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HEART RESEARCH

Open Journal

| December 2017 | Volume 4 | Issue 3 |

www.openventio.org

HEART RESEARCH

Openventio PUBLISHERS

ISSN 2377-164X

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Openventio PUBLISHERS

ISSN 2377-164X Open Journal http://dx.doi.org/10.17140/HROJ-4-141

Research

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Volume 4 : Issue 3
Article Ref. #: 1000HROJ4141

Article History
Received: July 23rd, 2017
Accepted: July 27th, 2017
Published: July 28th, 2017

Citation
Fusco A, Colarusso S, Piscopo M, et al. Short-term cardio-vascular risk score changes in type 2 diabetic patients on empaglifozin: A real-life clinical experience. *Heart Res Open J*. 2017; 4(3): 46-56. doi: 10.17140/HROJ-4-141

Short-term Cardio-Vascular Risk Score Changes in Type 2 Diabetic Patients on Empaglifozin: A Real-Life Clinical Experience

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ABSTRACT

Objective: The aim of the study was to test both efficacy and safety of empaglifozin in type 2 diabetic out patients followed-up within a single institution.
Materials and Methods: One hundred seventy-eight consecutive patients treated with empaglifozin for 6 months were compared to 356 fully matched control patients on alternative drugs: side effects were monitored and changes from baseline were evaluated for body weight, body mass index (BMI), blood pressure, circulating glucose, HbA1c, uric acid, triglycerides, total, high density lipoprotein (HDL) and low density lipoprotein (LDL)-cholesterol levels, as well as creatinine clearance rate. The 10-year Cardiovascular Risk score (10-y CV-RS) was also assessed in all patients according to the cryogenic underground observatory for rare events (CUORE) calculation charts. Weight, BMI, systolic and diastolic blood pressure, as well as, HbA1c, glucose, cholesterol, triglyceride and uric acid levels, significantly decreased after treatment, along with the 10-y CV-RS which is in fact strongly related to the above.
Results: Virtually no changes were observed in creatinine clearance rate and HDL levels. 45% patients reported polyuria, 15% genital itching, 8% genital infections, 12% urinary tract infections, no volume depletion or keto-acidosis episodes were described.
Conclusion: In conclusion, in diabetic patients experiencing no previous major cardiovascular events, empaglifozin significantly improved all cardiovascular risk factors, including glucose control, and the 10-y CV-RS. Moreover it was well tolerated, so that no treatment discontinuation was needed even in case of urinary tract infections.

KEY WORDS: Type 2 diabetes mellitus (T2DM); Cardiovascular risk; Cardiovascular risk (CVR); SGLT2i; Empaglifozin; Adverse effects.

ABBREVIATIONS: CV: Cardiovascular; T2DM: Type 2 Diabetes Mellitus; SGLT2i: Sodium-glucose co-transporter-2 inhibitors; CVD: Cardiovascular disease; CHD: Coronary Heart Disease; CVE: Cerebrovascular events; PAD: Peripheral Arterial Disease; HF: Heart Failure.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a progressive disease eventually leading to microvascular and cardiomacrovascular complications and to their consequent ever increasing economic burden.

Sodium-glucose co-transporter-2 inhibitors (SGLT2i) are new oral antidiabetic medications recently approved for the treatment of T2DM.[1-4] They enhance urinary glucose output and, through this mechanism, are known to significantly improve glycemic control,[5-7] and to cause both weight and fat mass loss.[8,9] Moreover, SGLT2 may add additional beneficial effects through decreasing systolic and diastolic blood pressure.[10] They are well-tolerated and mostly cause only mild adverse effects,[9] one of which, exacerbated by glycosuria, is represented by possible recurrent genitourinary infections.[11,12]

Empagliflozin is a highly selective SGLT2i improving glycemic control in patients with T2DM both *per se* and when added to metformin, sulfonylurea, or insulin.[13-15] In the Empa-Reg outcome study, when added to standard care in patients with T2DM and a high cardiovascular (CV) risk, empagliflozin reduced CV events, mortality and hospitalization as compared to placebo during a 48 month observation-period [Empa-Reg outcome].[14] Efficacy data reported so far for SGLT2 is derived from randomized clinical trials, while those from "clinical practice" are still missing.

Due to that, some of the most common questions posed by patients at the time of diagnosis, i.e. "*Will my disease be worse?"* or *"Will I get worse?*", in real life get highly variable answers by their doctors, who mostly talk about their own experience and, even when referring to large randomized controlled trials (RCTs), can only base their predictions on forecast risks derived from relatively short-duration (4 years at most) validated data.

The aim of our study was to verify the short-term efficacy and safety of empaglifozin under real life conditions in an attempt to provide appropriate answers to the above mentioned questions. To achieve that we evaluated our diabetic patients for 6-month changes in their metabolically relevant parameters and 10-year CV risk score (10-y CVrs). The latter was calculated according to the Italian CUORE risk-scoring equation,[16-19] considered to be more appropriate to the population under study than others based on populations with different eating habits and different CV mortality rates.

MATERIALS AND METHODS

Study Design

The present report is an observational study carried out in an outpatient setting. We analyzed a series of patients with T2DM followed-up by a network of 10 outpatient diabetes centers (DCs) from AID (Associazione Italiana Diabete) participating in the so called Associazione Medici Diabetologi (AMD) Annals Initiative, all having the same organization structure and previously documented to attain the same performance levels and therefore considered to be a single institution.[20] Participating DCs adopted the same software for everyday outpatient management. A dedicated software package allowed data extraction for further analysis (AMD Data File).

The latter, in fact, could import information exclusively from patients previously signing an informed consent to data anonymous utilization for better diabetes control and improved quality of life (QoL). All DCs received their local Ethics Committee approval for database utilization and the study conformed to the Helsinki Declaration.

The diagnosis of type 2 diabetes was made/confirmed within each participating DC according to criteria defined by the ADA Standards of Medical Care in Diabetes 2014.[21] The International Classification of Diseases, Clinical Modification (ICD-9-CM, V82.9 2014) was used to define T2DM diagnosis and comorbidities/complications.[22]

The DC network recorded blood pressure (BP), glucose, HbA1c, total, low-density lipoprotein and high-density lipoprotein cholesterol (TC, LDL-C and HDL-C, respectively), triglycerides (Tg) and serum uric acid (UA) as measured by high standard auto-analyzers in public laboratories successfully participating in nationwide quality control programs. All electro-medical devices used to evaluate patients were certified and periodically validated in accordance with the International Standards Organization (ISO) directive.[23]

Kidney function was assessed by both serum creatinine and urinary albumin excretion rate measurements. Estimated creatinine clearance rate (i.e., glomerular filtration rate, estimated glomerular filtration rate (eGFR) was equation.[24] calculated for each patient based on a standardized serum creatinine assay and the chronic kidney disease epidemiology collaboration (CKD-EPI). Only patients having at least one serum creatinine measurement and concordant eGFR values during the last 3 months were included in the study.

Inclusion criteria were: HbA1c higher than 7% despite metformin at maximal tolerated dosage and/or insulin therapy. BMI>25 kg/m^2, 35<age<69yrs, eGFR>60 ml/min/1.73m^2.

Exclusion criteria were: a history of recurrent urinary tract infections or previous urinary or pelvic surgery, poor adherence to therapy, severe liver disease, kidney failure, neoplasms, steroid therapy, previous cardiovascular events and clinical conditions favoring ketoacidosis.

The 7.0% (53 mmol/mol) HbA1c cut off was chosen according to both the algorithm by Ceriello et al[25] and ADA's "Standards of Medical Care in Diabetes", both meant at reducing the incidence of microvascular diseases without increasing the risk for hypoglycemia.[21] Less stringent HbA1c targets,[26-28]

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which would have been appropriate for specific, mostly fragile, populations were not taken into account, because, as stated above, critically-ill people or those with recent cardiovascular events or with rapidly progressing clinical conditions were not included in the study.

One hundred seventy-eight T2DM patients consecutively referring to the DCs and receiving add-on treatment with empaglifozin 10 mg (Treatment Group: TG) were enrolled. Empaglifozin 10 mg was added to metformin and/or insulin therapy according to prescribing criteria defined by the Italian Drug Agency (AIFA). As reference group 356 subjects with similar general clinical characteristics who were not taking empaglifozin were also enrolled in a 1:2 ratio, treatment *versus* control, respectively (Control Group: CG). All patients were followed-up for 6 months.

They were assessed for the above mentioned blood chemistry parameters, body weight (BW), body mass index (BMI), blood pressure, and eGFR at baseline as well as after 3 and 6 months. Drug safety and tolerability was also monitored in terms of aspartate aminotransferase (AST) (U/l), alanine aminotransferase (ALT) (U/l), γ-glutamyltransferase (γ-GT) (U/l), total bilirubin (mg/dL), alkaline phosphatase (U/l), eGFR (ml/min/khm^2), urea mg/dL (mg/dL), micro-macro-hematuria, cylindruria, ketonuria, urinary tract infections, genital infections, polyuria, other infectious diseases for safety and of asthenia, headache, nausea/vomiting, other gastrointestinal disorders, allergic reactions, mild or severe hypoglycemia for tolerability.

The individual 10-y CVrs was assessed using the CUORE project calculator. The CUORE project was an epidemiological and ischemic heart disease prevention project launched in 1998.[16] This score enables us to estimate the probability of experiencing any CV events (mainly myocardial infarction or stroke) for the first time over the next 10 years based on eight CV risk factors including age, gender, systolic BP (SBP), TC, HDL-C, diabetes mellitus, smoking habit and use of antihypertensive medications.[17-19] It was validated in patients 35 to 69 years of age without previous major cardiovascular accidents. It cannot be used in case of extreme risk factor values, including systolic blood pressure (SBP) higher than 200 mmHg or less than 90 mmHg, TC higher than 320 mg/dL or less than 130 mg/dL, HDL less than 20 mg/dL or higher than 100 mg/dL.

Statistical Analysis

Data are presented as mean values±standard deviation (M±SD). Categorical variables are given as frequencies and percentages. Repeated measures ANOVA was applied for intergroup and intragroup comparisons at the three chosen time points. *p* values <0.05 were taken as statistically significant. All analyses were performed using the STATA software, version 14 (Stata-Corp LP, College Station, Tex).

RESULTS

Clinical features of the whole study cohort population are reported in Table 1.

Table 1: Descriptive Features of the Enrolled Population. Data are Expressed as Mean+SD or as n. and Percent Rate in Case of Categorical Variables: 78 Subjects had more than a Single Complication.

