Vitamin A affects urothelial injury and regeneration in cyclophosphamide-induced cystitis

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Background
Vitamin A and its derivatives, the retinoids, are involved in tissue regeneration as they can modulate cell proliferation, differentiation, apoptosis (1) and the inflammatory response (2). A single injection of cyclophosphamide (CP) in rodents disrupts the blood-urine barrier of the urinary bladder by causing injury to the urothelium, which is followed by rapid regeneration (3–5). We used a mouse model for CP-induced urothelial injury, which is also a model for the inflammation of the urinary bladder known as cystitis. The aim of our study was to investigate the effects of a vitamin A-enriched diet on CP-induced cystitis. We analysed the proliferation and differentiation of urothelial cells as well as the transcriptome of the urinary bladder.

Methods
We randomly divided 80 female BALB/c mice (8 weeks old) into 8 groups of 10 mice each (permit No. U34401-4/2022/18). 4 groups received a vitamin A-enriched diet (containing 566081UI retinyl-acetate per kilogramme; VitA groups) 1 week before intraperitoneal (i.p.) injection of CP or saline (S) and the other 4 received a normal diet (N groups). Urinary bladders were collected 1 or 3 days after i.p. injection (VitA CP1d, N CP1d, VitA S1d, N S1d, VitA CP3d, N CP3d, VitA S3d, N S3d groups) and then dissected and prepared for RNA isolation followed by RNA sequencing (RNAseq) and for scanning electron microscopy (SEM). Half of each urinary bladder was embedded in paraffin for immunolabelling. The proliferation index was determined by quantification of Ki67-positive urothelial cells, while differentiation was determined by the percentage of uroplakins' (UPs) positive apical membrane length of superficial urothelial cells. We also determined the presence of highly differentiated superficial urothelial cells by immunolabelling of keratin 20 (Krt20). SEM was used to analyse the differentiation stage of the superficial urothelial cells by assessing the percentage of microridges (terminal differentiation stage) on the entire surface examined.

Data analysis was performed with ImageJ and statistically analysed with Excel and GraphPad Prism 8.01. Results are presented as mean values and standard deviations (mean ± SD) unless specified otherwise.

Results
The RNAseq results showed higher expression of genes involved in the cell cycle and PI3k-Akt signalling in the VitA CP1d group compared to the N CP1d group, indicating higher proliferation in this group. Genes involved in cell cycle (Bub1, Bub1a, Ccna2, Cdc25b, Cdk1, Knl1) and PI3k-Akt signalling (Itga3, Epha2, Spp1, Ereg, Areg and Il6) were significantly up-regulated, as revealed by Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analysis. The higher proliferation indices were subsequently confirmed in the urothelial cells of the VitA CP1d group (29.5% ± 20.5%) compared to the N CP1d group (19.6% ± 16.1%). In the VitA CP3d and N CP3d groups, the proliferation indices were 42.2%±17% and 38.4%±14.3%, respectively. In comparison, all groups with S injection (VitA S1d, N S1d, VitA S3d and N S3d group) had lower proliferation index of 1 to 2%.

All groups with S injection (VitA S1d, N S1d, VitA S3d and N S3d) had terminally differentiated superficial urothelial cells with positive UPs and Krt20 immunolabelling and microridges on their apical surface. One day after CP injection (VitA CP1d, N CP1d groups), the differentiation

stage of superficial urothelial cells was significantly lower compared to the S injection groups (VitA S1d, N S1d), as indicated by the presence of microridges. The VitA CP1d group exhibited a slightly higher differentiation stage (13.9%±25.1% of microridges) than the N CP1d group (9.1%±13.6% of microridges). On the other hand, 3 days after CP injection, the VitA CP3d group showed a slightly lower UPs-positive apical surface length of superficial urothelial cells (76.6%±15.4%) than the N CP3d group (88.1%±13.5%), while no difference was observed in the presence of microridges (VitA CP3d - 6.1%±14.8% and N CP3d - 7.0%±10.3%). There was no difference in Krt20 positive superficial urothelial cells between the CP-treated groups (VitA CP1d, N CP1d, VitA CP3d and N CP3d).

Conclusion
Our results suggest that increased vitamin A intake before the onset of CP-induced cystitis influences urothelial injury and regeneration. A vitamin A-enriched diet resulted in upregulation of several genes involved in the cell cycle and PI3k-Akt signalling and increased proliferation of urothelial cells during the initial stages of regeneration (day 1). These events accelerated the formation of the initial blood-urine barrier. We were able to demonstrate that proliferation remained increased in groups with VitA-enriched diet also in the later phases of regeneration (day 3), while no difference was observed in the differentiation stage of the superficial urothelial cells. We can conclude that vitamin A influences the initial regeneration, but its effects are no longer observed in the later stages of regeneration.

Keywords:

Retinoids, cyclophosphamide, urothelium, acute cystitis

References:

1. Carazo A, Macáková K, Matoušová K, Krčmová LK, Protti M, Mladěnka P. Vitamin A Update: Forms, Sources, Kinetics, Detection, Function, Deficiency, Therapeutic Use and Toxicity. Nutrients. 18. maj 2021;13(5):1703.
2. Huang Z, Liu Y, Qi G, Brand D, Zheng SG. Role of Vitamin A in the Immune System. Journal of Clinical Medicine. september 2018;7(9):258.
3. Gandhi D, Molotkov A, Batourina E, Schneider K, Dan H, Reiley M, idr. Retinoid signaling in progenitors controls specification and regeneration of the urothelium. Dev Cell. 16. september 2013;26(5):469–82.
4. Romih R, Koprivec D, Martincic DS, Jezernik K. Restoration of the Rat Urothelium After Cyclophosphamide Treatment. Cell Biology International. 2001;25(6):531–7.
5. Romih R, Veranic P, Jezernik K. Appraisal of differentiation markers in urothelial cells. Appl Immunohistochem Mol Morphol. december 2002;10(4):339–43.