Expanded Haemodialysis Therapy of Chronic Haemodialysis Patients Prevents Calcification and Apoptosis of Vascular Smooth Muscle Cells in vitro

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BACKGROUND

Vascular calcification is common in patients with chronic kidney disease and is associated with cardiovascular mortality. Vascular calcification is an active process promoted by hyperphosphatemia. This process is mediated in part by inflammatory processes in vascular smooth muscle cells (VSMC) and inhibited by anti-calcific proteins such as matrix Gla protein (MGP) and Fetuin-A under normal conditions. The increased risk of mortality is associated with increased levels of the proinflammatory cytokine growth differentiation factor 15 (GDF-15). Insufficient removal of certain cytokines using conventional hemodialysis may contribute to vascular calcification.

High cut-off (**HCO**) membranes showed a reduction in the serum levels of soluble TNF receptors, vascular cell adhesion molecule (VCAM) and other markers of inflammation, but this is accompanied by marked loss of albumin.

High retention onset (HRO) dialysis membranes, or middle cut-off, with a steeper cutoff were developed and have been shown to provide clearance for middle-sized molecules while limiting albumin filtration. The potential impact of expanded hemodialysis therapy with an HRO membrane on vascular calcification has not yet been reported.

OBJECTIVE

The study examined the impact of HRO dialysis membrane on the process of the in vitro calcification of VSMC in a well-established cell culture model.

METHODS

The study used serum samples from the Permeability Enhancement to Reduce Chronic Inflammation (PERCI II) clinical trial,[1] which included 45 patients. Every patient was dialyzed 3x/week using medium cut-off membrane (**MCO**-Ci 400) and high-flux (HF) membranes (**Revaclear** 400 dialyzer) for 4 weeks in a randomized order. After the second phase, an extension period of 8 weeks was added to assess the long-term effects. No dialysis performed. In a consecutive manner, half of the patients who were randomized to the HRO dialysis membrane group in the second phase were dialyzed using HRO membrane for 12 weeks.

Serum samples were collected before every first dialysis session of the week, centrifuged, frozen and then thawed for cell culture experiments. No freeze-thaw-refreeze cycles existed before the samples were used in the experiments.

Human VSMC were purchased from LifeLine Technology (Frederick, MD, USA). All cells used in these experiments came from the same donor. Cells were characterized as VSMC with alpha-SMA antibodies and passaged up to a maximum of 6 passages. During incubation, VSMC were stored in a humidified incubator at 37°C and 5% CO_2. Cells were cultured in 25-mL cell culture flasks until they were confluent. Cell number and viability were determined in a Neubauer counting chamber using Trypan blue, and cells were seeded at 100,000 cells/well on 24-well plates.

Induction and Determination of Calcification

An osteogenic medium was used to induce calcification in VSMC, and 5% of the serum samples collected from the 45 patients during the clinical trial was added. Calcification was evaluated using alkaline phosphatase (ALP) and alizarin red (AZR) after 7 and 10 days of incubation, respectively.

The water-soluble terazolium salt (WST-8) assay was used to measure the marker activity of viable cells, which served to normalize the activity of the calcification markers (ALP and AZR) of living cells.

Cell culture supernatants were collected at days 3 and 7 of incubation and later pooled, and the concentrations of calcification-associated proteins were measured.

Apoptosis rates caused by the different sera were measured via fluorescence spectroscopy after 7 days of incubation.

Results are presented as mean (standard error of the mean).

RESULTS

Calcification

ALP activity assay (Figure 1a):

- After 4 weeks of dialysis: HRO dialysis membrane induced 24% less calcification than HF dialysis (HF 2.91 (0.11) vs. HRO 2.21 (0.088), $p < 0.0001$)
- After 12 weeks of dialysis: HRO dialysis membrane induced 38% less calcification than HF dialysis (HF 3.14 (0.1) vs. HRO 1.96 (0.081), $p < 0.0001$)

AZR staining (Figure 1b)

- After 4 weeks of dialysis: HRO dialysis membrane induced 36% less calcification than HF dialysis (HF 0.31 (0.01) vs. HRO 0.19 (0.008), respectively, $p < 0.0001$)
- After 12 weeks of dialysis: HRO dialysis membrane induced 48% less calcification than HF dialysis (HF 0.30 (0.01) vs. HRO 12 weeks 0.16 (0.007), $p < 0.0001$)