Casuistry					
Variable	**Treatment Group**		**Control Group**		
	n=178		**n=356**		
	n.	**% (range)**	**n.**	**% (range)**	***p***
Female	102	57.4	199	55.9	ns
Age (years)	60.7±10.3	(41-68)	60.5±9.9	(40-67)	ns
BMI (kg/m^2)	30.6±4.5	(26.8-44.1)	30.4±4.6	(27.0±43.9)	ns
Normal weight	16	8.9	32	6.5	ns
Overweight	105	58.9	203	57.0	ns
Obese	57	32.2	130	36.5	ns
Diabetes duration (years)	9.2±7.1	(3-14)	9.4±7.7	(4-13)	ns
SBP (mmHg)	134,2±10,9	-	135.4±9.8	-	ns
DBP (mmHg)	81.7±7.3	-	80.9±6.9	-	ns
Subjects on Met alone	-	60.7	-	59.9	ns
Subjects on Insulin w/wo Met	-	39.3	-	41.1	ns
Biochemical parameters (M±SD)					
	n.	**%**	**n.**	**%**	***p***
Fasting blood glucose (mg/dl)	191.7±40.5	-	189.5±44.3	-	ns
HbA1c (%)	8.7±0.8	-	8.8±0.9	-	ns
Total Cholesterol (mg/dl)	179.3±36.4	-	175.7±33.9	-	ns
HDL Cholesterol (mg/dl)	43.9±8.9	-	44.8±9.6	-	ns
LDL Cholesterol (mg/dl)	103.2±29.5	-	107.5±25.9	-	ns

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Triglycerides (mg/dl)	165.9±61.4	-	171.4±59.6	-	ns
Creatinine (mg/dl)	0.9±0.6	-	0.8±0.5	-	ns
eGFR (ml/min/1.73m^2)	90.1±17.7	-	90.3±13.8	-	ns
Smokers (%)	-	47	-	50	ns
Lipid-lowering treatment (%)	-	60	-	62	ns
Antihypertensive treatment (%)	-	65	-	66	ns
Aspirin (%)	-	47	-	41	ns
Diabetes-related Complications					
	n.	**%**	**n.**	**%**	***p***
Overall	-	44	-	46.3	ns
Retinopathy BG	-	14.8	-	15.3	ns
Nephropathy*	-	13.1	-	12.5	ns
Autonomic Neuropathy	-	10.7	-	11.2	ns
Peripheral Neuropathy	-	14.7	-	15.1	

*Presence of microalbuminuria and/or eGFR>90 ml/min/1.73 m^2 or >60 <90 ml/min/1.73 m^2
SBP=Systolic Blood Pressure; DBP=Diastolic Blood Pressure; Met=metformin; w/wo=with or without.

Median age of investigated patients was 60 years in both the TG and the CG (interquartile (IQ) range: 41-68 and 40-47 years, respectively), 42.6% and 44.1% patients were male, respectively, and median diabetes duration was 9.2 and 9.4 years, respectively (IQ range: 3-14 and 4-13 years, respectively). Median BMI was 30.6 and 30.4 kg/m^2 (IQ range 26.8-44.1 and 27.0-43.9 kg/m^2, respectively). Glycemic control was poor, being median HbA1c>8.5% in both target group (TG) and control group (CG), with a fasting glucose level around 190 mg/dL, whereas lipid parameters and BP levels were moderately good, being LDL-C103 and 107 mg/dL, respectively, and mean BP close to 135/80 mmHg in both groups. Median eGFR was 90 ml/min/1.73 m^2 (IQ range 68-102 in both groups). In Table 1 also clinical characteristics are described including smoking habits, microvascular complications, use of aspirin, and lipid lowering or antihypertensive drugs.

Effect of Empaglifozin Treatment

No patients on empaglifozin added to metformin and/or insulin dropped off. Table 2 describes the effects of treatment.

Table 2: Parameter Changes within the Two Groups during the Follow-up Period.

	Treatment Group (n. 178)				**Control group (n. 356)**			
	Baseline T0	3 months T3	6 months T6	%Δ$^{\$}$	Baseline T0	3 months T3	6 months T6	%Δ$^{\$}$
Body weight (kg)	88.4±15.3	85.7±14.8*	84.8±14.8* °	-4.1	89.4±14.5	88.5±14.1^	88.06±13.2^	-1.5
BMI (kg/m^2)	31.7±3.8	30.7±3.6**	29.8±5.1* °	-5.4	30.4±4.6	30.4±4.4	30.2±3.9^	-0.5
Hb1Ac (%)	8.7±0.8	7.8±0.7**	7.5±0.7* °	-13.9	8.8±0.9	8.8±0.9^^	8.6±0.7^	-1.5
FPG (mg/dl)	191.7±40.5	143.1±2.3*	129.1±20.6* °	-32.7	189.5±44.3	182±31.5^	181.3±23.5^	-4.3
SBP (mm Hg)	134.2±10.9	126.7±6,9*	125.6±7.6* °	-6.4	135.4±9.8	135.2±9.6^	134.8±8.8^^	-0.4
DBP (mm Hg)	81.7±7.03	77.8±5.3*	77.5±5.2* °	-5.3	80.9±6.9	80.7±6.3	80.6±5.8^	-0.3
TChol (mg/dl)	179.3±36.4	175.6±24.8**	168.3±23.8** °°	-6.1	175.7±33.9	177.3±31.1	173.6±28.6^^	-1.2
HDL-C (mg/dl)	43.9±8.9	46.3±6.9**	45.8±10.6**	+4.4	44.8±9.6	44.3±7.2	43.8±7.5^^	-0.2
LDL-C (mg/dl)	103.2±29.5	100.4±27.1	89.9±25.6** °°	-12.9	107.5 ±25.9	106.4±24.6	105.1±22.6^	+2.3
Triglycerides (mg/dl)	165.9±61.4	144.3±49.2**	136.9±51.1* °	-17.5	171.4 ±59.6	169.4±44.2	167.9±46.6^	+2.2
Uric acid (mg/dl)	5.4±1.0	5.3 ±0.8	5.1±1.0$^{\$}$ °°	-5.6	5.5±1.1	5.4±1.0	5.4±0.9^^	-1.1
eGFR ml/min/1.73m^2)	90.1±17.7	90.5±16.7	92.9±14.7	+3.1	90.3±13.8	74.5±18.4^#	67.7±12.3^#	-25
10-y CV-RS	12.7±10.2	-	10.6±7.6*	-18.2	12.5±9.9	-	12.2±7.2	-2.2
*p<0.01 *vs.* T0; °p<0.01 *vs.* T3; **p<0.05 *vs.* T0; °°p<0.05 *vs.* T3; $^{\$}p$<0.05 *vs.* T3;					^p<0.01 *vs.* TG; ^^p<0.05 *vs.* TG; #p.0.01 *vs.* Baseline CG			
$^{\$}$ %Δ *vs.* baseline								

%Δ=percent difference observed between T6 and T0.
10-y CV-RS = 10 year individual cardiovascular risk score.

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Significant differences were observed in all biochemical and clinical parameters between control and empaglifozin groups. The latter displayed a significant and progressive improvement in all parameters with a rapid decrease in fasting blood glucose and HbA1c as shown in Figures 1 and 2, respectively.

BW changes are reported in greater detail in Figure 3, describing TG patients after splitting into metformin-only-treated and insulin-treated (with or without metformin) subgroups: both of them showed a significant BW decrease despite higher levels being consistently attained in the latter.

Figure 4 clearly shows significant differences ($p<0.01$) between TG and CG as compared to baseline in percent improvement of almost all investigated parameters, with the biggest differences involving fasting blood glucose (-32,4% *vs*. -4.3% in CG, respectively), HbA1c (-13.9% *vs*. -1.5%, respectively), Tg (-17.5% *vs*. +2.2%, respectively), LDL-C (-12.9% *vs*. +2.3%, respectively).

10-year CV Risk Score

At baseline the 10-y CV-RS score was 12.6±10.2 in the TG (Table 2 and Figure 5) and 12.5±9.9 in the CG (n.s.). After the 6-month follow-up a significant decrease was observed (-18.2%, $p<0.01$) in the TG *versus* an only slight decrease in the CG (-2.2%, n.s.) (Figure 4).

Table 3 describes the results of the multivariate Cox regression analysis: systolic BP and antihypertensive treatment

Figure 1: Comparison of Fasting Plasma Glucose (mg/dL) in the Treatment Group (TG) *versus* the Control Group (CG).*=$p<0.01$.

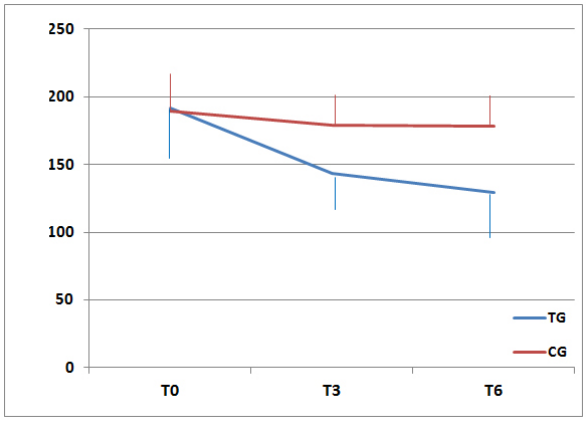


Figure 2: Comparison of HbA1c (%) in the Treatment Group (TG) *versus* the Control Group (CG).*=$p<0.01$.

