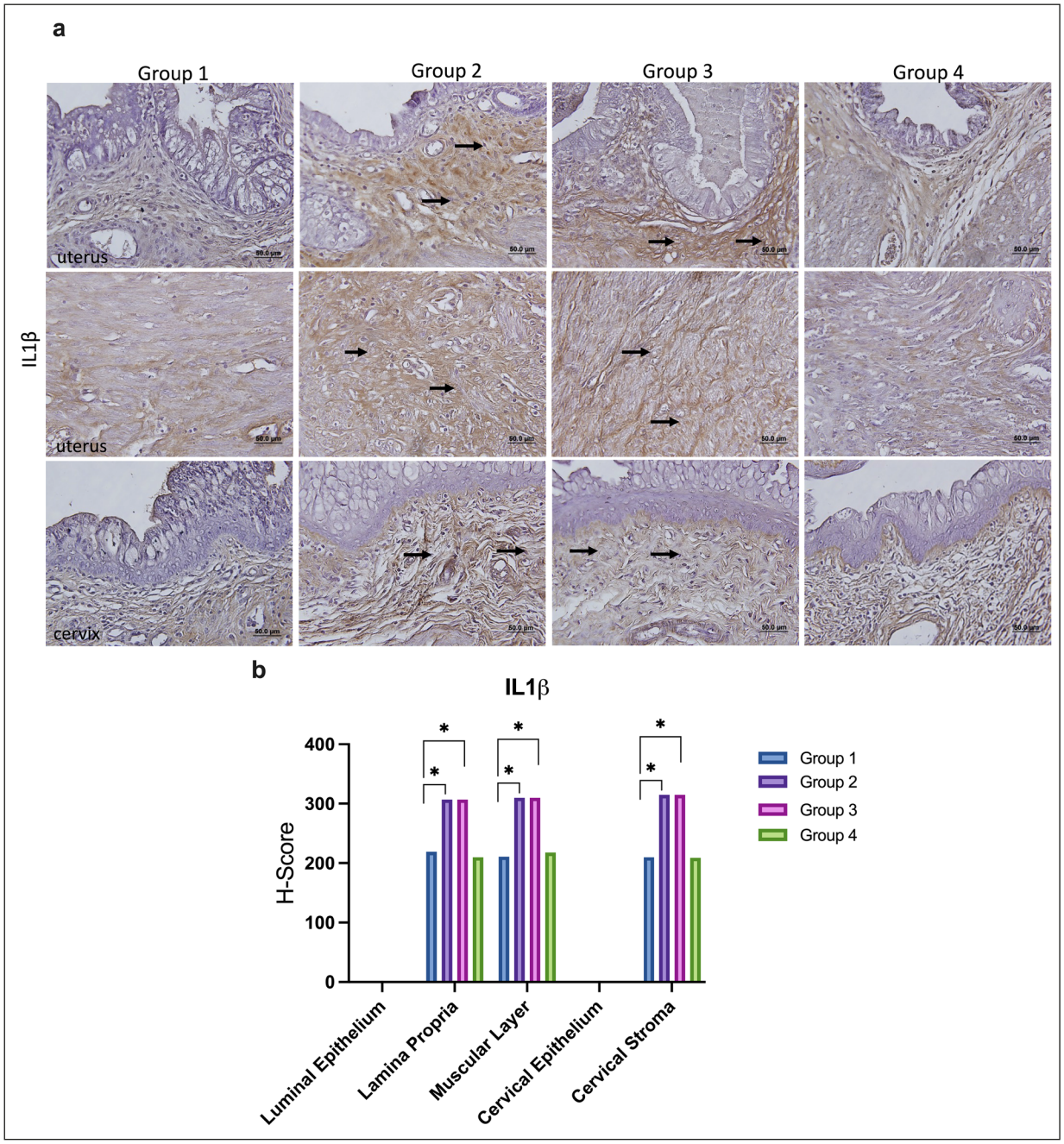

Figure 6. **a**, Immunohistochemical staining of IL1β in uterus and cervix tissues. Black arrows indicate IL1β staining. Magnification: 40×. Scale bar = 50 μm. **b**, H-score analyses of IL1β immunoreactivity. The asterisk indicates significant difference from control group ($p<0.05$).