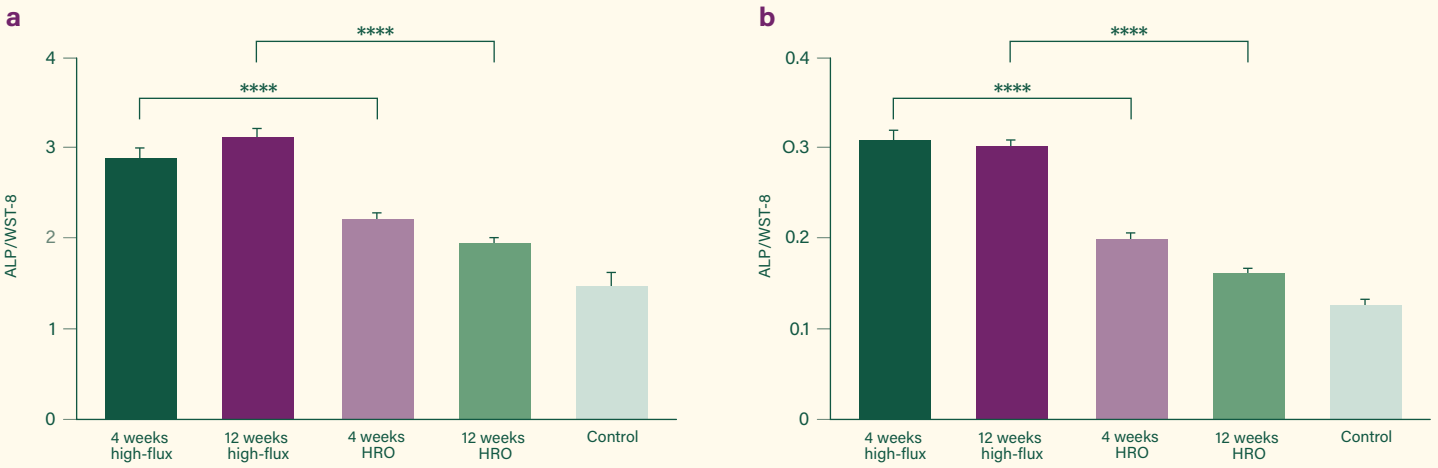

FIGURE 1. Ratio of ALP/WST-8 after 7 days of incubation **(a)** and AZR/WST-8 after 10 days of incubation **(b)**; n = 100 for 4 weeks High-Flux, 4 weeks HRO and 12 weeks HRO. n = 80 for 12 weeks High-Flux. n = 20 for control. **** p < 0.0001.

Calcification-Associated Proteins in Supernatants

MGP concentration (Figure 2a)

> MGP was 42% higher in the HF supernatants than HRO supernatants and 39% higher than the healthy control supernatants. MGP levels were comparable in the HRO and the healthy control supernatants.
> (HF 0.956 (0.045) vs. HRO 0.554 (0.018), $p < 0.0001$; HF 0.956 (0.045) vs. healthy serum 0.586 (0.01148), $p < 0.0001$)

OPN concentration (Figure 2b)

> OPN was 2.5 times higher in the HF supernatants than HRO supernatants and 4 times higher than the healthy serum group. (HF 0.238 (0.005) vs. HRO 0.083 (0.002), $p < 0.0001$; HF 0.238 (0.005) vs. healthy serum 0.060 (0.001), $p < 0.0001$

GDF-15 concentration collected at days 3 and 7 of incubation (Figure 3a and b respectively)

> GDF-15 was 27% lower in the HRO supernatant than HF supernatant at day 3 (HF 1,894.583 (158.4) vs. HRO 1,375.667 (48.68), $p < 0.01$)

> The absolute concentrations decreased towards day 7 in both groups, but the difference between HF and HRO groups remained stable at 32%
> (HF 402.5 (33.13) vs. 593.888 (34.30), $p < 0.05$)

