Cover Page for Protocol

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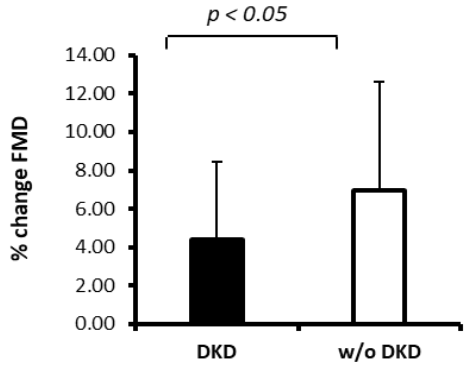
Background and significance

Patients with chronic kidney disease (CKD) have an exceptionally high risk for cardiovascular disease (CVD) and are 10 times more likely to die from CVD prior to requiring dialysis or kidney transplantation (1). Multiple studies have shown CKD to be an independent risk factor for cardiovascular events and mortality (2-6). Diabetes mellitus is the most common cause of CKD and is believed to contribute to a significant portion of the CVD with CKD (7). Indeed, patients with CKD and diabetes have a disproportionally higher risk for CVD when compared with patients with diabetes who do not have CKD (8). Thus, it is paramount that we explore therapies that may ultimately reduce the burden of CVD in those with CKD and diabetes.

Endothelial dysfunction via brachial artery flow mediated dilation (BA-FMD), is a reliable and non-invasive vascular assessment, which has been shown to predict CVD *prospectively* in a wide variety of 'at-risk' and diseased populations, including individuals with CKD (9). Preliminary data by our group demonstrates that endothelial function via BA-FMD test is significantly lower in individuals with CKD and diabetes as compared to those with CKD but without DM (**Fig 1**). These findings suggest that the etiology of DM within CKD patients appears to exacerbate the condition more so than CKD alone.

Figure 1: Patients with DKD exhibit lower BA-FMD compared to those with CKD without diabetes



Experimental studies, suggest that resveratrol (a widely-available nutraceutical) inhibits inflammation and restores endothelial nitric oxide (NO) *in vitro* (9-11) and *in vivo (12,13)*.

In this pilot study, we will evaluate if resveratrol supplementation improves endothelial function and oxidative stress in those with CKD and diabetes.

Study Design

25 subjects with stage 3 CKD and diabetes will be randomized to receive either resveratrol first then matching placebo or placebo first then resveratrol in a randomized cross over study. Resveratrol will be prescribed at 400 mg per day. The treatment with resveratrol or placebo will be for 6 weeks and separated by a 2 week washout period.

The inclusion criteria are shown in **Table 1**.

Table 1

Inclusion Criteria	Exclusion Criteria
Age 45-80 years old	Consuming >2 glasses/day red wine and/or taking resveratrol or vitamin C supplement in the past 12 months
CKD stage 3 (CKD-EPI eGFR: 30-60 mL/min/1.73m^2) on 2 occasions ≥3 months apart	Life expectancy <1 year
Type II DM	Pregnant, breastfeeding, or unwilling to use adequate birth control
angiotensin converting enzyme inhibitor or angiotensin II receptor blocker for ≥3 months prior to the study	Uncontrolled hypertension; blood pressure >140/90
Able to give informed consent	Severe liver disease
	Severe congestive heart failure
	Hospitalization within the last 3 months
	Active infection or antibiotic therapy
	Immunosuppressive therapy within the last year
	Uncontrolled DM with A1C > 8.5
	Currently taking anticoagulants including the following: coumadin, dalteparin, enoxaparin, heparin, and plavix.
	Severe systolic heart failure
	Currently partaking in another research study

Our rational for RSV dose is that: 1) it is available commercially at 400 mg and 2) recent studies utilizing RSV suggest that this dose is safe and does not result in serious side effects (14, 15).

The study is registered with clinicaltrials.gov

Institutional Review Board was obtained. The informed consent will detail the risks associated with the study drug and with all the study procedures.

- **Detailed study sessions**

All study procedures will be conducted at UIHC Clinical Research Unit (CRU) and all experimental testing measurements will be conducted after an overnight fast as well as refraining from exercise and caffeine prior to testing.

Session 1: Informed consent and screening questionnaire for inclusion and exclusion criteria including confirmation of stage 3 CKD on 2 separate occasions in the last 6 months. All women in child-bearing age will undergo pregnancy test at this visit. In addition, all participants will complete a 7-day physical activity recall.