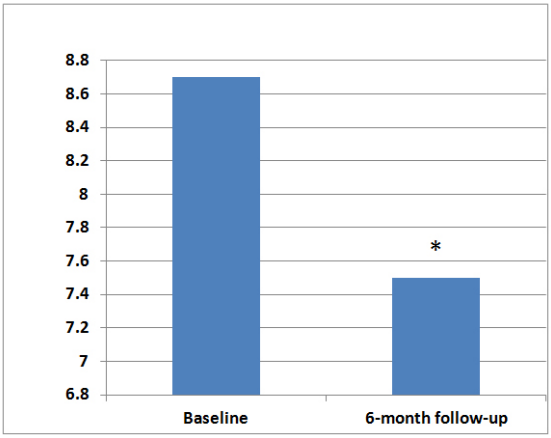
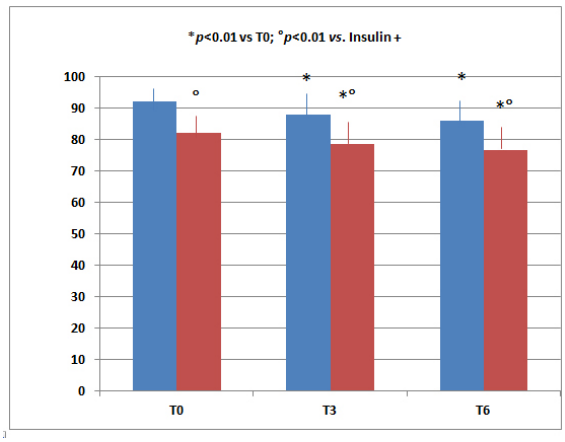
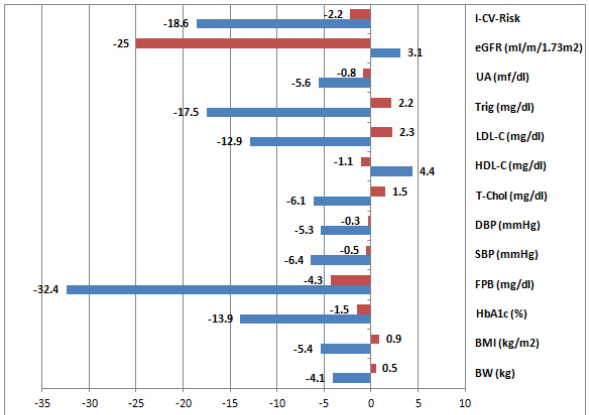


Figure 3: Body Weight Changes in Insulin Treated Patients (with or without metformin; Insulin +) as Compared to Those on Metformin only (Insulin -) within the Treatment Group (empaglifozin 10 mg/day).



Insulin+	*92*	*88*	*86*
Insulin-	*82*	*78,5*	*76,5*

Figure 4: Changes in Clinical Parameters from Baseline in Treatment Group (TG) Compared with Control Group (CG). All the differences were Statistically Significant ($p<0.01$).



	BW (kg)	BMI (kg/m2)	HbA1c (%)	FPB (mg/dl)	SBP (mmHg)	DBP (mmHg)	T-Chol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	Trig (mg/dl)	UA (mf/dl)	eGFR (ml/m/1.73m2)	I-CV-Risk
CG	0.5	0.9	-1.5	-4.3	-0.5	-0.3	1.5	-1.1	2.3	2.2	-0.8	-25	-2.2
TG	-4.1	-5.4	-13.9	-32.4	-6.4	-5.3	-6.1	4.4	-12.9	-17.5	-5.6	3.1	-18.6

I-CV-Risk=Individual CV Risk Score; UA= Uric Acid; Trig= Triglycerides; LDL-C=LDL-Cholesterol: HDL-C=HDL-Cholesterol; T-Chol=Total Cholesterol; DBP=Diastolic Blood Pressure; SBP=Systolic Blood Pressure; FPG=Fasting Plasma Glucose; BMI=Body Mass Index; BW=Body Weight.

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Figure 5: Changes in the 10-year CV Risk Score after 6-Month Treatment with Empaglifozin 10 mg, Compared with Baseline.

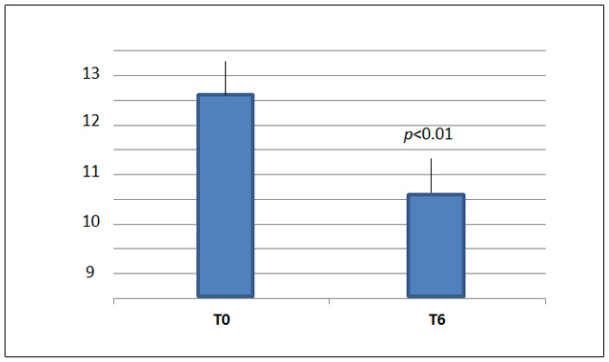


Table 3: Multivariate Cox Regression Analysis of all Parameters Contributing to the Final 10-y CV-RS (Individual 10-y Cardiovascular risk score in our Population.

	Hazard Ratio	*p*
Sex	1.085	<0.01
Age	1.090	<0.01
Diabetes	1.363	<0.001
Smoking	0.981	<0.05
Total Cholesterol	1.247	<0.001
LDL-C	1.121	<0.001
Systolic BP	1.395	<0.001
Antihypertensive Treatment	1.424	<0.001

were identified as major contributors to the individual 10-y CV-RS.

In greater detail, Figure 6 describes 10-y CV-RS changes in non-hypertensive and hypertensive TG patients. A significant difference (p<0.01) was deteted between them at baseline and at the end of follow-up, but, unlike the others, non-hypertensive subjects displayed only slight, non-significant intragroup changes.

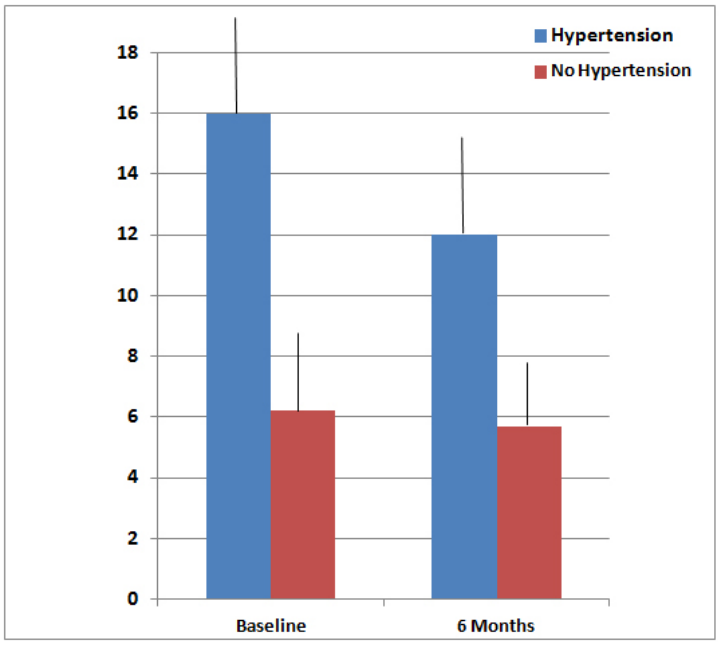
A similar result was observed when comparing insulin-treated to metformin-treated subjects (Table 4), who in fact displayed a significantly lower 10-y CV-RS than the others (p<0.01), being anyway a significant improvement observed (p<0.01) in both groups at the end of follow-up.

Safety Parameters and Side Effects

The upper part of Table 5 summarizes the results concerning safety parameters in the TG group: no significant differences were observed *versus* baseline in AST, ALS, γ-GT, total bilirubin, and blood urea; a similar behavior was observed for CG (not shown).

Unexpectedly, however, as seen in Figure 4 eGFR si-

Figure 6: Changes on 10 y CV Risk Score in T2DM People Treated with Empagnifozin when Splitted into Subjects with or without Hypertension, Respectively.



	Baseline		6 Months
Hypertension	16.0+9.0	*p*<0.01	10.0+7.8
No Hypertension	6.2+5.7	n.s.	5.7+4.6
	p<0.01		*p*<0.01

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Table 4: Comparison between the 10-y CV-RS (10 Year individual Cardiovascular Risk Score) in Empaglifozin Treated T2DM Subjects on Insulin with or without Metformin (Insulin +) or on Metformin alone (Insulin -) at Baseline (T0) and 6 Months (T6).

	10-y CV-RS		*p*
	T0	T6	
Insulin +	14.1±2.4	11.4±11.4	<0.001
Insulin -	9,7±1.9	7,8±1.8	<0.001
p	<0.01	<0.01	

Table 5: Safety Parameters and/or Adverse Effects in the Treatment Group. Data are Presented as Mean±DS or Number/Percent of Subject (%).

	Baseline	**T3**	**T6**
AST *(U/l)*	26±3	26±4	22±4
ALT *(U/l)*	24±4	25±3	24± 4
γ-GT *(U/l)*	22±8	22±9	21±6
Total Bilirubin*(mg/dl)*	0.9±0.3	0.6±0.4	0.8±0.2
Alkaline phosphatase*(U/l)*	41±6	38±8	40±5
eGFR*(ml/min/khm²)*	90.1±17.7	90.5±16.7	92.9±14.7
Urea mg/dl *(mg/dl)*	22±5	21±4	22±6
micro-macro-hematuria	0	0	0
Cylindruria	0	0	0
Ketonuria	0	0	0
Urinary tract infections	2 (1.1)	5 (2.8)	6 (3.4)
Genital infections	0	7 (3.9)	9 (5.6)
Polyuria	0	76 (42.7)	58 (32.5)
Other infectious diseases	0	0	0
Asthenia	0	0	0
Headache	0	0	0
Nausea/vomiting	0	0	0
Other gastrointestinal disorders	1 (0.6)	2 (1.1)	2 (1.1)
Allergic reactions	0	0	0
Mild hypoglycemia	1 (0.6)	2 (1.1)	2 (1.1)
Severe hypoglycemia	0	0	0

gnificantly worsened in the CG after 6 months *versus* both baseline and TG (-25% as compared to +3.1%, respectively; $p<0.01$).

In the TG no changes were observed in urine analysis except, as expected, for glycosuria. A slight non-significant increase was observed in urinary infection rate *versus* baseline (3.4 *vs.* 1.1 %), whereas genital infection rate significantly increased (+5.6% *vs.* baseline; $p<0.05$). Other possible side effects were either absent or superimposable to baseline. In greater detail, also the rate of recorded mild/moderate hypoglycemic episodes was quite low and, in any case, did not significantly change with respect to baseline at the end of follow-up. As also expected, no side effects were reported in the CG, either at baseline or at the end of follow-up.