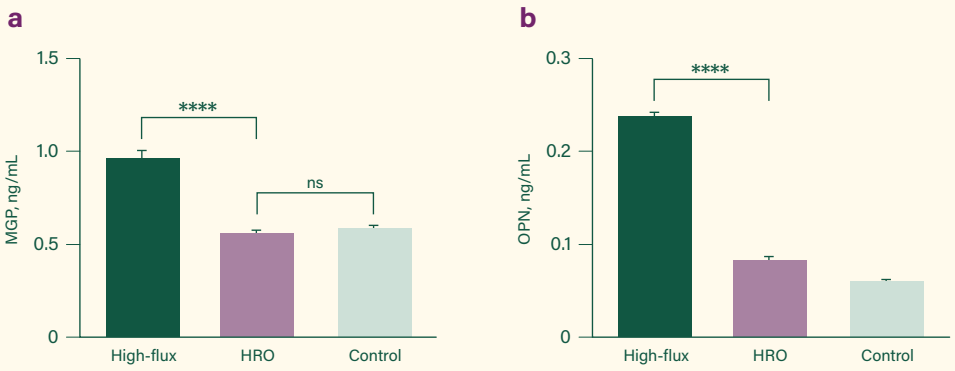

FIGURE 2. Production of Matrix-Gla protein **(a)** and OPN **(b)** by VSMC after incubation with HRO-serum, High-Flux-serum or serum from controls; $n = 18$ for High-Flux and HRO, $n = 12$ for control. **** $p < 0.0001$. ns, non significant.

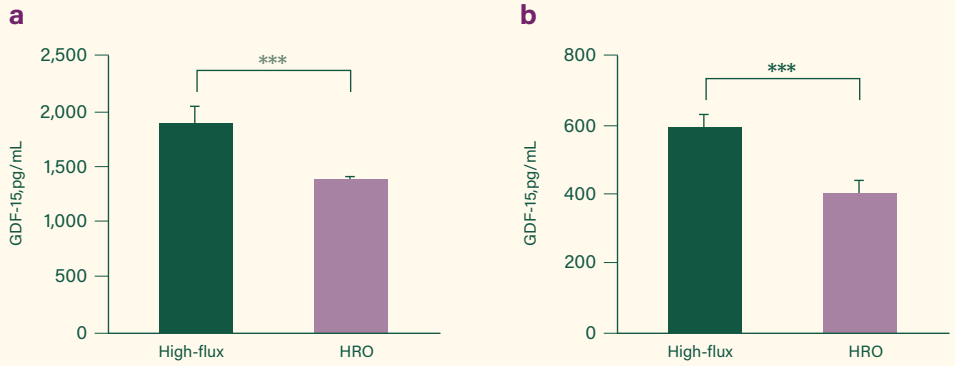

FIGURE 3. Concentration of GDF-15 in cell culture supernatants after 3 days (left, **a**) and after 7 days (right, **b**) of incubation with HRO-serum or High-Flux-serum; $n = 12$ (3 days), $n = 18$ (7 days). *** $p < 0.001$.

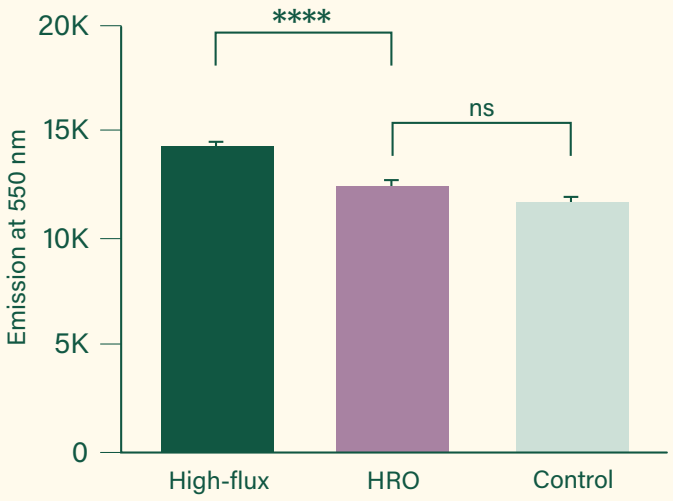

FIGURE 4. Apoptosis of VSMC after incubation with HRO-serum, **Revaclear**-serum or serum from controls. Apoptosis was determined by in situ cell death kit®; $n = 20$ for High-Flux and HRO, $n = 8$ for control. **** $p < 0.0001$. ns, non significant.

