Resveratrol in heart failure with reduced ejection fraction
Analysis of an investigator-initiated human clinical trial

Ph.D. thesis

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I. List of abbreviations

A	late diastolic ventricular filling velocity
ACE	angiotensin-converting-enzyme
AI	aggregation index
ALT	alanine aminotransferase
ARB	angiotensin receptor blocker
ARNI	angiotensin receptor-neprilysin inhibitor
AST	aspartate aminotransferase
ATP	adenosine triphosphate
CRP	C-reactive protein
EDTA	ethylenediaminetetraacetic acid
E	early diastolic ventricular filling velocity
e’	early diastolic mitral annular velocity
EF	ejection fraction
eGFR	estimated glomerular filtration rate
EI	elongation index
ESC	European Society of Cardiology
FEV_1	forced expiratory volume
FVC	forced vital capacity
GLS	global longitudinal strain
Hct	hematocrit
HFrEF	heart failure with reduced ejection fraction
ICD	implantable cardioverter defibrillator
IL	interleukin
IVC	inspiratory vital capacity
LDL	low density lipoprotein
LORCA	Laser-assisted Optical Rotational Cell Analyzer
M	Myrenne aggregation index
MRA	mineralocorticoid receptor antagonist
mRNS	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NO	nitrogen oxide
NT-proBNP	N-terminal prohormone of B type natriuretic peptide
NYHA	functional classification of heart failure (I-IV)
Pa	pascal
PV	plasma viscosity
RAAS	renin–angiotensin–aldosterone system
RBC	red blood cell
RES	resveratrol
ROS	reactive oxygen species
TAPSE	tricuspid annular plane systolic excursion
$t_{1/2}$	aggregation half time
WBV	whole blood viscosity
ULN	upper limit of normal
6MWT	six-minute walk test
γ (gamma)	smallest shear rate required for complete disaggregation

II. Introduction

1. Heart failure

Heart failure is defined, clinically, as a syndrome in which patients have typical symptoms and signs resulting from an abnormality of cardiac structure or function. Heart failure prevalence is continuously rising throughout the world. In developed countries, approximately 2% of the adult population has heart failure. The most common etiological factors of heart failure are hypertension and coronary artery disease both in men and in women.

Heart failure is divided into three different types: heart failure with reduced ejection fraction (HFrEF, EF<40%), heart failure with mild reduced ejection fraction (HFmrEF, EF 40-50%) and heart failure with preserved ejection fraction (HFpEF, EF<50%).

Despite modern evidence-based, non-pharmacological, pharmacological and device therapy, heart failure remains a serious condition with a large impact on life expectancy and quality of life. Heart failure-related health care expenses are also high. Treatment of HFrEF has improved enormously in the past two decades. Based on pathophysiological considerations, and clinical trials, a large body of evidence exists for the use of neuro-hormonal antagonists - ACE inhibitors, ARBs, MRAs, ARNI, and beta-blockers – to improve both symptoms and prognosis.

Although this approach improved the survival of HFrEF patients, the outcome of the disease still remained poor. Therefore, there is an overwhelming need for new therapies in heart failure. Novel drugs in experimental and clinical trials targeting myocardial contractility, cytokines, myocardial metabolism, or oxidative stress are promising and may present an alternative approach in the treatment of heart failure in the future.

2. Resveratrol and Heart Failure

Red wine contains high amount of polyphenolic compounds like resveratrol (RES), catechin, and quercetin, and RES is considered to be primarily responsible for the cardioprotective effect of red wine (French paradox). Resveratrol (3,5,4-trihydroxystilbene) is a nonflavonoid polyphenolic compound produced by plants (e.g. nuts, berries, and grapes) in response to environmental stress. Several mechanisms may be responsible for the cardioprotective effect of RES including reduction of oxidative stress, inflammation, and

pathologic hypertrophic signaling and improved Ca^{2+} handling. As these are important factors also in the pathogenesis of heart failure, we supposed that RES may also have protective effects in heart failure. Endothelial and vascular function can also be improved by RES treatment via decreasing the cholesterol and triglyceride levels, via increasing the endothelial NOS activity and NO level, as well as due to its anti-inflammatory effects.

Our workgroup demonstrated previously that RES supplementation in a post-infarct rodent model prevented the development of heart failure. RES improved left ventricular function, as well as decreased myocardial fibrosis, oxidative stress, and the amount of proinflammatory proteins. Other workgroups showed similar results in various animal heart failure models. However, in human clinical trials, the effect of RES has not yet been confirmed

3. Hemorheological alterations in Heart Failure

Hemorheology is focusing on the flow properties of the blood. Several studies have indicated that abnormal rheological factors should be handled as risk factors of cardiovascular diseases. The most important hemorheological parameters are the hematocrit, fibrinogen, plasma and whole blood viscosity, plasma proteins, red blood cell (RBC) deformability and aggregation.

Complex impairment of peripheral and coronary blood flow in HF including restricted microcirculation, attenuated regulatory mechanisms, and impaired hemorheological properties causes reduced oxygen utilization also contributing to the symptoms and progression of heart failure.

RBC aggregation and deformability have an important role in capillary blood flow including coronary microcirculation. Besides many clinical condition (e.g. ischemic heart disease, venous thrombosis), heart failure is known to be associated with increased RBC aggregation, which has a negative influence on the in vivo flow dynamics of blood.