DISCUSSION

In patients with T2DM, the main cause of morbidity and mortality is cardiovascular (CV) disease. Diabetic kidney disease, which develops in approximately 40% of patients with T2DM, further increases the risk of CV-related morbidity and mortality. The sodium SGLT2i empagliflozin, which is known to provide effective metabolic control both *per se*[29] or as an add-on to other glucose-lowering agents[29-34] in patients with T2DM, was also shown to improve CV and renal outcomes in the large, randomized, placebo-controlled EMPA-REG OUTCOME trial in patients at high risk of CV events.[13-14] The underlying mechanisms for the CV and renal protective effects of empagliflozin are not fully understood, but are likely to involve a combination of several mechanisms, including empagliflozin-associated body weight and blood pressure reduction, enhanced diuresis, modified substrate utilization and tubulo-glomerular feedback activation. The results of ongoing CV outcomes trials with other SGLT2 inhibitors will potentially confirm whether the beneficial CV and renal effects observed in EMPA-REG OUTCOME are unique to empagliflozin or reflect a drug class effect.[35]

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-141

Guidelines for cardio-vascular disease (CVD) prevention recommend the use of risk scores to identify adults at high CVD risk expected to benefit the most from preventive therapy. Several scoring systems exist to help clinicians assess the 10-year CHD risk, being the Framingham risk score the most widely used among them.[36,37]

The Framingham-Study researchers[37] also proposed a new model for use in the primary care setting, patently differentiated by gender. This "reclassification" of CVR was based on three basically sound ideas: first, it was easier to just use four parameters which had been recognized as the major risk factors (RF) in cardiovascular epidemiology, i.e. cholesterol, blood pressure (BP), diabetes mellitus and smoking; second, the model could predict all CVD events [coronary heart disease (CHD), cerebrovascular events (CVE), peripheral arterial disease (PAD) and heart failure (HF)] and provided calibration factors for each entity that may be of interest to the physician; and third, the concept of vascular or heart age was included, which could be calculated from the model.

Moreover, according to the 2009 Canadian Cholesterol Guidelines[38] all cardiovascular risk assessment calculators are defective *per se*, nevertheless the Framingham Risk Score (FRS) is recommended for total CVD anticipation. It was validated in Canada with the Cardiovascular Life Expectancy Model, and has been shown to increase adherence to therapeutic measures. Anyway, the FRS has been shown to underestimate the risk in specific categories of patients, especially the young, women, and eventually those with the metabolic syndrome.

By adding just 2 measurements (a family history of premature CAD and high hsCRP) to the Framingham model, the Reynolds Risk Score (RRS) seemed to further improve physician's CVD risk prediction ability, particularly for those people who in the past were perceived as being at moderate risk and was validated in men and women in an American population.[39-40]

There is reason to believe; however, that the FRS or the RRS, although practical, may not be applicable to southern Europe or Mediterranean populations, whose CVD prevalence is lower than observed in northern, central and eastern Europe.[41]

Within the CUORE project a risk score algorithm for assessing individual risk for cardio-vascular events was developed and validated in the Italian population.[16-19] It is a simple tool to estimate the probability of undergoing a major cardiovascular event (myocardial infarction or stroke) over the next 10 years, provided the levels of eight risk factors are known, including sex, age, diabetes, smoking habit, systolic blood pressure, total cholesterol, HDL-cholesterol and antihypertensive treatment. The score provides a more accurate assessment than risk cards elaborated in collaboration with the Italian Institute of Health (ISS) under the same CUORE Project.[42]

Our data, obtained in an outpatient setting in real-life conditions during a relatively short follow-up period is in agreement with those coming from major trials in terms off ast improvement in glycemic control and of beneficial effects on blood pressure, lipids and body weight, counter balanced by only slight genitourinary troubles. Accordingly, in a recent large multinational intervention study based on de-identified health records across six countries (US, Norway, Denmark, Sweden, Germany and the UK) all available SGLT-2 is [canagliflozin (53%), dapagliflozin (42%), and empagliflozin (5%)] were associated with lower heart failure and mortality rates than other Glucose-Lowering Drugs. The homogeneity of the results obtained across countries, despite geographic variations in the use of a specific SGLT-2i, suggests that in real-world practice the benefits previously reported with empagliflozin in the context of a randomized clinical trial may be applicable to all SGLT is in a broad population of patients with T2DM and reflect a class effect.[43]

Our goal was in fact to convincingly answer above mentioned patient questions on possible treatment-related cardiovascular complications.

Using the Cox regression analysis we weighed single factors used by the individual CV risk calculator and we could realize arterial hypertension and antihypertensive treatment to be the leading ones. In fact, as seen in Table 4, age, sex and diabetes were highly significant *per se* but, as non-modifiable factors, could not be expected to contribute to risk reduction over time. Among modifiable risk factors, smoking habits were also significant, as expected, but less than the others in our hands and total cholesterol e LDL-C, which proved to be highly significant, had a much lower HR than systolic-BP and antihypertensive treatment. We cannot provide an explanation for such observation as our investigation was not powered enough for a meaningful statistical analysis of this finding which, being unexpected, had not been included among the goals of the study. However, we may hypothesize a possible mechanism behind it to be insulin resistance, which is known to be a strong factor contributing to arterial hypertension in people with T2DM. Further *ad hoc* designed studies are warranted to validate such hypothesis.

LIMITATIONS

The main limitations of our study are the small number of enrolled subjects compared to large RCTs and the relatively short follow-up period.

However, it should be considered that cardiovascular outcomes were already found to be positively affected in the EMPAREG OUTCOME study within less than 6 months and that, by using our population-based CV-Individual Risk Score within the apparently short observation period we chose, we could anyway confirm those favorable effects in terms of single CV risk factors contributing to the final score.

CONCLUSION

In conclusion, our results confirmed empaglifozin efficacy in terms of persistent control of both glucose and virtually all other

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-141

cardiovascular risk factors (weight, BMI, blood pressure, lipids, uric acid). As a consequence of that, the 10-year Cardiovascular Risk (CRA) score significantly decreased in diabetic patients without previous major cardiovascular events. Finally, the drug was well tolerated and, when present, genitourinary tract infections mostly resolved with specific medications without any need for treatment discontinuation.

AUTHORS' CONTRIBUTIONS

All authors contributed to study conception and design, data acquisition, analysis and interpretation, manuscript preparation and critical revision.

COMPLIANCE WITH ETHICAL STANDARDS

The study was organized and supported by the AID Study Group (non-profit organization for the study of endocrine and metabolic disorders), Naples, Italy in conformance with good clinical practice (GCP) standards. Written informed consent was obtained from all participants before enrollment; the study was conducted in accordance with the Declaration of Helsinki and was approved all the Ethics Committees of the Centers participating in the study.

HUMAN AND ANIMAL RIGHTS

All followed procedures were in accordance with the ethical standards of the institutional and national responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Research

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Volume 4 : Issue 3
Article Ref. #: 1000HROJ4142

Article History
Received: July 5th, 2017
Accepted: August 28th, 2017
Published: August 29th, 2017

Citation
EL Desoky ES, Hassan AKM, Salem SY, Fadil SA, Taha AF. Cardioprotective effect of Losartan alone or in combination with remote ischemic preconditioning on the biochemical changes induced by ischemic/reperfusion injury in a mutual prospective study with a clinical and experimental animal arm. *Heart Res Open J*. 2017; 4(3): 57-65. doi: 10.17140/HROJ-4-142

Cardioprotective Effect of Losartan Alone or in Combination with Remote Ischemic Preconditioning on the Biochemical Changes Induced by Ischemic/Reperfusion Injury in a Mutual Prospective Study with a Clinical and Experimental Animal Arm

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ABSTRACT

Background and Aims: Losartan is an angiotensin receptor II antagonist used clinically to reduce blood pressure and treat hypertension or hypertrophic cardiomyopathy. It is also proposed to suppress cardiac injury following reperfusion. We evaluated the potential effect of Losartan alone and in combination with remote ischemic preconditioning (RIPC), an established treatment, on the biochemical changes induced by ischemic/reperfusion injury (I/R) in both humans and rabbits.

Methods: Thirty consecutive patients undergoing elective percutaneous coronary intervention (PCI) were divided into three groups (10 each): group 1 (control group without any preconditioning), group 2 (patients who were maintained on losartan (50-100 mg /day) for one month before PCI), group 3 (similar to group 2 but PCI was preceded by RIPC). On the other hand, sixty adult male New Zealand white rabbits were divided into 6 groups (10 each): group I (control), group II (sham), group III (I/R as 30 min ischemia followed by 120 min reperfusion), group IV (regular losartan 20 mg/kg for 40 days orally followed by I/R), group V (I/R preceded by RIPC) and group VI (similar to groups IV but I/R was preceded by RIPC). Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), nitric oxide (NO), troponin I (cTnI), creatine kinase MB (CK-MB) and C-reactive protein (CRP) were measured in blood for all study groups.

Results: Clinical and experimental parts showed that groups with RIPC combined with losartan pretreatment enhanced cardioprotective effects of RIPC against I/R injury as evidenced by significant reduction ($p<0.001$) in the levels of IL-6 and cTnI; also the level of NO was significantly ($p<0.$ 0.001) increased compared with other groups. However, this effect was not significant regarding the level of TNF-α, CK-MB nor CRP.

Conclusions: Pretreatment with losartan enhances the cardioprotective effects of RIPC in ischemic reperfusion injury.

KEY WORDS: Ischemic reperfusion; Losartan; Remote ischemic preconditioning; Biochemical markers; PCI.

ABBREVIATIONS: RIPC: Remote ischemic preconditioning; cTnI: troponin I; NO: Nitric Oxide; IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor- α; CRP: C-reactive protein; PCI: Percutaneous Coronary Intervention; CAD: Coronary Artery Disease; ROS: Reactive Oxygen Species; I/R: Ischemic Reperfusion.

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INTRODUCTION

Sudden reperfusion of the ischemic myocardium, initiates a cascade of cellular events that lead to additional cellular damage and death called ischemic reperfusion (I/R) injury.[1] Investigational studies have identified several factors that mediate the harmful effects of I/R injury such as the pH paradox**,** intracellular and mitochondrial Ca^{2+} overload**,** accumulation of reactive oxygen species **(**ROS) due to massive input of oxygen by reperfusion and down regulation of basal nitric oxide (NO) in the cell.[2-4] Percutaneous coronary intervention (PCI) has become the principal coronary revascularization strategy for stable and unstable coronary artery disease (CAD) patients.[5-7] However, (I/R) injury post PCI may represent either an acceleration of the pathological processes initiated during ischemia or new pathophysiological changes that develop after reperfusion. The injury can mediate cell death, microvascular damage, myocardial stunning, and potentially lethal arrhythmias which worsen the clinical outcomes after PCI.[8,9]

Ischemic preconditioning (IPC) concept is now well known[10] where the application of brief episodes of ischemia reperfusion before sustained ischemia creates a condition of adaptive responses of cardiac cells to various kinds of stress induced by I/R.[6] Further research in the area led to the development of remote ischemic preconditioning (RIPC) technique where the application of brief cycles of ischemia reperfusion in organs remote from the tissue of sustained ischemia can reduce the I/R injury in that ischemic tissue on reperfusion.[11] In addition, some drugs which have unique pleotropic effects presented as anti-inflammatory, immunomodulatory and anti-oxidative properties have been proposed to reduce myocardial I/R injury. Examples are statins and angiotensin II receptor blockers (ARBs).[12-14]

In our first report, we evaluated the cardioprotective effects of the statin atorvastatin.[15] This second part of our study aimed to evaluate a possible cardioprotective effect of the angiotensin II receptor blocker, Losartan, alone and in combination with RIPC on patients undergoing PCI and in experimental rabbits which were exposed to I/R injury. The serum levels of the biochemical parameters interleukin-6 (IL-6), Tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), Troponin I (cTnI), nitric oxide (NO), and Creatine kinase MB (CK-MB) were assessed as biochemical markers of cardiac injury and inflammation.

