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CYTOKINE EXPRESSION AFTER VAGINAL DISTENSION OF DIFFERENT DURATIONS IN VIRGIN SPRAGUE-DAWLEY RATS

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Abstract

Purpose—To investigate the effect of duration of vaginal distention (VD) on the differential expression of stem cell homing and tissue repair cytokines and cytokine receptors in order to identify factors most important for recovery from injury.

Methods—Twenty 10-week-old virgin Sprague-Dawley rats were divided into 4 groups: 1-hour VD, 4-hour VD, 6-hour VD, and anesthetized shams. Vagina, bladder, urethra and rectum were harvested immediately after VD and Real-Time PCR was used to determine relative expression of cytokines and receptors of interest. Mixed models analysis was used to determine associations between expression level and VD duration.

Results—Positive associations between VD duration and level of urethral expression were found for one of the receptors of MCP-3 [CCR1 ($p=0.0001$)], as well as, for MCP-3 ($p=0.025$), CCR5 ($p=0.032$), and HIF1- α , ($p=0.023$). A positive relationship between VD duration and MCP-3 expression was also observed in rectal tissue ($p=0.035$). Urethral expression of CCR2, another receptor for MCP-3, approached significance ($p=0.066$). An inverse relationship between VD duration and expression of IL8 was found in the bladder ($p=0.0008$). No association was demonstrated between VD duration and expression of SDF-1, CXCR4, CCR2, CCR3, and VEGF in any pelvic organs.

Conclusions—These data support a relationship between VD duration and subsequent expression of MCP-3 and one of its associated receptors (CCR1) in the urethra immediately following VD. The increase in HIF1- α expression in the urethra with prolonged VD suggests a limited role of tissue ischemia in the immediate response of pelvic organs to VD.

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Keywords

stress urinary incontinence; vaginal childbirth; MCP-3; SDF-1; IL8; HIF; Real-time PCR; female

Introduction

Stress urinary incontinence (SUI) and other related disorders of the pelvic floor are a significant epidemiologic problem. Vaginal delivery of children is the greatest risk factor for SUI.¹ The objective of this experiment was to determine whether duration of simulated childbirth injuries resulted in differential expression of cytokines and cytokine receptors involved in tissue repair, inflammatory responses, and mesenchymal stem cell (MSC) homing. Improved understanding of healing mechanisms after delivery will provide opportunities for development of novel treatments and preventions for SUI.

Vaginal distension (VD) has been used extensively as an experimental model of simulated childbirth injuries in rats.²⁻⁴ Previous studies have demonstrated increased tissue damage with increasing duration of VD.^{3,5} We have previously demonstrated that one stem cell homing factor, monocyte chemoattractant protein-3 (MCP3), is upregulated in vaginal and urethral tissues immediately after VD in virgin rats.⁴ However, studies comparing molecular markers of tissue repair after VD of differing durations are lacking.

Several biochemicals were selected for investigation in this study, including MCP-3, a chemotactic agent in MSC homing⁶ as well as its receptors (CCR-1, -2, and -3). We also included hypoxia inducible factor-1 α (HIF-1 α), which is expressed in response to hypoxic conditions, and two of its downstream modulators: stromal-derived factor-1 (SDF-1) and its receptor CXCR-4, which are also associated with stem cell homing.^{7,8} Interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) were also studied for their known roles in inflammatory responses⁹ and because they are significantly upregulated at the time of cervical effacement and dilation.^{10,11}

Materials and Methods

This project was approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic. Twenty 10-week old virgin Sprague-Dawley rats (206-232 g) underwent: 1, 4, or 6 hours of VD or anesthesia without VD for 4 hours. All animals were euthanized immediately following the procedure for assessment of cytokine and receptor expression in pelvic organ tissues by Real-time PCR.

Vaginal Distension (VD)

All rats were anesthetized with a mixture of intraperitoneal (ip) ketamine (100mg/kg) and xylazine (10mg/kg). Groups 1-3 underwent vaginal accommodation using lubricated bougie dilators (24F to 32F), followed by placement of a lubricated, modified 10F Foley catheter into the vagina. The balloon then was slowly inflated with 3ml sterile water and a single stitch (3-0 silk) was placed in the skin near the vagina to secure the catheter in place, as we have done previously.^{3,5} After the chosen duration, the stitch was removed, the balloon deflated, and the catheter removed.