Session 2:
• Blood pressure at rest
• Endothelial cell sampling
• Blood sampling for fasting lipid profile
• Blood sampling for hemoglobin A1C
• Blood sampling for levels of RSV and its metabolites
• Blood sampling for basic metabolic profile and estimated GFR including cystatin C
• Blood sampling for circulating markers of inflammation and oxidative stress (oxLDL)
• Urine sampling (random) for albumin/creatinine ratio (ACR)
• Evaluation of EDD and EID by measurements of BA-FMD and nitroglycerin-mediated dilation (NMD)
• Start study drug after this visit

Session 3: will include the same measurements as Session 2. In addition, it will include the following:
• Physical activity questionnaire
• Pill count
• Safety monitoring:
    - Completion of an adverse event questionnaire
    - Update medication list

Sessions 4 and 5: will involve the exact same procedures as Sessions 2 & 3 above except it will involve the other study drug being ingested for 6 weeks (resveratrol or placebo).

- **Outcome measurements**

1. Measurements of endothelial dependent and independent dilation: *Endothelial dependent dilation* (EDD) of the brachial artery (BA-FMD) will be measured at the Cardiovascular Research Lab in the Clinical Research Unit (CRU) at the University of Iowa by principal investigator using high-resolution Doppler ultrasonography as described originally by Celermajer et al (16,17). Electrocardiogram (ECG) gated end-diastolic ultrasound images and Doppler flow of the artery will be acquired during baseline and FMD and conditions. Briefly, and after 15 minutes of quiet supine rest, the vascular reactivity of the brachial artery will be assessed as based on the following: 1) two minutes of baseline hemodynamics followed by inflation of a rapidly inflating pneumatic pediatric cuff wrapped around the maximal circumference of the forearm to a pressure of 240 mmHg for 5 min, and 2) rapid deflation of the cuff after five minutes. A commercially available software package will be used to acquire and analyze ECG-gated brachial artery diameters. *Endothelial independent dilation* (EID) will also be determined by measuring brachial artery dilation for 10 minutes after administration of sublingual nitroglycerin (0.4 mg). BA-FMD

(pre- vs. post-intervention) will be calculated as the % change from baseline and compared against the placebo condition.

2. Systemic markers of oxidative stress: oxidized low density lipoprotein (LDL) cholesterol (ALPCO Diagnostics, Catalog #30-7810) will be quantified using commercially available enzyme-linked immunosorbent assays performed according to kit instructions.

3. Endothelial cell sampling and cell protein expression: This technique will be done by a trained/qualified individual at the UI CRU. Briefly, J-wires will be advanced into an antecubital vein and withdrawn. Cells will be recovered by standard techniques (18). Collected cells will be fixed with 3.7% formaldehyde and plated on slides. After blocking nonspecific binding sites with 5% donkey serum, cells are incubated with the primary antibody and with CY3-conjugated secondary antibodies. Slides will be systematically scanned to identify endothelial cells (positive VE-Cadherin) and nuclear integrity confirmed using 4',6'-diamidino-2-phenylindole hydrochloride staining. Once endothelial cells with intact nuclei are identified, images will be captured and then analyzed to quantify the intensity of CY3 staining (i.e., average pixel intensity). Values for each sample will be reported as ratios of endothelial cell protein expression/human umbilical vein endothelial cell (HUVEC) to account for any variation in the staining procedure. Sirtuin 1 expression will be evaluated since this has been reported to increase with resveratrol.

4. Other measurements: High performance liquid chromatography coupled with tandem mass spectrometry will be used to determine plasma and urine levels of resveratrol and its metabolites. In addition, urinary albumin/creatinine ratio, serum creatinine and estimated GFR will be assessed at the University of Iowa Clinical Lab.

- **Statistical analysis**

Normally distributed data are presented as mean±SD, non-normally distributed data as median (interquartile range), and categorical data as count (percentage of patients). To determine the effects of resveratrol supplementation on our primary and secondary outcomes, a generalized linear mixed effects model was used to examine the significance of condition, time, and their interaction for normally distributed variables, and a gamma mixed effects model was used for non-normally distributed variables. In each model, a random intercept was built for each patient to account for inherent between-patient variability. A complete case analysis was utilized when analyzing primary and secondary outcomes. A Wilcoxon Rank Sum Test was used to test differences in resveratrol metabolites between the resveratrol and placebo arms. Significance was set at an alpha level of 0.05. All analyses were performed in R, version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria).

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