III. The aim of the study

The thesis is based on our single-centre, double-blind, randomized, placebo-controlled human clinical study. We sought answers to two different questions (preplanned) in the same population, and our results were published in the form of two scientific papers.

The major aim of our study was to assess the effects of RES treatment on left ventricular function, on exercise capacity, on quality of life as well as on biomarkers and inflammatory cytokine levels in heart failure with reduced ejection fraction.

A further aim of this study was to test the hypothesis that RES can improve the hemorheological parameters, thereby the microcirculation in patients with heart failure with reduced ejection fraction. In addition, we investigated the relationship between hemorheological factors and exercise capacity of the patients.

IV. Materials and Methods

1. Study design, main baseline characteristics of the population, eligibility

The thesis based on a single-center, double-blind, randomized, placebo-controlled study. 60 stable outpatients (ages: 66.7 ± 2.01 years, 17 women and 43 men) with HFrEF in NYHA (New York Heart Association) class II or III were enrolled between 01/03/2016 and 30/11/2017 into our study (ejection fraction (EF) <40% and ischemic/non-ischemic origin: 34/26). They were randomized into two groups (RES group and placebo group). One-hundred milligrams of RES was administered orally (2×50 mg) for 3 months in the RES group (n=30) and placebo capsule in the other group (n=30).

The main exclusion criteria were acute cardiovascular or cerebrovascular event or major cardiac surgery or intervention within 30 days prior to randomization, renal failure (estimated glomerular filtration rate (eGFR) < 20 mL/1.73 m^2/min) or hepatic impairment ALT or AST ≥ 2x ULN at baseline).

The patients had baseline and follow-up visits after 1 month and after 3 months of treatment. During the whole study period, subjects were in stable clinical conditions and received unchanged medical therapy. There were no significant differences in demographic characteristics between the placebo and RES-treated groups at the baseline. All involved patients were on evidence-based drug treatment for HFrEF based on the actual ESC heart failure guidelines (2016).

On the day of randomization and 3-months-later physical examination, blood pressure and weight measurement, echocardiography, lab test, 6MWT, spirometry and quality of life questionnaire (QoL test) were performed. During the 1-month follow-up visit, only a physical examination, blood pressure, weight measurement and lab test were done

The following hemorheological parameters were determined at randomization and 3-months later: hematocrit, plasma and whole blood viscosity, red blood cell deformability and aggregation. The hemorheological parameters of RES and placebo groups were compared to age matched control group (mean age: 67.15±1,01 years, female/male: 11/9), without heart failure (ejection fraction >50%), and with moderate cardiovascular risk profile.

2. Statistical analysis

SPSS statistical software, version 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was used to conduct a descriptive analysis and to describe the sample. After using the Kolmogorov-Smirnov test to check the normality of the data distribution, differences of the mean values within the groups were analyzed by repeated-measures ANOVA with a Greenhouse–Geisser correction. Differences between the groups were calculated by one-way ANOVA test. Data are expressed as mean ± S.E.M. Significance level was defined as $p < 0.05$. The homogeneity of the groups was tested by Levene's F-test. The nonparametric Friedman test (post-hoc analysis with Wilcoxon signed-rank test) was applied to analyse the potential changes of the quality of life of the patients. Impact of rheological parameters on 6MWT results were analysed by Pearson correlation analysis.

3. Methods

3.1. Blood Pressure and Body Weight Measurement

Body weight and blood pressure were measured during randomization at 1-month and at 3-month follow-up visits.

3.2. Laboratory Test

Laboratory analysis performed on blood samples that were extracted from the cubital vein after a 12-h-long fasting period. From blood samples, NT-proBNP, fasting lipid levels (total cholesterol, low-density lipoprotein (LDL) - cholesterol, high-density lipoprotein (HDL) -cholesterol and triglycerides), troponin T, glycosylated hemoglobin-HgbA1c, serum albumin, renal and liver function, serum iron parameters and quantitative and qualitative blood cell counts were measured in the Department of Laboratory Medicine, University of Pecs, Pecs, Hungary. Special parameters (galectin-3, interleukin (IL)-1 and IL-6) were determined using an ELISA method in the Szentagothai Research Centre, University of Pecs.

3.3. Echocardiography

Transthoracic echocardiography was performed for noninvasive evaluation of cardiac structure and function at baseline and after the 3-month-long treatment. We used a GE Vivid E9 ultrasound imaging device (GE Healthcare, Chicago, IL, USA). Structures were visualized from parasternal long axis; short axis and apical four, two and three-chamber views. M-mode (one-dimensional mode), 2D-mode, 3D-mode, PW (pulsed-wave), CW (continuous wave) and tissue Doppler mode imaging were used to determine the left ventricular ejection fraction (EF, %), the diameters (mm) and volumes (mL) of the left ventricle and left atrium, the diastolic function (E/A and E/e'), the global longitudinal strain of the left ventricle (GLS, %), the wall thicknesses of the left ventricle (mm) and the right ventricular parameters. The EF was measured by two different methods ("Simpson" and "Quinone"). The investigators were blinded to the protocol; thus, they did not know whether the patient was taking RES or placebo.