Dissection and tissue preparation

Pelvic dissection was performed immediately after VD with attention to dissection and tissue handling. The vagina, urethra, bladder, and rectum were harvested. The bladder neck and

trigone were excluded from the bladder specimens. All tissues were flash-frozen and stored at -80°C.

Real Time Polymerase Chain Reaction (Real-time PCR)

Real-time PCR was conducted to evaluate expression of 5 biochemicals and 5 receptors associated with tissue injury and stem cell homing, listed in the table. Total RNA was extracted from the tissues using Rneasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer instructions. Quantitative Real-time PCR was performed using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). The reaction mixture contained TaqMan Universal PCR master mix (Applied Biosystems Inc., Foster City, CA), each primer at 900 nM, probe at 200-250nM, and 50ng of cDNA. After activation of the AmpliTaq Gold (Applied Biosystems, Foster City, CA) for 10 minutes at 95°C, 40 cycles were carried out with each cycle (15 seconds at 95°C, then 1 minute at 60°C). Standard curve method was used for TaqMan Real-time PCR. Primers/probe sets (SDF-1, CXCR-4, CCR-1, CCR-2, VEGF, CCR-3, and CCR-5) for TaqMan Real-time PCR were obtained from Applied Biosystems Inc. TaqMan MGB probes for MCP-3, HIF-1 α and IL-8 were synthesized. The sequences used were: 6FAMTGCTGCTCACAGCTG-MGBNFQ, 6FAM-AAAGAGCCCCGATGCC-MGBNFQ and 6FAMCTCATCTTGGTGGTGAACA-MGBNFQ, respectively. Relative mRNA levels were determined by comparison to the GAPDH internal control.

Data analysis

Data is presented as the median $\pm$ 25th/75th percentile of Real-time PCR results normalized to mean of anesthetized sham values. The data were analyzed using a mixed models ANOVA (SAS v9.1; SAS Institute Inc., Cary, NC) to test for a significant relationship between VD duration and biochemical expression, followed by a Dunnett's posthoc test to determine statistically significant differences compared to control. $P < 0.05$ was considered statistically significant.

Results

Trends in expression of CXCR-4, CCR-2, CCR-3, SDF-1, and VEGF over increasing durations of VD were not significant for any of the tissues examined (data not presented).

Positive associations between duration of distension and level of tissue expression were found in the urethra in CCR-1 ($p=0.0001$; Figure 1), CCR-5 ($p=0.032$; Figure 2), MCP-3 ($p=0.025$; Figure 3), and HIF-1 α ($p=0.023$; Figure 4). In all 4 cases, only after 6 hours of distension was expression significantly different from control. When normalized to the control, MCP-3 expression in the urethra was 3.2-, 8.2-, and 16.7-fold in the 1-, 4-, and 6-hour VD groups, respectively ($p=0.025$). Urethral expression of CCR-2 approached significance ($p=0.066$; data not shown).

A relationship between duration of distension and MCP-3 expression was also identified in rectal tissue ($p=0.035$; Figure 3). When normalized to the control group, MCP-3 expression in the rectum was 3-, 21.5- and 15.8-fold in the 1-, 4-, and 6-hour VD groups, respectively ($p=0.035$). Only expression in the rectum after 4 hours of distension was significantly different from control with posthoc pairwise analysis.

Normalized vaginal expression of MCP-3 for the 1-, 4-, and 6- hour groups were 2.8-, 9.1- and 17-times control. However, this association was not significant ($p=0.27$), likely owing to the large variability in tissue expression of MCP-3 within each vaginal tissue experimental group.

An inverse relationship between duration of distension and IL-8 expression was found in the bladder tissue ($p=0.0008$; Figure 5). Expression after both 1 hour and 6 hours of distension

were significantly different from controls. No other tissues examined demonstrated an association between IL-8 expression and duration of distension.