MATERIALS AND METHODS

Clinical Part

Patient recruitment: As presented in details previously,[15] we performed a prospective non-randomized parallel groups comparison at the catheterization laboratory of Assiut University Hospitals (AUH).

Patients between 18 and 80 years of age, scheduled to undergo an elective PCI and able to give an informed consent were eligible for enrollment in the study (Table 1). Elective PCI was defined as any coronary revascularization in a low-risk, haemodynamically stable patient who presents to the facility for a planned PCI or for a coronary angiogram followed by ad hoc PCI. Exclusion criteria were (1) emergency PCI; (2) diabetic patients on regular treatment (3) nicorandil or glibenclamide use; (4) peripheral vascular disease affecting upper limbs; and (5) patients with severe renal impairment or on regular dialysis and (6) patients not taking Losartan 50-100 mg for at least one month pre PCI in patients groups, (7) more than 3 hours time gap between RIPC application and the PCI procedure.

Consecutive patients undergoing elective PCI between September 2014 and April 2015, were invited to participate in the study during their attendance at a routine preadmission clinic. Thirty eligible patients, after obtaining informed consent, were systematically allocated to the study groups according to our daily based allocation technique.

Study design: Patients were divided into three groups (10 patients in each group):

Group 1: Control group which includes patients who were exposed to PCI without any preconditioning.
Group 2: Patients who were maintained on Losartan at a dose 50-100 mg/day for at least one month before undergoing PCI.
Group 3: Patients who were maintained on Losartan at a dose 50-100 mg/day for at least one month before the day of application of PCI technique. At that day, PCI was preceded by RIPC.

The study protocol was approved by the Ethical Committee of Assist Faculty of Medicine and a written informed consent was obtained from all participants. The consent form was designed with an explanation on the purpose and conduction of this research study.

Induction of remote ischemic preconditioning (RIPC): RIPC was induced by inflating a standard 9 inch blood pressure cuff to 200 mmHg on the left upper arm for five minutes (transient ischemia) for three cycles and each was separated by a reperfusion period of five minutes, during which the cuff was kept deflated.[16] The protocol was commenced on the day of PCI procedure and was timed around an hour before the intervention procedure. If the intervention was delayed more than three hours after RIPC, the patient was excluded from the study as the endothelial protection conferred by RIPC disappears after 4 hours of preconditioning stimulus.[16]

PCI was performed using femoral approach in all our patients using 6 French guiding catheters. Participants were pre-treated with aspirin 150 mg and clopidogrel 600 mg orally before the procedure, and intra procedural intravenous heparin bolus 10,000 IU. Glycoprotein IIb/IIIa inhibitors, stent implantation and all other medication was given at the discretion of the primary operator, adhering to best conventional clinical practice. Participants were managed conventionally following PCI, including a period of in-patient observation, with 150 mg/day

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-142

Table 1: Demographic and Clinical Data of Patients used Losartan Alone or in Combination with Remote Ischemic Preconditioning and Control Groups before Percutanious Coronary Intervention.

Variable	Control PCI Group 1 (N=10)	Losr. Group 2 (N=10)	Losr. +RIPC Group 3 (N=10)	*p*
Age (years)	56±10	56±8	57±8	NS
Male Gender, n (%)	6 (60%)	6 (60%)	8 (80%)	NS
Risk factors				
Smoking, n (%)	5 (50%)	7 (70%)	6 (60%)	NS
Hypertension, n (%)	4 (40%)	4 (40%)	5 (50%)	NS
Dyslipidemia, n (%)	3 (30%)	4 (40%)	4 (40%)	NS
BMI, median (IR)	27.1 (25.1-30.2)	26.3 (25-30.1)	26.2 (25 -30.1)	NS
Clinical details				
LVEF, %	59±9	60±10	60±10	NS
Time since the latest angina before PCI<24 h, n (%)	2 (20%)	2 (20%)	3 (30%)	NS
Angina CCS grade III/IV, n (%)	2 (20%)	3 (30%)	2 (20%)	NS
Hemoglobin (mg/dL)	12±2	12±3	12±3	NS
Base-line SCr, (mg/dL)	0.95±0.31	0.98±0.41	0.98±0.39	NS
Medications in last month, n (%)				
Aspirin therapy	10 (100%)	10 (100%)	10 (100%)	NS
Clopidogrel therapy	4 (40%)	6 (60%)	6 (60%)	NS
Losartan 50-100 mg	0	10 (100%)	10 (100%)	NS
Atorvastatin 80 mg	0	0	0	NS
Beta blockers	8 (80%)	9 (90%)	8 (80%)	NS

Data are presented as mean±standard deviation, number (%) of patients or median (interquartile range). BMI: Body Mass Index; CCS: Canadian Cardiology Society; CKD: Chronic Kidney Disease; CABG: Coronary Artery Bypass Graft; Losr.: Losartan; LVEF: Left Ventricular Ejection Fraction; RIPC: Remote Ischemic Preconditioning; NS: Not Significant; PCI: Percutanious Coronary Intervention; SCr: Serum Creatinine.

of aspirin and 75 mg/day of clopidogrel for 1 month in case of a bare-metal stent and for 1 year in case of a drug eluting stent as described in details in our previous report.[15]

Blood sampling and serum preparation: The blood samples were drawn from a blood vessel in the arm immediately before and 24 hr after PCI procedures. ELISA kits were used for determination of serum concentrations of IL-6 (Orgenium Laboratories, Finland), TNF-α (ASSAYPRO, USA), and Troponin-I (Monobind-inc., USA), C- reactive protein serum level was determined by latex agglutination test kit produced by Lab Care Diagnostics, India. Nitric oxide serum level was determined by kit produced by Biodiagnostic, Egypt, which is used for colorimetric determination of nitrite in serum. Creative kinas-MB serum level was determined by kit produced by Chema Diagnostic, Italy which is used for the spectrophotometric quantitative determination of CK- MB inserum or plasmas described in details in our previous report.[15]

Peri-procedural parameters: Without prior knowledge of the study allocation of the participants as described in details in our previous report.[15]

Angiographic parameters: The target vessel characteristics and the final result of PCI (predilation, postdilation, stent diameter, stent length and number) were noted. Angiographic lesion characteristics were classified. Preprocedural and postprocedural assessments of coronary blood flow (Thrombolysis in Myocardial Infarction flow score) were performed as described in details previously.[15] Other angiographic complications occurring during PCI (artery dissection, perforation, or jailed side branch with compromised flow) and contrast dose were noted.

Experimental Part

Animals: Adult male New Zealand white rabbits weighing 2000-2500 grams were purchased from the animal care unit of the Faculty of Medicine, Assiut University and cared for in compliance with the Guide for the Care and Use of Laboratory Animals published by National Institute of Health (NIH) as described in details in our previous report.[15] The experimental animal part of the study was approved by the Pharmacology Department Council of the Faculty followed by approval by the Ethics Committee of Assiut University, Assiut Governorate, Egypt.

Chemicals: Losartan was purchased as amosar tablet 100 mg from Amoun Drug Company, Egypt. The tablets were crushed and grinded by a mortar to form powder for suspensions in distilled water in concentrations 2% for Losartan which given to the rabbits orally (as mentioned below in the experimental design). Urethane 20% was used for anesthesia and it was dissolved in sterile saline before injection.

Experimental design: The rabbits were divided into 10 groups (n=10 each) as follows:

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-142

Group I: Control which took distilled water.
Group II: Sham which was exposed to all surgical procedure except the induction of ischemia.
Group III: Model of myocardial ischemia reperfusion (i.e., I/R group).
Group IV: I/R preceded by remote ischemic preconditioning (RIPC) (i.e., RIPC+ I/R).
Group V: Losartan treated; the rabbits were administered Losartan orally once daily at a dose of 20 mg/kg for 6 weeks[12,13] followed by induction of I/R (i.e., Losartan + I/R).
Groups VI: were exactly similar to groups V in exposure to Losartan and I/R respectively I/R was preceded by RIPC.

Experimental Procedure

Anaesthesia and endotracheal intubation: Was described in details in our previous report.[15]

Induction of I/R injury: Left anterior descending (LAD) coronary artery occlusion and reperfusion were performed according to the method described before.[15]

Induction of RIPC: RIPC was carried out as described before.[15]

Blood sampling and biochemical analysis: As described in our primary report.[15]

Statistical Analysis

Data were expressed as mean±standard error. Statistical analysis was performed by one way ANOVA followed by *post hoc*-Tukey's test to compare more than two groups. In case of comparison of two different groups, unpaired student *t*-test was used. All statistical analyses were performed using Graph Pad Prism (version 5.0, Graph Pad Software Inc., San Diego, California, USA) and significance was considered at $p<0.05$.

RESULTS

Clinical Part

The 3 clinical groups were comparable regarding demographic, clinical and procedural characteristics as presented in Tables 1 and 2. Table 3 showed that patients who received pretreatment with Losartan before undergoing PCI in group 2 and patients who received the drug and exposed to RIPC before doing PCI in group 3 developed significant reduction in serum levels of IL-6, TNF-α and CRP associated with significant increase in serum level of NO compared with corresponding values in patients who were exposed directly to PCI only.The synergistic effects of adding RIPC to Losartan pretreatment in group 3 was more prominent in the form of significant reduction in the values of IL-6 and cTn compared to Losartan group only. On the other

Table 2: Angiographic and Periprocedural Data of Patients used Losartan Alone or in Combination with Remote Ischemic Preconditioning and Control Groups Before Percutaneous Coronary Intervention.