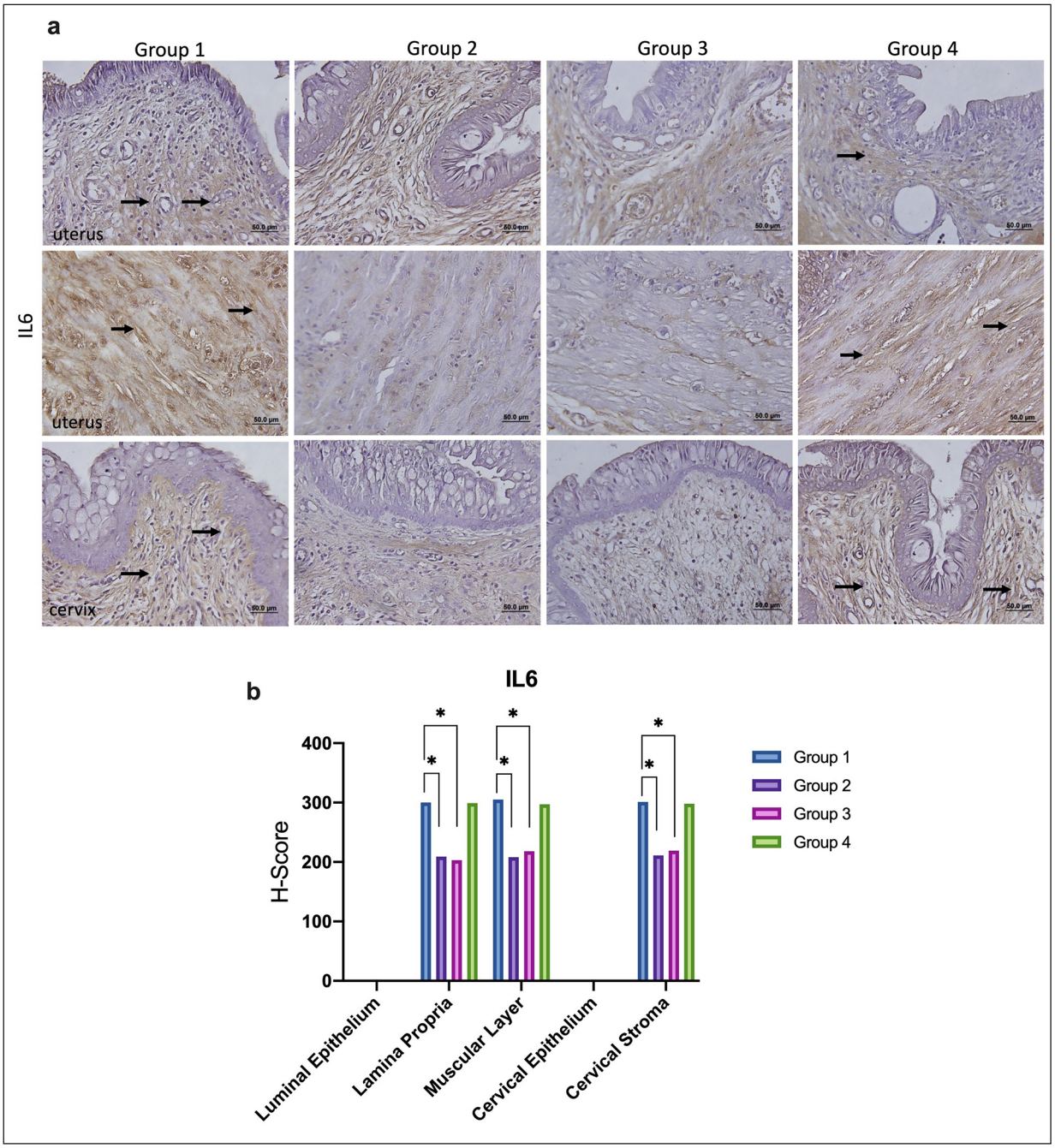

Figure 7. **a**, Immunohistochemical staining of IL-6 in uterus and cervix tissues. Black arrows indicate IL-6 staining. Magnification: 40×. Scale bar = 50 µm. **b**, H-score analyses of IL-6 immunoreactivity. Asterisk indicates significant difference from control group ($p<0.05$).

fects, such as proangiogenic[4] and proinflammatory properties[28,29]. The increased formation of low molecular weight HA, along with a deficiency of cervical HA, increases the risk of preterm birth due to increased infection. Experiments[30] in mice have identified a mechanism by which pathogen-facilitated loss of HA in the lower reproductive tract may be achieved, contributing to increased susceptibility to infection and preterm birth.

As inflammatory biomarkers play a key role in the cervical changes before delivery, we investigated their expression in our model of PTB induction to understand the effect of HMW-HA. When cytokines, including TNF-α and IL1β, upregulate prostaglandin synthesis during par-

turition - *via* increased expression of inducible prostaglandin H synthase-2 - uterine contractility enhances and parturition is promoted. The significant role of prostaglandins in term of parturition was demonstrated by scholars[31,32] who showed that prostaglandin synthase inhibitors delay labor. Prostaglandin F2α (PGF2α), along with both IL1β and TNF-α, by upregulating decidual VEGF transcription and translation, potentially leads to an increased chemotaxis of inflammatory cells[33]. In our experiments, mifepristone treatment increased TNF-α and IL1β mRNAs: low dose of HMW-HA significantly decreased the synthesis of IL1β, high dose of HMW-HA significantly decreased the synthesis of both, thus counteracting their effects in inducing PTB. This confirms the anti-inflammatory activity and immunomodulatory effect of HMW-HA[34]. IL-6 is another key factor involved in the inflammatory cascade and is usually considered a marker of PTB. Nevertheless, IL-6 involvement often depends on the nature of the stimuli and is influenced by compensatory actions of TNF-α and IL1β. This is the reason, for example, because IL-6 null mutant mice exhibit a normal response to LPS but an impaired reaction to turpentine or local tissue damage[35]. IL-6 often acts non in parallel with other proinflammatory cytokines because it may exert both proinflammatory and anti-inflammatory effects[36]. It is constitutively expressed in the uterine tissues during gestation, and its action depends on the balance of other signaling molecules[37]. Furthermore, even if this cytokine is known to play a role in childbirth, its action not always correlates with cervical shortening, that recently has become a clinical marker of PTB risk[38]. PTB remains controversial and complex, and experimental evidence has shown[38,40] that only systemic infusion of IL1β and TNF-α can induce PTB in mice. In our experiments both PCR and IHC analysis evidenced that IL-6 did not act in parallel with IL1β and TNF-α; in fact, it did not increase in PTB, and its level remained also reduced in low HMW-HA dose group compared to the control group. However, high dose of HMW-HA partially reversed the downregulation of IL-6 in PTB, by increasing its level.