3.4. Six-minute walk test (6MWT)

The 6MWT is a submaximal exercise test that measures the walking distance for 6 min. The test was performed on a 30-m-long section of the corridor in our department according to the guidelines of the American Thoracic Society (ATS).

3.5. Spirometry

We used a PISTON PDD 301/s spirometer (Piston Ltd., Budapest, Hungary) for measuring the basic resting lung parameters of the patients at baseline and 3 months later. We determined the most common parameters in the percentage of the reference values, such as FVC; FEV1; FEV1/FVC ratio; forced expiratory flow 25–75% (FEF 25–75); maximal expiratory flows (MEF 25%, MEF 50% and MEF 75%) and IVC.

3.6. Quality of Life Questionnaire (QoL test)

For the assessment of QoL, we used the "Euro QoL five-dimension" (EQ-5D) questionnaire. This scoring system consists of five dimensions (mobility, self-care, usual activities, pain/discomfort and anxiety/depression), each with three levels of response or severity (no problems—2 point, some problems—1 point or extreme problems—0 point). In addition to the index-based scoring system, the visual analogue scale (VAS) component of the EQ-5D enables the patient to place their current health state on a range from 0 (worst imaginable health state) to 100 (best imaginable health state).

3.7. RNA Isolation, RNA-Seq Library Preparation and Sequencing and Analysis

QIAamp RNA Blood Mini Kit was used for purification of total RNA from the whole blood. During the QIAamp procedure for purification of RNA from blood, erythrocytes are selectively lysed, and leukocytes are recovered by centrifugation. RNA concentrations were measured using Qubit 3.0 (Invitrogen, Carlsbad, CA, USA). The RNA quality was verified on an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Differential expression of genes at the end of the study was assessed in each experimental group compared to baseline expression values while controlling cross-checking of the patient IDs of paired data. Differentially expressed genes (DEGs) were filtered out, using a threshold of fold change (FC) absolute value > 1.5 and false discovery

rate (FDR)-adjusted p-value < 0.05. DEGs were visualized on a heat map using CLC Genomics Workbench 12.

3.8. Hemorheological parameters

Blood samples were taken on the day of randomization and after 3 months from the antecubital vein after a 12-hour fasting. Samples for hemorheological measurements were collected into 2x6 ml EDTA-coated Vacutainer tubes with a 21-gauge butterfly infusion set. Hemorheological measurements were carried out within 2 hours after blood sampling

Hct was measured by Haemofuge microhematocrit centrifuge (Heraeus Instr.; Germany). Measurements were performed at room temperature (22±1°C).

PV and WBV values were determined by Hevimet 40 capillary viscometer (Hemorex Ltd., Hungary). Plasma was collected after blood sample centrifugation for 10 minutes at 1500 G. Apparent WBV values interpolated to 90 s-1 shear rate were reported. Measurements were performed at 37 °C

Red blood cell aggregation measurements were carried out with a LORCA aggregometer (Laser-assisted Optical Rotational Cell Analyzer; R&R Mechatronics, Hoorn, The Netherlands) based on syllectometry. The aggregation behavior of blood sample is characterized by the aggregation index (AI) calculated from the first 10 seconds of the syllectogram after the shape recovery period, and by the time that elapses until intensity is reduced to half of the peak amplitude (t1/2). The smallest shear rate required for complete disaggregation (γ: gamma (1/s)) was also determined. RBC aggregation was measured also by Myrenne aggregometer (MA-1 Aggregometer, Myrenne Ltd., Germany), applying the light transmission method of Schmid-Schönbein et al. This method calculates aggregation index according to the change in intensity of transmitted infrared light during aggregation either at zero shear (M0) or low shear (M1 at 3 s-1) after disaggregation.

For the deformability measurement with LORCA ektacytometer, 25 µl blood was suspended in high viscosity (32.6 mPas) polyvinylpyrrolidone solution. RBCs were sheared by shear stress from 0.3 Pa to 30 Pa, and their deformation was visualized by laser-diffraction. The isointensive points of the diffraction pattern draw an ellipse with a longer A and a shorter B diameter. Deformation is characterized by the elongation index (EI) calculated by (A–B)/(A+B). Measurements were performed at 37°C.

V. Results

1. The Effect of Resveratrol on Laboratory Parameters

Laboratory parameters did not show significant changes in either group after one month of treatment compared to the baseline values (data not shown). There was no significant change in renal function, liver function, cardiac troponin T, iron parameters, HgbA1c and quantitative and quality blood counts in either group after the three-month follow-up period compared to the baseline values. The total cholesterol (4.74±0.22 mmol/L vs. 4.52±0.24 mmol/L, $p<0.05$) and LDL-cholesterol (3.09±0.23 mmol/l vs. 2.79±0.23 mmol/L, $p<0.05$) levels were significantly lower in the RES group by the end of the follow-up period; however, in the placebo group, no significant alterations could be seen compared to the baseline values.

2. The Effect of Resveratrol on Inflammatory parameters

The acute-phase proteins (CRP and ferritin) and white blood cell count did not show significant changes in either group during the three-month-long follow-up period. The levels of IL-1 (95.61±17.74 pg/mL vs. 140.65±24.04 pg/mL, $p<0.05$) and IL-6 (5.42±0.35 pg/mL vs. 6.76±0.56 pg/mL, $p<0.05$) measured by ELISA, however, were significantly lower in the RES-treated group at the end of the study period compared to the placebo group (Figure 1).