Discussion

The chemokines and receptors we examined belong to two families, CC (CCR-1, CCR-2, CCR-3, CCR-5, MCP-3) and CXC (IL-8, CXCR-4, SDF-1). Although it was initially believed that members of the CXC-cytokine family were primarily involved in neutrophil chemotaxis⁹ and members of the CC-cytokine family were primarily involved in monocyte chemotaxis,¹² research has demonstrated their complex roles and interrelationships. MCP-3 has been shown to impact neutrophil chemotaxis, but with much lower efficacy than IL-8.¹² In addition, it has been demonstrated in mice that CCR-1 has a critical role in both neutrophil and monocyte responses.¹³ CCR-1 has been demonstrated to be upregulated in cutaneous wound healing, although it is not required for adequate wound healing.¹⁴ HIF-1 α appears to play a central role as a transcription factor mediating the downstream production of several genes involved in the cellular response to hypoxia, including VEGF and SDF-1.⁷ IL-8 was included because it is a potent known mediator of an acute inflammatory polymorphonuclear leukocyte response.^{9,15}

Our findings confirm our previously reported observations showing increased expression of MCP-3 in the urethra immediately after VD.⁴ However, we did not confirm the prior observation of SDF-1 downregulation after VD. This may be due, in part, because we did not include unanesthetized control or sham groups, since Woo et al found that MCP-3 expression was significantly increased 20-fold in both shams and controls.⁴ We observed a significant trend in MCP-3 expression in rectal tissue after VD of increasing durations, suggesting that stem cell homing could play a role in rectal healing after delivery, a risk factor for fecal incontinence.¹⁶ These findings could therefore indicate that treatments or preventatives based on facilitating stem cell homing may be applicable to fecal incontinence as well as SUI.

In contrast to our results, Woo et al. reported an 8-fold increase in MCP-3 expression in the vagina as compared with unanesthetized controls ($p=0.028$), but they did not demonstrate significant upregulation of MCP-3 after VD in rectal or bladder tissues.⁴ In addition to the factors mentioned above, slight differences in dissection techniques may account for the differences observed. For example, the entire mid-vagina (anterior and posterior) was collected for this experiment, while only the anterior wall was collected in the study by Woo et al.⁴ Nonetheless, both experiments point to a critical role of MCP-3 early after VD.

Our study further investigates this association by comparing tissue expression of relevant receptors and associated biochemicals. A strong association is demonstrated in the urethra with one of the known receptors for MCP-3: CCR-1, and a weaker one with another receptor: CCR-2 ($p=0.066$). The finding of nonsignificance in expression of CXCR-4, a monogamous receptor for SDF-1, in all tissues investigated, further substantiates the previously reported findings that SDF-1 is not likely to play a role in recovery from VD,⁴ at least immediately afterward. All these findings again strongly suggest that MCP-3 is the factor most likely to be responsible for stem cell homing early after VD, and that VD of increasing distention results in greater expression of this cytokine.

The urethra demonstrated greater upregulation of biochemicals associated with tissue repair compared with neighboring organs. This could be because the urethra is the only organ evaluated that does not physiologically undergo large stretch to accommodate solids (vagina, rectum) or fluids (bladder). Therefore, the observed biochemical response may represent a pathologic response to supranormal tissue stress. The urethra also has the least redundant vascular supply since the other three organs possess arterial supply from multiple branches of

the major pelvic vessels,¹⁷ so the urethral response may reflect a response to acute urethral hypoxia.

IL-8 is strongly associated with polymorphonuclear leukocyte activity.^{9,15} We were surprised to find an inverse relationship between expression of IL-8 and VD duration in the bladder. Interestingly, while Damaser and colleagues reported a clear reperfusion response in the vagina and urethra after deflation of the vaginal balloon, they observed slow return of blood flow to the bladder after VD,¹⁸ suggesting an adaptive response of this organ in response to distension. Further research into mechanism of this response could resolve this question.

