The effect of uremic conditions on smooth muscle cells cultured on PEG hydrogels

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Background

Chronic kidney disease (CKD) is one of the leading causes of death in the United States.

- Approximately 37 million Americans are living with CKD, and ~3% of those cases are classified as end-stage, which requires **hemodialysis treatment**.[1]
- Surgical implantation of an arteriovenous fistula or graft is necessary for access, but the access site is prone to inflammation, infection, and thrombosis, the leading causes of dialysis-related morbidity.[2]

Inflammation has been correlated to high levels of **uremic toxins**, including phosphate and urea, and may cause **phenotypic switching** of smooth muscle cells into an osteogenic state.

- SMC can alternate between a contractile and synthetic state, which is beneficial for vascular remodeling. This phenotypic switching is a response to biochemical and biomechanical cues from the extracellular environment, and it is characterized by a reduction in expression of SMC markers such as αSMA and SM22.[3]
- High levels of phosphate (>3.5 mM) can induce osteogenic behavior in SMC and lead to calcification of arterial walls.[4,5]

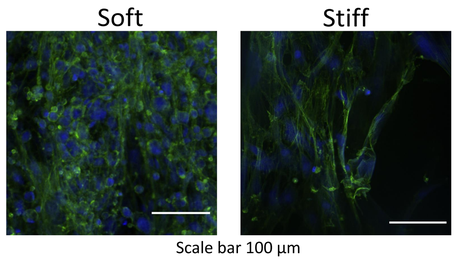
Arterial stiffening is another condition of CKD; many methods have been proposed to explain the mechanism of arterial stiffening, including the impact of calcification. Stiffness of healthy arterial tissue measures ~5 kPa. Diseased tissue is reported as >60 kPa.

The goals of this study were to evaluate the responsiveness of SMC to a PEG-based hydrogel system and to recapitulate the effects of uremic sera in an *in vitro* environment. The *in vitro* effects of substrate stiffness and uremic levels of phosphate and urea on smooth muscle cells are presented here.

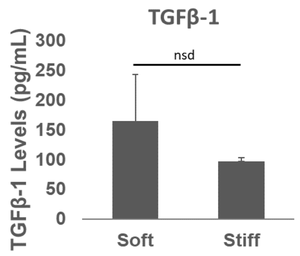
Results

Effect of substrate stiffness on cell behavior

SMCs attached to the surface of both soft (5 kPa, healthy tissue) and stiff (60 kPa, diseased tissue) PEG hydrogels with tethered RGD. There was no attachment to hydrogel constructs without RGD (data not shown). During culture, cell spreading in both conditions was apparent; however, more cells appeared attached on the soft substrate.



Scale bar 100 µm



Active TGFβ1 was produced from cells seeded on soft and stiff substrates. Mean levels were higher on the soft but was not statistically significant (p=0.6).

Materials and Methods

Hydrogel formation Stiff hydrogels: 7-9 wt% 8-arm PEG-norbornene macromer (10 kDa) (PEG-NB) and PEG-dithiol crosslinker (1 kDa) (PEG-dSH) were combined at 0.95 thiol:ene with 5 mM RGD tethered and 0.05% photoinitaitor Irgacure 2959 (I2959). Soft hydrogels: 4 wt% PEG-NB (10 kDa) and PEG-dSH (1 kDa) were combined at 0.95 thiol:ene with 5 mM RGD tethered and 0.05% I2959. Hydrogel constructs were cured under 352 nm light at 5 mW for 8 minutes, then swollen in PBS and placed in 96 well plates.

Cell culture Studies were conducted using immortalized human aortic smooth muscle cells (SMC) from ATCC (CRL-1999). SMC were expanded in growth media (F12K supplemented with 10% FBS, 1% Pen/Strep, and 0.3 mg/mL ECGS) until 70-90% confluent. Cells were seeded onto soft (5 kPa) PEG hydrogels, stiff (60-100 kPa) PEG hydrogels, or onto TCPS, at 180,000 cells/cm^2 in healthy media (F12-K supplemented with 1% or 10% FBS, 0.3 mg/mL ECGS, 1% Pen/Strep, 50 µg/mL ascorbic acid, 1% ITS+, and 10 mM sodium pyruvate) or osteogenic media (healthy media plus varying concentrations of β-glycerophosphate (β-GP) or urea).

ELISA TGFβ1 levels were measured in the media using a DuoSet ELISA kit.

PCR RNA was extracted from cells using kits from QIAGEN and converted to cDNA. Samples were analyzed for gene expression using SYBR reagent and with beta-2-microglobulin (B2M) as the housekeeping gene.

Fluorescent and brightfield imaging Cells were stained with Phalloidin/DAPI to observe attachment and cell spreading and imaged using a confocal fluorescent microscope. Morphological differences were monitored using a brightfield microscope.

References and Acknowledgements

1. National Institute of Health https://www.niddk.nih.gov/health-information/health-statistics/kidney-disease. 2. Vanholder, *et al*. *Kidney Intl.* **63**, S6-S10 (2003). 3. Owens, *et al*. *Physiol. Rev.* **84**, 767–801 (2004). 4. Zhang, D. *et al*. *Kidney Blood Press. Res.* **42**, 1205–1215 (2017). 5. Shioi, A. *et al. Arterioscler. Thromb. Vasc. Biol.* **15**, 2003–2009 (1995). 6. Ding, Y. *et al. Acta Biomater.* **105**, 68–77 (2020).

This work was supported by NIH #R01HL119371 and the DOEd GAANN fellowship.