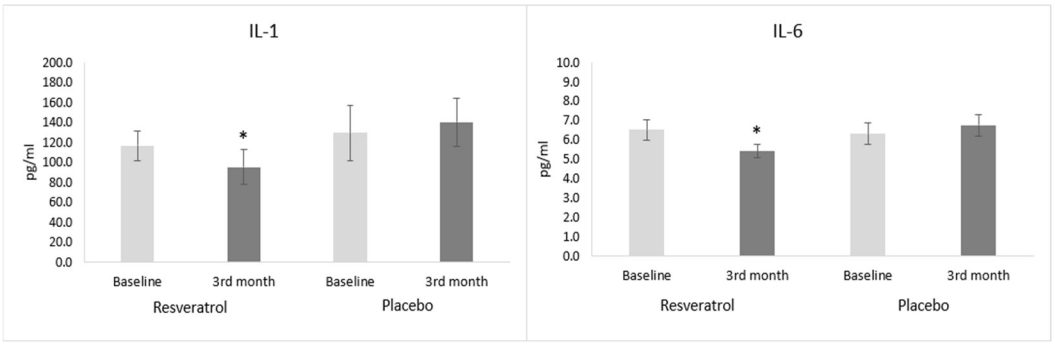

Figure 1. Effect of resveratrol on inflammatory cytokines in heart failure patients. Values are expressed as mean ± SEM. * $p < 0.05$ vs. placebo group at the 3rd month. Baseline: measured values at randomization in RES or in placebo group and 3rd month: patients treated with RES or placebo for 3 months. IL: interleukin.

3. The Effect of Resveratrol on Biomarkers of Heart Failure

There was no significant difference in the NT-proBNP level between the RES and placebo groups at the baseline (2998±507 pg/mL vs. 3139±446 pg/mL, NS); however, by the end of the treatment period, the NT-proBNP level was significantly lower in the RES group than in the placebo group (2760±346 pg/mL vs. 4054±577 pg/mL, $p<0.05$). This change suggests that RES inhibited the elevation of the plasma NT-proBNP level, a parameter which showed the severity of heart failure. In parallel with NT-proBNP, the galectin-3 level also showed a significant difference between RES and the placebo group at the end of the study (5.61±0.42 ng/mL vs. 6.98±0.54 ng/mL, $p<0.05$)

4. The Effect of Resveratrol on BMI, Blood Pressure, Heart Rate and ECG Parameters

BMI alterations were not significant in either the RES (29.31±0.99 kg/m^2 vs. 29.06±1.01 kg/m^2, NS) or in the placebo group (31.04±1.46 kg/m^2 vs. 31.34±1.39 kg/m2, NS) after three months compared to the baseline. Similarly, no significant alterations could be seen in the blood pressure, in the heart rate and in the ECG parameters after one month and at the end of the study.

5. The Effect of Resveratrol on Echocardiographic Parameters

The echocardiographic parameters of the patients did not differ significantly between the groups at the beginning of the study. The left ventricular EF measured by two different methods was improved significantly due to RES administration by the end of the treatment period compared to the baseline (Quinone method: 29.19±1.04% vs. 33.40±1.20%, $p<0.01$ and Simpson method: 30.06±1.04% vs. 34.60±1.44%, $p<0.01$) (Table 1). Global longitudinal strain (GLS,%) of the LV showed a significant improvement (−8.40 ± 0.62 % vs. −9.58 ± 0.73%, $p < 0.05$) in the treated group by the end of the follow-up compared to the baseline. In contrast, in the placebo group, no significant change could be observed. In the case of LV diastolic function, a significant difference in the E/A (1.18±0.15 vs. 1.54±0.19, $p < 0.05$) and E/E' ratios (15.55±1.43 vs. 19.94±1.43, $p<0.05$) was observed between the RES and placebo groups after the three-month-long treatment.

In addition, the LAESV, LA long axis, RVIDd, RVIDs and RAEDV were also decreased significantly in the RES group compared to the RES baseline values, as well as LAEDV, RAESV, IVC and TAPSE, which also showed significant differences between the RES and placebo groups after three

Table 1. Left ventricular echocardiographic parameters.

	Baseline		3rd month	
	Resveratrol	**Placebo**	**Resveratrol**	**Placebo**
EF (Quinones, %)	29.19±1.04	30.16±1.10	33.40±1.20** #	29.79±0.95
EF (Simson, %)	30.06±1.04	31.70±1.27	34.60±1.38** #	30.41±1.36
LVIDd (mm)	65.50±1.55	62.83±1.12	65.09±1.44	62.62±1.12
LVIDs (mm)	54.87±1.67	51.90±1.06	52.88±1.62	51.67±1.06
LVEDV (ml)	182.4±11.73	157.3±7.92	176.7±10.05	160.1±8.01
LVESV (ml)	129.0±9.37	106.7±5.80	116.3±8.00**	111.4±5.60
SV (ml)	52.85±2.82	50.69±3.38	60.46±3.13** #	48.56±3.84
SW (mm)	11.47±0.28	11.83±0.31	11.43±0.29	11.83±0.31
PW (mm)	11.03±0.17	11.17±0.20	11.20±0.17	11.14±0.21
GLS (%)	-8.40±0.62	-8.80±0.75	-9.58±0.73* #	-8.45±0.75
E (m/s)	0.89±0.04	0.98±0.05	0.80±0.05* #	1.00±0.05
A (m/s)	0.78±0.04	0.71±0.05	0.78±0.04	0.76±0.06
e' (m/s)	0.05±0.002	0.05±0.003	0.06±0.003	0.05±0.003
E/A ratio	1.26±0.12	1.57±0.20	1.18±0.15#	1.54±0.19
E/e' ratio	17.65±1.31	19.32±1.21	15.55±1.43#	19.94±1.43