Significant individual variation in the biochemical response to VD is evident by relatively large variance, particularly with increasing duration of VD, suggesting that some rats may respond more strongly to tissue injury than others. Relatively small numbers of animals were used in this study, but significant differences were observed nonetheless. This variation may help explain why some women may be more or less predisposed to the development of pelvic floor disorders than others and, perhaps, provide prognostic information for women at risk.

The biochemical changes occurring in the pelvis at the time of vaginal delivery are quite complex. A number of studies have demonstrated high levels of expression of tissue-repair growth factors and cytokines in tissues of the female genitourinary tract at the time of parturition. In particular, evidence suggests that IL-8, VEGF, MCP-1, MCP-2, MCP-3, ILGF-1, and a number of other proteins are upregulated in the cervix at the time of effacement and dilation.^{10,11,19,20} In addition, MCP-3 and IL-8 are two of several cytokines that are overexpressed in amniotic fluid, cervical mucus, and contracting myometrial cells at the time of delivery.¹⁹⁻²¹ Future studies comparing cytokine expression in virgin rats and post-gestational rats need to be conducted to validate the model.

Conclusion

Our investigation demonstrates a relationship between duration of VD and subsequent expression of the stem cell homing factor, MCP-3, and one of its associated receptors in the urethra immediately following VD. Rectal MCP-3 expression was also associated with increased duration of VD. These findings point to a need for further investigation of factors that may potentiate the stem cell homing response and improve healing to treat or prevent SUI after vaginal delivery.

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Figure 1.

Relative expression of CCR1 in **A.** urethra, **B.** vagina, **C.** bladder, and **D.** rectum after anesthesia for 4 hours (anesth X4hr), vaginal distension for 1 hour (VDX1hr), vaginal distension for 4 hours (VDX4hr), or vaginal distension for 6 hours (VDX6hr). Each symbol represents the median $\pm$ 25th/75th percentile of data from 5 animals. Significance is indicated by the p-value above the graph. P=NS indicates that changes with vaginal distension were not significant ($p > 0.05$).

Figure 2.

Relative expression of CCR5 in **A.** urethra, **B.** vagina, **C.** bladder, and **D.** rectum after anesthesia for 4 hours (anesth X4hr), vaginal distension for 1 hour (VDX1hr), vaginal distension for 4 hours (VDX4hr), or vaginal distension for 6 hours (VDX6hr). Each symbol represents the median $\pm$ 25th/75th percentile of data from 5 animals. Significance is indicated by the p-value above the graph. P=NS indicates that changes with vaginal distension were not significant ($p > 0.05$).

Figure 3.

Relative expression of MCP-3 in **A.** urethra, **B.** vagina, **C.** bladder, and **D.** rectum after anesthesia for 4 hours (anesth X4hr), vaginal distension for 1 hour (VDX1hr), vaginal distension for 4 hours (VDX4hr), or vaginal distension for 6 hours (VDX6hr). Each symbol represents the median $\pm$ 25th/75th percentile of data from 5 animals. Significance is indicated by the p-value above the graph. P=NS indicates that changes with vaginal distension were not significant ($p > 0.05$).

Figure 4.

Relative expression of HIF1-alpha in **A.** urethra, **B.** vagina, **C.** bladder, and **D.** rectum after anesthesia for 4 hours (anesth X4hr), vaginal distension for 1 hour (VDX1hr), vaginal distension for 4 hours (VDX4hr), or vaginal distension for 6 hours (VDX6hr). Each symbol represents the median $\pm$ 25th/75th percentile of data from 5 animals. Significance is indicated by the p-value above the graph. P=NS indicates that changes with vaginal distension were not significant ($p > 0.05$).

Figure 5.

Relative expression of IL8 in **A.** urethra, **B.** vagina, **C.** bladder, and **D.** rectum after anesthesia for 4 hours (anesth X4hr), vaginal distension for 1 hour (VDX1hr), vaginal distension for 4 hours (VDX4hr), or vaginal distension for 6 hours (VDX6hr). Each symbol represents the median $\pm$ 25th/75th percentile of data from 5 animals. Significance is indicated by the p-value above the graph. P=NS indicates that changes with vaginal distension were not significant ($p > 0.05$).