Fluorescent imaging was performed at the BioFrontiers Institute Advanced Light Microscopy Core (RRID: SCR_018302).

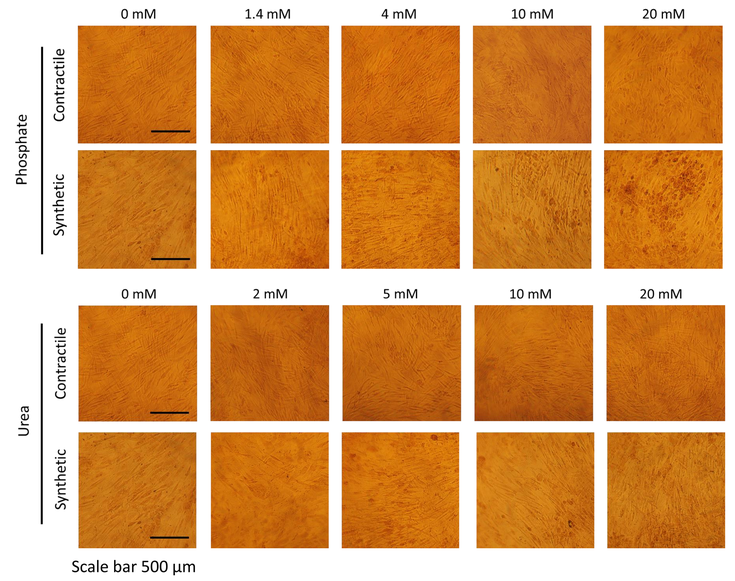
Results and Discussion

Effect of urea and phosphate on cell morphology

Cells were serum-starved in 1% FBS to induce the contractile state and compared to cells in the synthetic state, which were treated with 10% FBS.

Cells undergo morphological differences after 14 days of culture on TCPS in uremic media with 1% or 10% FBS.

Morphological differences were more apparent in the high phosphate conditions than in high urea conditions. Between the phenotypic states, cells in the synthetic state displayed more of a response to the uremic media.



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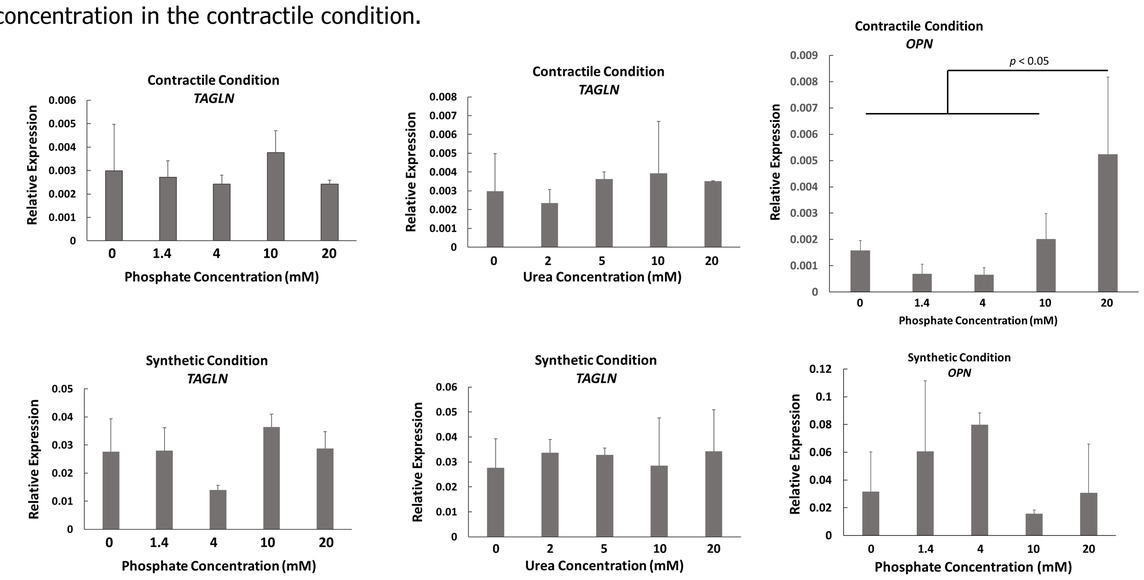
Effect of urea and phosphate on gene levels

After two weeks of culture with uremic toxins, SM22 (*TAGLN*) levels were consistent in both the contractile and synthetic states and across toxin levels. Interestingly, relative expression was higher in the synthetic state.

Relative expression for osteogenic marker osteopontin (*OPN*) showed a significant increase with phosphate concentration in the contractile condition.



These results show that phosphate does initiate a change in the osteopontin levels of the contractile condition, which is confirmed by the morphological changes shown above. The *OPN* levels of the synthetic condition are not significantly different from the physiological level of 1.4 mM, but stark morphological changes were observed in the above images.

Conclusions and Future Directions

Smooth muscle cells attach to PEG-based hydrogels of varying stiffness; additionally, the difference in stiffness does **not affect TGFβ1 production**.

On TCPS, SMC were affected by high phosphate concentrations (20 mM) at the gene level and showed morphological differences at all non-physiological levels (≥4 mM). The results suggest **phosphate is a stronger uremic toxin** than urea alone. Interestingly, immortalized cells are **slower to respond** to uremic levels of phosphate compared to previously published studies with primary cells.[4,5]

Follow-up studies are ongoing to identify the combined effects of substrate stiffness and uremic toxins on smooth muscle cells. Additional future work will investigate the **impact of H_2S** and other antioxidants on mitigating the effects of the toxins.