Values are expressed as mean ± SEM. * p<0.05, 3rd month values of the RES group compared to the baseline values. ** p <0.01, 3rd month values of the RES group compared to the baseline values. # p <0.05, RES vs. placebo group at the 3rd month. A: late diastolic ventricular filling velocity, A': late diastolic mitral annular velocity, GLS: global longitudinal strain of the left ventricle, E: early diastolic ventricular filling velocity, e': early diastolic mitral annular velocity, EF: ejection fraction, LVEDV: left ventricular end-diastolic volume, LVESV: left ventricular end-systolic volume, LVIDd: diastolic left ventricular inner diameter, LVIDs: systolic left ventricular inner diameter, PW: posterior wall thickness, SV: stroke volume and SW: septal wall thickness

6. The Effect of Resveratrol on Exercise Capacity

The six-minute walk distance increased significantly in the RES group (from 275±19 m to 298±22 m, p<0.05) by the end of the treatment period compared to the baseline; however, in the placebo group, there was no apparent change (NS).

7. The Effect of Resveratrol on the Respiratory Parameters

The treatment with RES resulted in significantly improved FVC (75.38±3.31% vs. 79.27±3.07%, $p<0.05$) by the end of the study period. Other expiratory parameters did not show any changes compared to the baseline values. However, the inspiratory vital capacity (IVC) also increased significantly by RES administration (83.85±3.65% vs. 90.27±3.39%, $p<0.05$) compared to the baseline.

8. The Effect of Resveratrol on the Quality of Life

Our nonparametric statistical analysis showed significant improvement in the mobility ($Z=-2.236$, $p<0.05$), usual activities ($Z=-2.646$, $p<0.05$) and anxiety/depression ($Z=-2.236$, $p<0.05$) but did not reveal any significant difference in self-care and pain/discomfort of the patients in their RES-treated group after three months compared to the baseline values. Summary, RES administration resulted in a significantly improved percentage of the subjective health state (54.86±2.78% vs. 61.17±2.72%; $Z=-3.146$, $p=0.002$), but there was no significant change in the placebo group (49.43±3.15% vs. 48.17±3.34%; $Z=-0.631$, NS).

9. Sequence Analysis and Differential Expression

The main finding in this trial was that the RES treatment suppressed the expression of 7 genes of mitochondrial ETC (electron transport chain) members in leukocytes, including MT-ATP6 (ATP synthase F0 subunit 6-complex V), MT-CYB (cytochrome b), MT-ND1 (NADH dehydrogenase, subunit 1-complex I), MT-ND2 (NADH dehydrogenase, subunit 2-complex I), MT-ND4 (NADH dehydrogenase, subunit 4-complex I), MT-ND5 (NADH dehydrogenase, subunit 5-complex I) and MT-ND4L (NADH dehydrogenase, subunit 4L-complex I). However, the genes of complex II and complex IV were not affected by RES.

10. Hemorheological alterations after resveratrol treatment

10.1. Hemorheological parameters

The apparent WBV was increased ($p<0.05$) in heart failure patients, but Hct and PV didn't show any difference in either RES or placebo groups compared to the control group at baseline. According to our results, RES had no effect on Hct, PV or WBV and no difference was observed between the two groups (RES and placebo) either at baseline or after the 3-month follow-up period.

The AI and γ were significantly higher and $t_{1/2}$ was significantly lower in both RES and placebo groups compared to the control group at baseline ($p<0.05$). The M1 measured by Myrenne ($p<0.05$), the LORCA AI ($p<0.05$) and LORCA γ ($p<0.05$) decreased significantly after RES treatment. Furthermore, $t_{1/2}$ measured by LORCA also demonstrated a significant ($p<0.05$) alteration after 3 months compared to baseline (Table 2).

The deformability of RBCs at any measured shear stresses didn't show significant changes in either groups after 3 months compared to baseline, though an increasing tendency of EI at high shear stress could be seen in the RES group.

10.2. Relationship between 6-minute walk distance and hemorheological variables

Hct ($r=0.343$, $p<0.05$) and WBV ($r=0.308$, $p<0.05$) had a moderate positive correlation with the 6MWT (correlation indices of the baseline pooled group). In the RES group significant correlation could be revealed between the 6-minute walk distance and the RBC aggregation after 3 months: lower M1 aggregation index ($r=-0.269$, $p<0.05$) and lower γ (-0.417, $p<0.05$) were associated with longer walk distance. In addition, in this subgroup significant positive correlation was found between 6MWT and EI at high shear stresses (EI30: $r=0.480$, $p<0.005$)

Table 2. Effect of resveratrol on hemorheological parameters.