Parameter	Control PCI Group 1 (N=10)	Losr. Group 2 (N=10)	Losr.+RIPC Group 3 (N=10)	*p*
Angiographic parameters				
Target vessel, n (%)				
LM	0	0	0	
LAD	5 (50%)	5 (50%)	4 (40%)	NS
LCx	2 (20%)	2 (20%)	3 (30%)	NS
RCA	3 (30%)	2 (20%)	3 (30%)	NS
Side Branch >2mm, n (%)	4 (40%)	3 (30%)	4 (40%)	NS
CTO, n (%)	1 (10%)	0	1(10%)	NS
Subtotal occlusion, n(%)	2 (20%)	3 (30%)	2 (20%)	NS
Lesion type (AHA/ACC), n (%)				NS
A	4 (40%)	6 (60%)	5 (50%)	
B	4 (37%)	3 (30%)	3 (30%)	
C	2 (20%)	1 (10%)	2 (20%)	
PCI-related parameters				
Pre dilation	4 (40%)	4 (40%)	4 (40%)	NS
Post dilation	8 (80%)	8 (80%)	9 (90%)	NS
Number of DES	4 (40%)	5 (50%)	5 (50%)	NS
Mean number of stents per case	1.9±0.5	1.9±0.7	1.8±0.7	NS
Mean stent diameter per case, mm	3.0±0.7	2.9±0.8	3.0±0.8	NS

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Mean stent length per case, mm	31±17	30±17	30±17	NS
Contrast, mL	150±90	160±80	150± 80	NS
Glycoprotein IIb/IIIa inhibitors	2 (20%)	1 (10%)	1 (10%)	NS
Clinical state during angioplasty				
SBP, mm Hg	125 ± 20	132 ± 15	133 ± 17	NS
DBP, mm Hg	78±12	76±13	76±11	NS
HR, beats per minute	70±14	75±12	75±11	NS
After stent implantation, n (%)				
TIMI flow score 3	9(90%)	10(100%)	10 (100%)	NS

Data are presented as mean±standard deviation, or number (%) of patients. CTO: Chronic Total Occlusion; DBP: Diastolic Blood Pressure; DES: Drug Eluting Stents; HR: Heart Rate; LAD: Left Anterior Descending Artery; LCx: Left Circumflex Artery; LM: Left Main Artery; Losr.: Losartan; NS: Not Significant; PCI: Percutaneous Coronary Intervention; RCA: Right Coronary Artery; RIPC: Remote Ischemic Preconditioning; SBP: Systolic Blood Pressure; TIMI: Thrombolysis in myocardial infarction.

Table 3: Effect of Losartan Alone or Preceded with Remote Ischemic Preconditioning on Serum Levels of Different Biomarkers in Patients Undergoing Percutaneous Coronary Intervention Compared with Control Group.

Parameters	PCI (Group 1)	Losartan+PCI (Group 2)	Losartan +RIPC+PCI (Group 3)	Statistical analysis			
				ANOVA#		Post hoc##	
				F	*p* value	(Tukey's test) a	b
IL-6 (pg/mL)	16.24±0.54	13.65±0.55*	8.86±0.49	51.61	0.002	0.002	0.02
TNF-α (pg/mL)	35.11±0.57	17.5±0.88*	26.17±0.51	53.86	<0.001	0.01	<0.001
CRP (mg/L)	16.80±1.96	8.40±1.83*	8.80±1.93	54.68	<0.001	<0.001	0.31
cTnI (ng/mL)	2.80±0.09	2.50±0.11	1.03±0.09	54.55	0.01	<0.001	<0.001
NO(M mol/L)	46.62±1.29	52.96±0.95*	57.07±0.61	38.00	<0.001	<0.001	<0.001
CK-MB (U/L)	75.79±3.59	73.50±1.19	72.67±1.86	34.52	0.85	0.85	0.47

Results are represented as mean±SE, n=10 for each group.
ANOVA test between the three groups followed by *posthoc* Tukey's test
group 3 *versus*: group 1 [a], group 2 [b]
* Unpaired *t*-test between group 1 *vs*. group 2 Significance at $p<0.05$

hand significant increase in NO production in the group 3 compared to group 1 and 2 ($p<0.001$) (Figure 1). However, no treatment had any effect on levels of CK-MB compared to the control group.

Experimental Part

The administration of Losartan alone for 6 weeks before induction of I/R achieved significant reduction in the serum levels of IL-6, TNF-α and CRP, associated with significant elevation in NO level (Table 4). cTnI and CK-MB however, were unchanged. These findings collectively suggest a possible biochemical cardioprotective effect of Losartan alone against I/R injury.

Table 5 illustrated that the application of RIPC before induction of I/R induced significant reduction of IL-6, TNF-α while NO serum level significantly increased in comparison with their corresponding values in I/R group. Figure 2, showed that the addition of RIPC to Losartan before induction of I/R compared to Losartan only, achieved positive addition to its effect on the serum levels of different biochemical parameters mainly IL-6, TNF-α, CRP, cTnI and NO.

DISCUSSION

The main finding of the present study with its clinical and experimental animal parts, were that Losartan in addition to RIPC have beneficial cardioprotective effect against ischemic reperfusion injury, assessed by measurement of circulating markers. This beneficial effect is defined from the biochemical point of view through reduction of some inflammatory markers associated with cardiac injury like IL-6, TNF-α and CRP associated with increase in NO level.

The changes reported in our study as regards the serum levels of different biochemical parameters in the I/R model compared with control and sham group coincide with I/R injury and previous studies reported similar results.[17,18]

In our study; Losartan pretreatment in animal model of I/Rat a dose of 20 mg/kg/day for 6 weeks before induction of I/R achieved a biochemical cardioprotective effect through reduction of I/R induced elevation of IL-6, TNF-α and CRP. Angiotensin II is described as a potent pro-inflammatory mediator causing up regulation of macrophages to induce inflam-

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Table 4: Effect of I/R Preceded by Daily Administration of Losartan 20 mg/kg for 6 Weeks on the Serum Levels of Different Biochemical Parameters in Comparison with I/R Group of Rabbits.

Parameters	I/R group	I/R preceded by losartan group	*p*-value#
IL-6 (pg/mL)	10.49±0.49	8.46±0.25 [b]	0.0026
TNF-α (pg/mL)	11.79±0.38	4.19±0.50 [b]	<0.0001
CRP (mg/L)	652.8±58.7	341.3±28.2 [b]	0.0002
cTnI (ng/mL)	1.13±0.04	1.12±0.04	0.86
NO (M mol/L)	27.59±0.60	30.59±0.66 [b]	0.004
CK-MB (U/L)	1173±53.3	1159±54.6	0.85

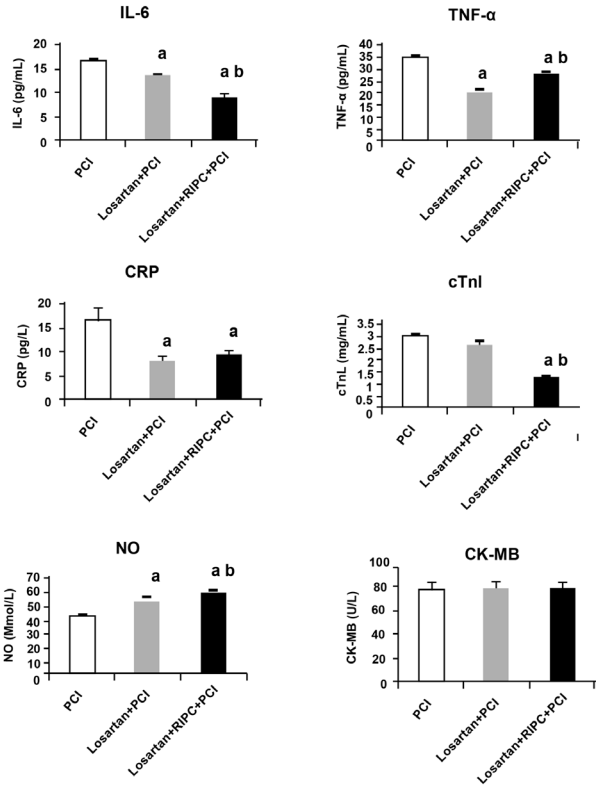
Results are represented as mean±SE, n=10 for each group.
[#] Unpaired *t*-test. Significance at $p<0.05$.[b]: *vs.* I/R group.

Table 5: Effect of I/R Preceded by 3 Cycles of RIPC on the Serum Levels of IL-6, TNF-α, CRP, cTnI, NO and CK-MB in Comparison with I/R Alone on Rabbits.

Parameters	I/R group	I/R preceded by RIPC group	*p*-value#
IL-6 (pg/mL)	10.49±0.49	8.38±0.32[b]	0.0089
TNF-α (pg/mL)	11.79±0.38	7.42±0.51[b]	<0.0001
CRP (mg/L)	652.8±58.7	704.0±64.0	0.58
cTnI (ng/mL)	1.13±0.04	1.13±0.06	0.96
NO (M mol/L)	27.59±0.60	32.54±0.87[b]	0.0003
CK-MB (U/L)	1173±53.3	1214±70.4	0.65

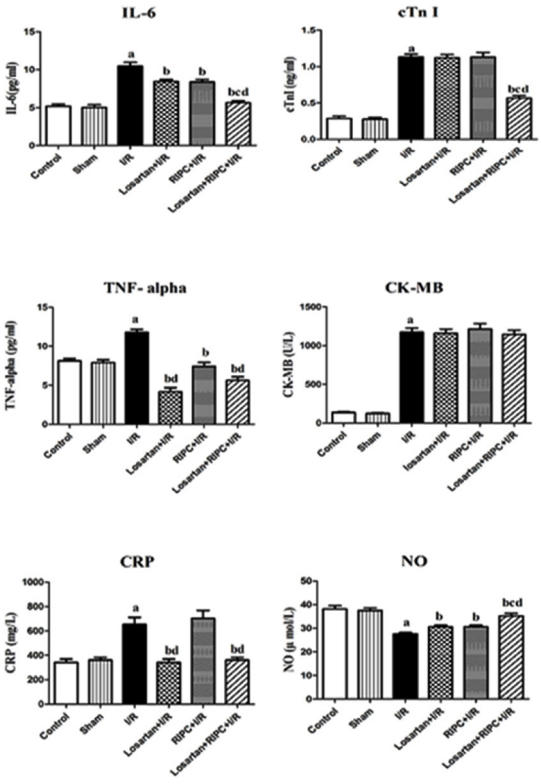
Results are represented as mean ± SE, n=10 for each group.
[#]Unpaired *t*-test. Significance at $p<0.05$.[b]: *vs.* I/R group.

Figure 1: The Effects of Pre-Treatment with Losartan (50-100 mg/day for at Least One Month) only and Plus Remote Ischemic Pre-Conditioning (RIPC) on Serum Levels of Different Biochemical Parameters in Patients undergoing Percutaneous Coronary Intervention (PCI).