Conclusions

Considering the results found in our experimental model, we strongly believe that further investigation in animals and humans are necessary to deeply understand the mechanism by which HMW-HA acts in the management of PTB. However, considering the importance of immunotolerance for successful pregnancies, the anti-inflammatory activities of HMW-HA are strongly encouraging, and we think that the HMW-HA-induced delay in rat preterm birth is very promising for human trials. In our opinion these findings

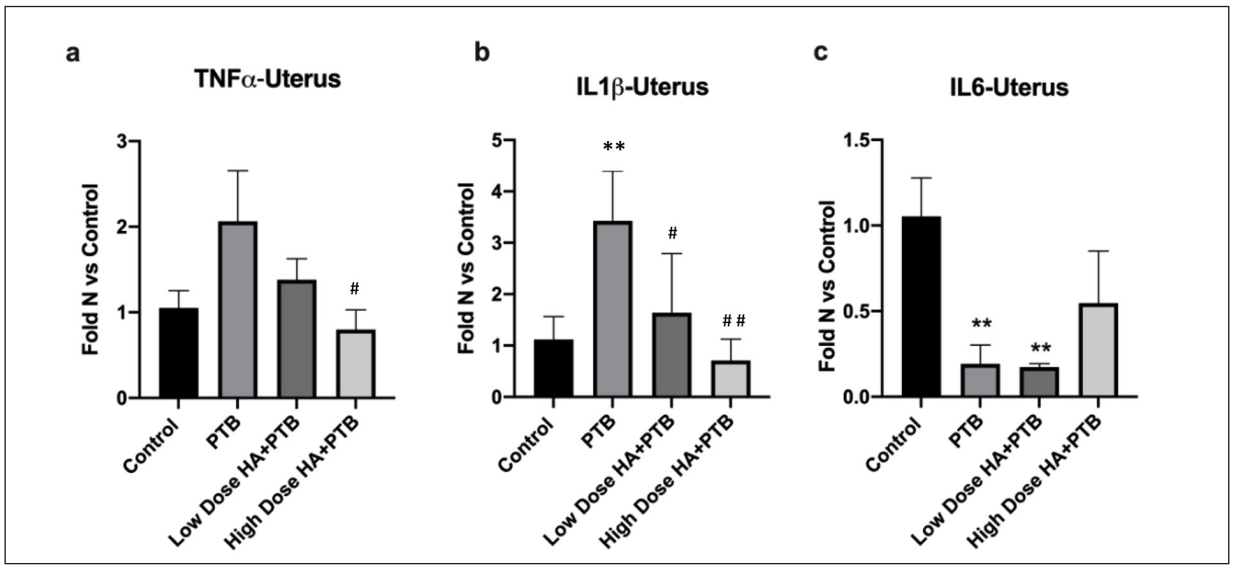

Figure 8. Real time polymerase chain reaction (PCR) expression analysis of TNF-α (**a**), IL1β (**b**), IL-6 (**c**) mRNAs in uterine tissue. Results are presented as fold increase *vs.* control value assumed as 1. Mann-Whitney U test was used to compare groups; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *vs.* PTB; $^{**}p<0.01$ *vs.* control.

have a significant implication for human health, by supporting the oral use of HMW-HA in obstetrics as a new approach to protect the physiological pregnancy.

Conflict of Interest
The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Sara Proietti and Vittorio Unfer are employees at LoLi Pharma srl; other authors are consultants at LoLi Pharma srl.

Authors' Contributions
Dr. Serap Cilaker Micili conceived the idea and contributed to project development, data analysis and manuscript writing. Ozan Tarı contributed to experimental model, laboratory studies and data collection. Dr. Isabella Neri contributed to data analysis and manuscript revision. Dr. Sara Proietti contributed to data analysis and manuscript writing. Dr. Vittorio Unfer contributed to project development and manuscript revision.

Ethics Approval
The study was approved by the Ethics Committee of the Research of Laboratory Animals, Dokuz Eylül University Medical School, Turkey (Protocol number 02/2021).

Informed Consent
Not applicable.

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Availability of Data and Materials
All data generated or analyzed during this study are included in this published article.

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