	Baseline			3rd month	
	Control N=20	**Resveratrol N=30**	**Placebo N=30**	**Resveratrol N=30**	**Placebo N=30**
Hct (%)	43.35±0.63	45.03±0.98	44.93±0.98	44.76±0.93	44.10±1.02
WBV (mPas)	4.04±0,07	4.59±0.13#	4.55±0.14#	4.45±0.13	4.42±0.13
PV (mPas)	1.28±0.02	1.34±0.02	1.31±0.02	1.32±0.02	1.32±0.02
Aggregation (Myrenne):					
M	5.91±0.25	6.02±0.36	6.38±0.28	5.90±0.31	6.36±0.28
M_1	13.77±0.78	13.96±0.42	14.14±.0.47	13.46±0.40*	13.84±0.51
Aggregation (LORCA):					
AI	69.37±1.1	73.36±1,02#	72.08±1.04#	70.55±0.99*	70.15±1.14
$t_{1/2}$ (s)	1.57±0.11	1.23±0.08#	1.34±0.09#	1.45±0.08*	1.51±0.11
γ (1/s)	112.7±4.43	161.9±11.02#	160.4±12.54#	133.7±7.25*	138.6±9.12

Values are expressed as mean± SEM. #=significant difference resveratrol or placebo groups compared to control group at baseline; * = significant difference 3rd month values of resveratrol group compared to the baseline values of resveratrol group ($p< 0.05$). Baseline: measured values at randomization in resveratrol or in placebo group; 3rd month: patients treated with resveratrol or placebo for 3 months; AI: aggregation index; t1/2 (s): aggregation half time; M and M1 values: aggregation indices at different rotation speed of the aggregometer; PV: plasma viscosity; RBC: red blood cell; WBV: whole blood viscosity; γ (1/s): threshold shear rate.

VI. Discussion

1. Resveratrol Improves Heart Function by Moderating Inflammatory Processes

Our present work firstly proved that RES beneficially influences heart failure in a randomized double-blind clinical trial (RCT). The major findings of our trial are that the RES treatment improved heart function, exercise tolerance, several spirometry parameters and quality of life and decreased the level of cholesterol and inflammatory cytokines in systolic heart failure patients.

It is well-established that RES has various beneficial effects on the cardiovascular system. It has a marked antioxidant effect due to its scavenger capability and due to enhancing the antioxidant enzyme production (SOD, CAT and eNOS), as well as decreasing the amount of prooxidant enzymes (NOXs and MPO). Therefore, RES also has a marked anti-inflammatory and antiplatelet effect. However, these data are derived almost completely from preclinical studies.

It was published previously by our workgroup that RES in a murine post-infarct heart failure model improves the heart function and decreases myocardial fibrotic remodeling via its anti-inflammatory effect and via blocking the profibrotic intracellular signaling routes. Other workgroups showed similar results in various animal heart failure models; however, in a human clinical trial, the effect of RES has not been confirmed yet.

In this single-center double-blind RCT, the baseline characteristics of symptomatic HFrEF patients were well-balanced due to the used randomization method (adaptive minimization). Guideline-directed medical treatment (GDMT) was administered in the maximal tolerated dose, and almost every patient was given ACEI or ARB and beta-blockers, and only one-quarter of the patients was not on MRA treatments.

In our heart failure patients, RES supplementation improved the systolic left ventricular function expressed as the ejection fraction (EF). The EF was measured in two different ways, by using the Quinones and the Simpson methods. However, the volumetric Simpson method is by far the most commonly used method for quantifying left ventricular function. In the case of both methods, a similar increase could be seen in the RES-treated group; however, in the placebo group, no change was observable.

GLS is another measure of LV global function that also correlates with the extent of myocardial fibrosis in patients with HFrEF. In the placebo-treated group, the GLS value remained unchanged by the end of the treatment period. The RES treatment, however, caused an improvement of the GLS value in parallel with other measures indicating systolic heart function. Moreover, GLS can show the extent of myocardial remodeling, especially myocardial fibrosis. In parallel with the improvement of systolic heart function, diastolic heart function also got better (i.e. the E/e' ratio decreased) in the RES group. In the placebo group the diastolic function was unchanged.

Natural polyphenols could improve heart function in a wide variety of experimental heart failure models. However, in human clinical trials, there are only few data regarding the effect of RES treatment on heart function. Our workgroup proved previously that a low-dose RES treatment (10 mg/day) improved the diastolic function in patients with coronary artery disease (CAD), while, in the case of systolic LV function, only a favourable tendency was present. In another small trial also conducted in CAD patients using a higher dose (100 mg/day) of RES, both the systolic and diastolic function improved.

Not only the heart function, but also the exercise tolerance determined by the 6MWT showed significant improvement in RES-treated patients by the end of the three-month-long treatment period. This result is in accordance with the preclinical results carried out in several murine models. However, there are no clinical data regarding the effect of RES on the physical exercise capacity in heart failure patients yet. In an interesting trial, Voduc N. and his co-workers proved that RES itself in healthy people has no positive effect on exercise capacity and on the maximal oxygen consumption (VO2max); moreover, Glieman L. et al. demonstrated that, in aged men, the RES treatment blunted the positive cardiovascular effects of exercise and moderated the increase of VO2max caused by the training program. Therefore, the increase of exercise capacity can be predominantly the consequence of the improvement of heart function in heart failure patients.