IL-6: Interleukin 6, TNF-alpha: Tumor Necrosis Factor-alpha, CRP: C-Reactive Protein, cTnI: Cardiac troponin-I, NO: Nitric Oxide, CK-MB: Creatine kinase-MB. Results are represented as mean±SE, n=10 for each group. ANOVA test between all groups followed by post hocTukey's test. [a]=$p<0.05$ *vs.* PCI, [b]=$p<0.05$ *vs.* Losartan +PCI.

Figure 2: Influence of Remote Ischemic Pre-Conditioning (RIPC) Plus Pre-Treatment with Losartan on Biochemical Parameters in Ischemic/ Reperfusion (I /R) Animal Model.



IL-6: Interleukin 6, TNF-alpha: Tumor Necrosis Factor-alpha, CRP: C-Reactive Protein, cTnI: cardiac troponin-I, NO: Nitric Oxide, CK-MB: Creatine kinase-MB. Results are represented as mean±SE, ANOVA test between all groups: $p<0.05$, followed by post-hocTukey's test.
[a]=$p<0.05$ *vs.* control, [b]=$p<0.05$ *vs.* I/R,[c]=$p<0.05$ *vs.* Losartan+I/R, [d]=$p<0.05$ *vs.* RIPC+I/R

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-142

matory cytokines during various pathological cardiovascular insults.[19] Furthermore, angiotensin II has been found to have a dual effect on macrophages through activation of the mitogen-activated protein kinase (MAPK) pathway and up-regulation of early growth response-1 (Egr-1) gene expression which both master the switch for vascular inflammatory responses.[20,21] So, by blocking Ang II signaling, Losartan reduces the inflammatory response as presented in different studies concerned with heart failure treatment outcomes[22-24] Losartan induced elevation of NO level in the animal model could reflect the ability of this drug to improve endothelial functions. Losartan by inhibiting angiotensin II receptor may decrease the release of ROS, keeping NO which improves the endothelial function.[25]

The elevated CK-MB and cTnI levels in I/R model reflected ischemic injury. Losartan pretreatment in our model did not show any change in either CK-MB or cTnI following I/R. This finding coincides with the findings of a previous study on rat model of I/R, losartan at a dose of 2 mg/kg administered intravenously 10 minutes before induction of ischemia did not affect either cTnI or CK-MB. On the contrary, lowering of CK-MB levels was reported in the effluent liquid of an isolated heart model linked to langend off reperfused apparatus where the heart was pretreated with valsartan before induction of I/R.[26] The discrepancy in losartan effect on CK-MB levels between the two animal models may be ascribed to the difference in study design where our model was *in vivo,* while the other study[26] was *in vitro*. *In vitro* model of I/R allowed direct perfusion of the ang II blocker (valsartan) to the heart while in our study losartan was administered orally, so *in vitro* model may achieve better availability of the drug to the heart than *in vivo* model.

The change in cTnI serum level may require longer duration of reperfusion to be affected by these drugs at a measurable level and in our experiment the reperfusion period was limited by the available conditions for survival of animals; however, in the clinical part the level of cTnI level was significantly reduced in the pretreated group as it was measured 24 hr post PCI which is the peak elevation of troponin post I/R.[27] However, the synergistic effects of Losartan with RIPC compared to Losartan alone were able to reduced cTnI levels.

In this part of the study, the application of RIPC before induction of I/R showed possible beneficial biochemical effect against cardiac injury either in the experimental or clinical parts also as previously reported in our 1st report.[15] It has been reported that application of RIPC before myocardial I/R suppresses the pro-inflammatory gene expression in circulating leukocytes leading to inhibition of cytokine synthesis, leukocyte chemotaxis, adhesion and migration, exocytosis, innate immunity signaling pathways, and apoptosis.[28] Onthe other hand, RIPC induces NOS expression leading to increase in NO levels with subsequent reoxygenation in post ischemic myocardium during reperfusion.[29-30]

It has been reported before that infants undergoing repair of simple congenital heart defects through open heart surgery-related cardiopulmonary bypass developed lowered serum concentrations of IL-6, IL-8, IL-10 and TNF-α when RIPC was induced pre-operatively *via* three cycles of 5 minutes of ischemia followed by 5 minutes reperfusion on the left upper arm using a blood pressure cuff compared with control group who were exposed to that surgery without preceding RIPC.[31] Furthermore, a metaanalysis of randomized clinical trials to establish the cardioprotective effect of RIPC in patient on patients with myocardial infarction who were exposed to RIPC before PCI showed significant reduction in cardiac troponin level but no change in CRP.[32] Also the use of RIPC<2 h before PCI, reduces the incidence of PCI-related MI and improves ischemic symptoms in patients undergoing elective PCI in our previous report on 200 patients undergoing PCI.[33]

In the current study, the additive cardioprotective biochemical effects of RIPC combined with Losartanin the animal model of I/R and Losartanin the clinical part encourages a suggestion to use the RIPC plus pharmacological preconditioning (Losartan in this study) for cardiac patients before doing PCI at least to guard against periprocedure cardiac injury.

CONCLUSIONS

The present study with its clinical and experimental animal parts revealed that pretreatment with Losartan combined with RIPC exert cardioprotective effects in both humans and animal models, reducing both inflammatory responses and myocardial damage.

ACKNOWLEDGMENTS

We received 10,000 pounds as a research support from Assiut University Faculty of Medicine for this research. Especially thanks to Dr. Ghobrial Abdalla Mekhaeel, Assistant Lecturer of Anatomy Department, for helping anatomical exploration of coronary and femoral arteries in the experimental part of the study.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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ISSN 2377-164X Open Journal http://dx.doi.org/10.17140/HROJ-4-143

Mini Review

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Volume 4 : Issue 3
Article Ref. #: 1000HROJ4143

Article History
Received: September 27th, 2017
Accepted: October 3rd, 2017
Published: October 4th, 2017

Citation
Sun Z. Three-dimensional printing in cardiovascular disease: Verification of diagnostic accuracy of 3D printed models. *Heart Res Open J*. 2017; 4(3): 66-70. doi: 10.17140/HROJ-4-143

Three-Dimensional Printing in Cardiovascular Disease: Verification of Diagnostic Accuracy of 3D Printed Models

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ABSTRACT

This article discusses the diagnostic accuracy of patient-specific 3D printed models in delineating cardiovascular anatomy and disease, with a focus on a recent paper published in the *American Journal of Roentgenology* about the accuracy of 3D printed hollow models of visceral aneurysms. Three aspects will be discussed in this review: first, 3D printed physical models are accurate in assessing the sizes and shapes of visceral aneurysms and related arteries; second, a more reliable method was implemented in this study for measurement of the diagnostic accuracy of 3D printed model to further validate the precision of 3D printing technique; and finally, 3D printed models serve as a reliable tool for replicating anatomical structures and pre-surgical simulation of endovascular treatment of aneurysmal disease.

KEY WORDS: Accuracy; Aneurysm; Cardiovascular disease; Model; Simulation three-dimensional printing.

ABBREVIATIONS: 3D: Three-dimensional; CT: Computed Tomography; MRI: Magnetic Resonance Imaging.

Three-dimensional (3D) printing is a rapidly developed technique showing great promise in medicine with increased applications reported in the cardiovascular disease.[1-12] 3D printed physical models based on computed tomography (CT) or magnetic resonance imaging (MRI) imaging data show high accuracy in replicating complex anatomic structures of cardiovascular system, ranging from delineation of anatomical details of cardiovascular system to detection of pathologies.[1-9] Furthermore, 3D printed realistic models have been shown to play an important role in pre-surgical planning and simulation of complex cardiovascular disease, in both adult and pediatric patients through clear illustration of cardiac pathologies and facilitation of the simulation.[13-17]

Before 3D printed models are recommended for routine clinical applications, it is important to ensure the accuracy of 3D printed models. This is especially important for dealing with cardiovascular disease due to the complexity of cardiovascular anatomy and a variety of cardiovascular diseases, which requires high precision of 3D printed models. Accuracy of patient-specific 3D printed models has been reported in the maxillofacial surgery using anatomic landmarks.[18-20] Similarly, good to excellent agreement has been reached between 3D printed models and original source 2D images for dimensional measurements of aortic valve, aortopulmonary artery, and aortic aneurysms.[21-25] However, in a recent study, Ho et al have indicated that the variances in aortic diameter measurements between 3D printed models and 2D contrast-enhanced CT images exceeded 1.0 mm, which is beyond the standard deviation of 1.0 mm.[26] This highlights the potential limitations of using anatomic landmarks to measure accuracy of 3D printed hollow models, such as heart models or aneurysmal models. By taking into account both sizes and shapes of the visceral aneurysms, the accuracy of 3D printed hollow models has been validated to further confirm the reliability of 3D printing technology.[27]

In their recent study, Shibata et al retrospectively analyzed 11 patients having a total

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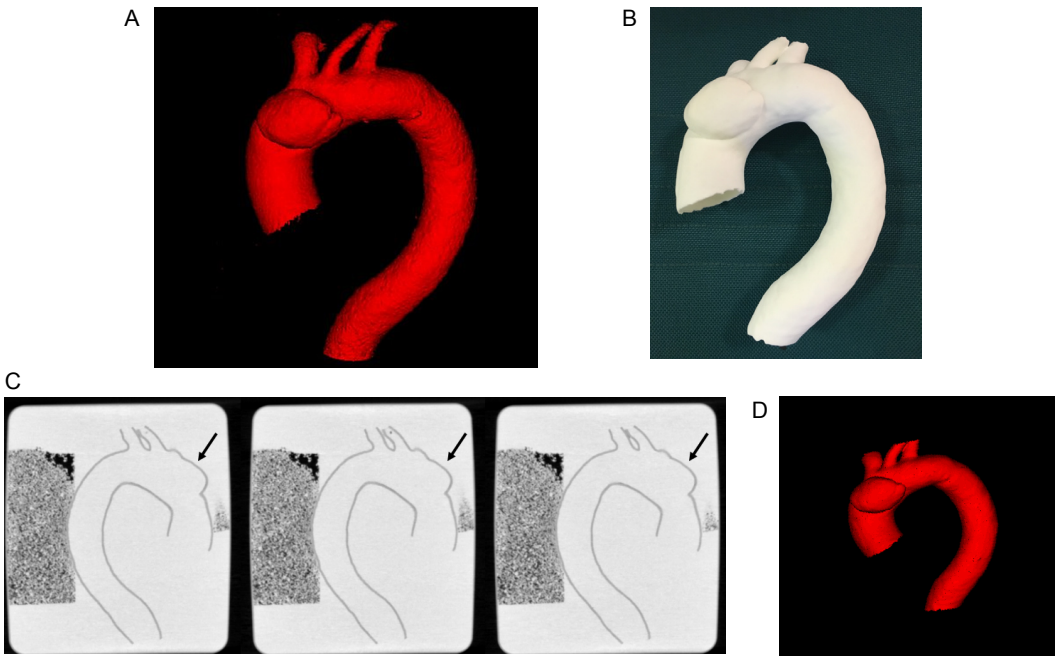
ISSN 2377-164X
http://dx.doi.org/10.17140/HROJ-4-143