Apoptosis

The apoptosis rate of cells incubated with HRO was 15% lower than in HF-incubated cells (HF 14,410.15 (127.9) vs. HRO 12,452.9 (244.3), $p < 0.0001$). Apoptosis in the control group was similar to the HRO group (control 11,740.12 (225.5) vs. HRO 12,452.9 (244.3), p=ns) and 19% lower than the HF group ($p < 0.0001$). (Figure 4)

DISCUSSION

HCO dialyzers can reduce inflammatory markers and improve vitro vascular calcification, however the relevant loss of albumin prohibits long-term use in chronic dialysis patients. The current in vitro study using samples from the PERCI II trial showed that HRO dialyzer membranes maintained the beneficial effect on vitro calcification, while albumin loss was limited.

Vascular calcification in vitro was significantly reduced by 24% (ALP) and 36% (AZR) after 4 weeks of HRO dialysis membrane and by 33% (ALP) and 48% (AZR) after 12 weeks of dialysis using HRO membranes compared to HF dialysis. This observation suggests that the effects of HRO dialysis could involve long-term regulation of the pro-calcific properties inherent in dialysis patients.

The concentrations of MGP and OPN were significantly elevated after incubation with HF serum compared to HRO serum and healthy controls. OPN has been shown to help prevent several consequences of chronic kidney disease, including uremia, calcium deposition and especially vascular calcification. MGP might interfere with both calcification signaling pathways and mineralization and act as an inhibitor of cardiovascular calcification. The production of these molecules upon exposure to serum from dialysis patients could be a compensatory mechanism in response to inflammatory signals and enhanced calcification.

The release of GDF-15 in culture supernatants was significantly decreased after incubation with HRO serum. GDF-15 has been identified as a biomarker for cardiovascular events and risk, several vascular diseases, as well as renal and cardiac damage in general. It may also have an active role in atherosclerosis and other vascular pathologies. Whether the decrease in GDF-15 associated with HRO is causally involved in vascular calcification is unknown at this point.

Apoptosis was significantly lower in the HRO group. Apoptosis is important in the early process of vascular calcification, and VSMC apoptosis is known to be promoted by TNF-α and IL-6. The reduction of these molecules using HRO dialysis membrane may have led to the lower apoptosis rate that was comparable to healthy serum.

Limitations

First, we did not assess in vivo calcification but only the influence of serum samples on calcifying VSMC. The effects of reduced calcification were observed under cell culture conditions that were somewhat extreme with very high concentrations of procalcifying substances. Whether a comparable reduction can be observed in vivo under less extreme conditions has to be examined in future trials that include clinical end points. Second, even though we noted clear effects on in vitro calcification, we fail to provide a clear mechanism that explains the observed reduction in calcification. As discussed in previous publications, molecules of the TNF superfamily and other proinflammatory molecules are indeed reduced with HRO and provide a possible explanation. Finally, the HRO membranes used in our trial still allow relevant filtration of albumin, with a significant decline in serum albumin concentrations after 4 weeks of HRO dialysis. There was however an increase of serum albumin again after 12 weeks. Hence, in future trials, a further reduction in cut-off to reduce albumin filtration has to be considered to make these filters a treatment option for chronic dialysis patients.

CONCLUSION

The study results suggest that expanded hemodialysis therapy has beneficial effects on the calcific potential of uremic serum. This indicates that dialysis with HRO membranes is a possible option to modify vascular calcification in end-stage renal disease. With a markedly reduced albumin filtration compared to high cut-off dialysis, use of the HRO dialysis membrane may provide a treatment option for chronic dialysis patients to reduce the progression of vascular calcification.

Expanded hemodialysis therapy reduced in vitro markers of calcification, which could mean a reduction in vascular calcification and corresponding cardiovascular mortality in patients with chronic kidney disease.

1. Zickler D, Schindler R, Willy K, et al. Medium Cut-Off (**MCO**) Membranes Reduce Inflammation in Chronic Dialysis Patients-A Randomized Controlled Clinical Trial. *PLoS One*. 2017;12(1):e0169024. Published 2017 Jan 13. doi:10.1371/journal.pone.0169024