The analysis of cardiac biomarkers strengthens the findings regarding heart function and exercise tolerance. Galectin-3, which is secreted by macrophages, has been known for its significant role in mediating cardiac fibrosis and inflammation. These data were also proved in a human clinical examination. In our trial, the RES treatment decreased the galectin-3 level, but in the placebo group no change could be seen. This result supports the beneficial changes that occurred in the case of GLS, because GLS is directly proportional to the extent of interstitial fibrosis.

NT-proBNP is the most widely used biomarker in the diagnosis and risk stratification of heart failure. It shows a strong correlation with the severity of heart failure, although its value can be variable due to alterations in the volume status of patients. In our trial, the RES supplementation decreased the level of NT-proBNP (NS), and in the placebo group, a worsening tendency could be seen, so by the end of the treatment period, a significantly lower natriuretic peptide level was achieved in the RES group than in the placebo group. In a study conducted in stable CAD patients, Militaru and co-workers found that RES decreases the NT-proBNP level even without overt heart failure. The continuously increasing level of heart failure biomarkers (NT-proBNP and galectin-3) in the placebo group can be a consequence of the progression of the disease. According to the literature, there was a linear relationship between NT-proBNP and the number of hospitalizations, as well as mortality, due to heart failure.

The improvement of left ventricular heart function in the RES-treated group is in-line with the decreased volume retention and with the decongestion of organs. It is known that extravascular fluid accumulation in the lungs is accompanied by the alteration of several spirometry measures, e.g. FVC and IVC. Moreover, Gehlback et al. showed that, after heart transplantation, not only the ventilation volumes but the airflow velocity (FEV1) was also better. Due to RES supplementation in the present trial, FVC and IVC increased significantly by the end of the treatment period. However, in the case of airway obstruction parameters, no significant changes could be seen in this study. FEV1 showed only a mild, nonsignificant improvement in the RES group.

The level of inflammatory cytokines, IL-1 and IL-6 decreased in the RES-treated heart failure patients compared to the placebo group. Similar results were seen in the literature. This favourable change is in parallel with the level of galectin-3β that mediates inflammation. The levels of inflammatory cytokines produced by the activated leukocytes are inversely proportional to the systolic left ventricular function. The increasing level of IL-1 and IL-6 in the placebo group can be explained by the slow progression of heart failure in our enrolled population. The correlation between inflammation and adverse cardiovascular outcomes in heart failure was documented. Essentially, heart failure progression was attributed to sustained proinflammatory cytokine signaling, based on the observation that proinflammatory cytokines were elevated and continuously worsened during the progression of the disease. Interestingly, the mRNA profile analysis of leukocytes revealed a significant decrease in genes encoding several mitochondrial respiratory proteins. RES, however, did not interfere with the expression of various proteins playing a part in the mitochondrial

quality control. Moderating the production of ETC proteins in leukocytes can decrease the oxidative phosphorylation in leukocytes, which is directly proportional to their activity. The decreased production of proinflammatory cytokines in this work can be a sign of the moderated activity of leukocytes. This anti-inflammatory effect can be an important aspect of the RES treatment in heart failure patients, besides its well-known mitochondrial protective effects in cardiomyocytes.

2. Hemorheological alterations in patients with heart failure with reduced ejection fraction treated by resveratrol

The altered hemorheological factors in heart failure may play an important role in the complex impairment of microcirculation and the progression of heart failure. In previous studies, hemorheological parameters (WBV, plasma fibrinogen level, RBC aggregation and deformability) were found significantly worse in heart failure than those of healthy people. Reduced peripheral blood flow, oxygen transport (hypoxemia) and increased oxidative stress in heart failure are described to cause RBC disorders. Damage to red blood cells by ROS results in abnormalities in the function, morphology, and metabolism of erythrocyte including RBC aggregation and deformability. Cytoskeletal and membrane proteins and lipids are oxidized by ROS, what possibly increases the tendency of 'damaged' erythrocytes to adhere with other erythrocytes thereby increasing RBC aggregation. Our results demonstrated increased erythrocyte aggregation in heart failure patients compared to age matched patients with moderate cardiovascular risk profile without heart failure. Moreover, hypoxemia induced rise in hematocrit (increased erythrocyte production) and elevated blood viscosity were observed in heart failure, while in advanced stages of heart failure hematocrit level often decreases due to increased plasma volume (hemodilution), iron deficiency and/or bone marrow depression caused by excessive cytokine and ROS production.

According to literature, RES may have protective role against the development of cardiovascular diseases. Primarily RES is thought to be responsible for the cardioprotective effect of red wine (French paradox). The bioactive polyphenol RES possesses antioxidant properties, reduces oxidative stress in animal models and may contribute to the preservation of cardiac structure and function in animals. Animal studies showed that RES can stabilize erythrocytes by the reduction of erythrocyte osmotic fragility. RES was also described to maintain vascular endothelial function and to dilate blood vessels. A previous human study of our working group assessing endothelial dysfunction detected significant improvement in

vasorelaxation in RES treated patients. Other authors reported that RES can change the properties of plasma proteins and preserve the structure of fibrinogen from conformational alterations, which may influence RBC aggregation as well.

The effects of RES on hemorheological parameters have already been evaluated in some animal and human trials, however, they have not been studied in heart failure.

The main findings of our present trial after a 3-month follow-up period are as follows: (1) RBC aggregation was decreased significantly in patients treated with RES; (2) macrorheological parameters didn't change significantly; (3) the 6-minute walk distance was increased significantly in RES treated patients; (4) relationship was detected between the 6-minute walk distance and some hemorheological variables (erythrocyte aggregation, erythrocyte deformability, hematocrit and whole blood viscosity).

We found no significant change in Hct, WBV and PV values after the 3-month RES treatment. Similar results were seen in a previous study of our workgroup.

On the other hand, we could demonstrate a significant decrease of RBC aggregation in our patients after RES treatment via several parameters. Based on our results - which confirm a previous study - RBC aggregation is deteriorated significantly in patients with heart failure, while in this study RES treatment partially reversed these changes of RBC aggregation. Though some difference could be seen between the results of LORCA and Myrenne, these are probably the consequences of the different principles of operation and precision of the instruments.

The decrease in aggregation may be a consequence of the antioxidant properties of RES and also the modifications of plasma proteins. According to one of the accepted theories, RBC aggregation is due to the bridging between adjacent cells by specific plasma proteins (e.g., fibrinogen, large molecular weight globulins) and influenced by the concentration and specific binding to the erythrocyte membrane of fibrinogen. It is known that polyphenols are bound to plasma proteins due to their poor water solubility. RES may change the properties of plasma proteins and RBC surface molecules, thus reducing the ability to form cross links between cellular components and decreasing erythrocyte aggregation. The reduction of RBC aggregation may have a positive effect on the flow properties of coronary microcirculation, what can be especially important in heart failure.

In this trial, we could not demonstrate the change of RBC deformability after RES treatment by any ektacytometry parameters.

6MWT is a routine diagnostic procedure to quantify exercise capacity of heart failure patients. In this double-blind, randomized placebo-controlled study, RES treated patients

had longer walk distance after the 3-month follow-up period, which is concordant with the results of a previous study. Furthermore, positive correlation was found between the functional capacity and the favourable hemorheological alterations as well. The improving hemorheological parameters may partly contribute to the longer walk distance in patients treated with RES.

VII. Conclusion

In this human clinical trial, the positive effects of RES were proved in HFrEF patients added to the standard therapy. The RES treatment improved several parameters of heart function, exercise tolerance and quality of life. Moreover, RES exerted an anti-inflammatory effect measured by the decrease of levels of inflammatory cytokines (IL-1 and IL-6). According to our results, the decreased activity of leukocytes can be an important mechanism of RES, and it can contribute to its cardioprotective effect in heart failure. Moreover, in our in vivo human study, we confirmed the beneficial effect of RES on erythrocyte aggregation in heart failure. Decreased RBC aggregation by RES may positively influence the microcirculation, tissue perfusion and oxygen supply, what may contribute to the improved coronary and peripheral blood flow and probably increases the exercise capacity of patients with heart failure with reduced ejection fraction.

VIII. Summary of the new scientific results

1. Our present work firstly demonstrated that moderate dose of RES treatment has a beneficial effect on heart failure (HFrEF) patients in a randomized double-blind human clinical trial (RCT).
2. We have proved that RES supplementation improved the systolic and the diastolic left ventricular function of HFrEF patients.
3. In parallel with heart function, the RES treatment improved exercise tolerance, quality of life and several spirometry parameters as well.
4. In our trial we demonstrated the antiinflammatory effect of RES measured by the decrease of levels of inflammatory cytokines (IL-1 and IL-6).
5. The mRNA profile analysis of leukocytes revealed significant change in expression profile of several genes after RES supplementation.
6. According to our results, the decreased activity of leukocytes can be an important mechanism of RES, and it can contribute to its cardioprotective effect.
7. In our trial we firstly confirmed the beneficial effect of RES on erythrocyte aggregation in human clinical study in HFrEF.
8. In addition, we found a positive correlation between functional capacity and favourable hemorheological alterations. According to our hypothesis, the improvement of the microcirculation may contribute to the increasing exercise capacity of patients.

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X. Publications of the author

1. Publications supporting the dissertation

1. **GAL R**, DERES L, HORVATH O, EROS K, SANDOR B, URBAN P, SOOS S, MARTON Z, SUMEGI B, TOTH K, HABON T and HALMOSI R. Resveratrol Improves Heart Function by Moderating Inflammatory Processes in Patients with Systolic Heart Failure. *Antioxidants*, 2020;9(11):1108.
   Impact factor: 5.014 (Q1)
2. **GAL R**, PRAKSCH D, KENYERES P, RABAI M, TOTH K, HALMOSI R and HABON T. Hemorheological Alterations in Patients with Heart Failure with Reduced Ejection Fraction Treated by Resveratrol. *Cardiovascular Therapeutics.* 2020;2020:7262474.
   Impact factor: 2.538 (Q2)

2. Other published papers

3. **GÁL R**, HALMOSI R. A szívelégtelenség aktualitásai a terápiában 2020-ban. Kardio-Vaszkuláris Iránytű. 2020;2(4):49-54.
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