of 15 true visceral aneurysms consisting of splenic aneurysms (n=8), gastric aneurysms (n=2), hepatic aneurysms (n=2), epigastric, gastroduodenal and posterior superior pancreaticoduodenal aneurysms (1 for each of these arterial aneurysms).[27] 3D printed models were created from contrast-enhanced CT angiographic images which were acquired on a 320-slice CT scanner. These hollow models of visceral aneurysms were manufactured with nylon material with a layer thickness of 0.2 mm using a fused deposition melting 3D printer. The 3D printed models were scanned on a 320-slice CT scanner with slice thickness of 0.5 mm, reconstruction interval of 0.25 mm, and pixel size of 0.234×0.234 mm. The sizes and shapes of segmented aneurysms and related arteries in terms of diameters at x, y and z-axis dimensions as well as volume of the aneurysms from both patient's CT and 3D printed model images were analyzed and compared using the Dice coefficient index which allows for more accurate assessment of diagnostic accuracy. Their results showed no significant difference in aneurysm volumes between measurements from CT angiography (mean, 5168±5808 mm^3, range 138 to 18,691 mm^3) and those from 3D printed model data (5271±6279 mm^3, range 149 to 21,570 mm^3) (p=0.56). The percentage differences in volume measurement between patient's CT data and 3D printed model data ranged from 0.05 to 15.4%. A high-level of accuracy has been shown with all of the 3D printed models with the Dice coefficient index between 84.2% and 95.8%.

There are three observations that bear discussion from Shibata and colleagues' study. First, 3D printed models are highly accurate in replicating complex vascular anatomy and aneurysm when compared to the original CT angiographic images. Previous studies have reported the accuracy of 3D printed models of cardiovascular disease with good to excellent correlation between 3D printed models and original CT or MRI data.[21-25] However, these studies are limited in assessment of diameter changes in the selected anatomical regions, which failed to consider the whole shapes of vascular structures or aneurysms. Further, according to a recent systematic review, majority of the current studies compared 3D printed models with original source CT or MRI data,[12,21-24] while only a few of them compared images of the scanned 3D printed models with original source imaging data (Figure 1).[25,26] Discrepancy in measuring these dimensional diameters was shown in one of the studies with the maximal difference in vessel diameter being 3.2 mm, highlighting the limited accuracy of using diameter measurements.[26]

This limitation has been addressed by using a more accurate method to determine the accuracy of 3D printed models, which is the second observation from Shibata et al study. Authors used the Dice coefficient index, a reproducibility validation metric which is represented by the spatial overlap index. The Dice coefficient index is a reliable method measuring the anatomic accuracy of the models, with value 0 indicating no overlap and 1 showing identical and complete overlap between segmented geometries (Figure 2).[27-29]

Figure 1: Aortic Aneurysm in the Ascending Aorta. A: 3D Reconstruction CT Image Showing an Aneurysm in the Ascending Aaorta just Inferior to the Innominate Artery. B: 3D Printed Model Bbased on Segmentation of CT AngioGraphic Data with use of White Strong and Flexible Material. C: Coronal Reformatted Images Showing the CT Images of the 3D Printed Model Filled with Contrast Medium with Clear Delineation of the Aneurysm (Arrows). D: 3D Reconstruction CT Image of the Model Showing the Aneurysm with the same Dimension to that from the Original CT Angiographic Images.



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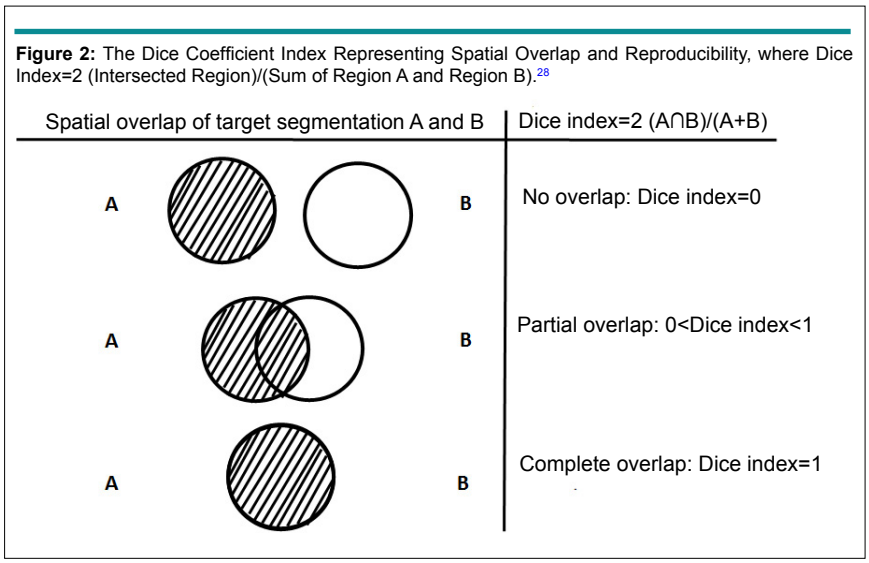

Figure 2: The Dice Coefficient Index Representing Spatial Overlap and Reproducibility, where Dice Index=2 (Intersected Region)/(Sum of Region A and Region B).[28]

This method has been used to validate the segmentation accuracy in white matter lesions and the peripheral zone of the prostate gland.[28,30,31] Shibata and colleagues adopted the Dice coefficient index in their study to perform statistical shape analysis of the aneurysms and related arteries, with findings further validating the high accuracy of 3D printed models. The similar approach was used by Frolich et al who analyzed shapes of cerebral aneurysms with a high-level of accuracy in aneurysm volume measurements between 3D printed models and 3D rotational angiographic data.[29] The Table 1 summarizes the study characteristics of comparing 3D printed models and original patient's imaging data based on these two studies which used the Dice index approach to determine accuracy of 3D printed models.

The third observation lies in the fact that high accuracy of 3D printed models of complex aneurysms enables it to serve as a useful tool for pre-operative planning and simulation of cardiovascular disease. With 3D printed models integrated into pre-operative planning, the efficiency of surgical interventions will be increased by decreasing operating/or interventional procedural time, reducing radiation exposure to patients during these procedures, thus improving patient outcome with reduced complications. 3D printing has been considered a valuable tool in pre-surgical planning and simulation of cerebral aneurysms according to both quantitative and qualitative assessments.[32] Further, 3D printed hollow models of cerebral aneurysms help the design of catheter device prior to the interventional procedure, therefore, contributing to development of a patient-specific treatment plan through identifying optimal micro catheter shape for coiling an aneurysm.[33] Due to difficulty in fully understanding complex cardiovascular anatomy, radiologists or cardiologists can improve their knowledge or operating skills by performing simulation of treatment procedures on the 3D printed models. Thus, a high-level of accuracy of 3D printed model is essential to achieve this goal by precisely demonstrating the anatomical details and pathological changes. This is confirmed by Shibata and Frolich's studies, although further research is needed to employ their method in the diagnostic assessment of accuracy of 3D printed models with different cardiovascular diseases.

In summary, patient-specific 3D printed models can accurately replicate cardiovascular disease, with diagnostic accuracy further enhanced with use of a more reliable method, the Dice coefficient index. This is of paramount importance for assessment of complex aneurysmal diseases due to presence of different vascular sizes and shapes. High accuracy of 3D printed models comprises an essential component for interventional radiology (ELIMINATE radiological procedures) because of widespread use of less invasive procedure, endovascular treatment in the management of the aneurysms.[34] The 3D printed models serve as a valuable tool for pre-operative planning and simulation of endovascular procedures through accurately and precisely replicating anatomical structures. Further studies with inclusion of large cohort of patients are necessary to validate these findings.

Table 1: Study Characteristics of Using Dice Coefficient Index for Assessment of 3D Printed Model Accuracy.

Studies	No. of aneurysms	Imaging technique for 3D printing	Patient aneurysm volume (mm^3) (mean±SD)	Model aneurysm volume (mm^3) (mean±SD)	Dice coefficient index (%) (mean±SD)
Shibata et al[27]	15 visceral aneurysms	CT angiography	5168±5808	5271±6279	91.1±4.1
Frolich et al[29]	10 intracranial aneurysms	3D rotational angiography	570.1±976.9	554.1±949.1	93.6±2.4

3D: Three-dimensional; SD: Standard Deviation; CT: Computed Tomography.

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-143

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ISSN 2377-164X Open Journal http://dx.doi.org/10.17140/HROJ-4-144

Mini Review

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Volume 4 : Issue 3
Article Ref. #: 1000HROJ4144

Article History
Received: September 24th, 2017
Accepted: October 5th, 2017
Published: October 5th, 2017

Citation
Suzuki G, Young RF, Suzuki H. Where are cell-based therapies heading? Current limitations and future directions. *Heart Res Open J*. 2017; 4(3): 71-77. doi: 10.17140/HROJ-4-144

Where are Cell-Based Therapies Heading? Current Limitations and Future Directions

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ABSTRACT

Congestive heart failure (CHF) secondary to chronic coronary artery disease is a major cause of morbidity and mortality worldwide. Its prevalence is increasing despite advances in medical and device therapies. Adult stem cell therapies have emerged as a promising treatment generating new cardiomyocytes and vessels, and are anticipated to reverse functional deterioration in patients with congestive heart failure for whom heart transplantation is the only cure. This field was enthusiastically studied in last two decades, revealing that the major beneficial outcomes from cell therapy are associated with paracrine effects rather than direct differentiation. Accordingly, paracrine factors (e.g., growth factors, cytokines and microRNAs) secreted from stem cells reduce scar volume and myocyte apoptosis, increase myocyte proliferation and activate endogenous cardiac stem cells to produce new myocytes. Moreover, functional efficacy of progenitor cells isolated from the bone marrow, adipose tissue and the heart have shown promising effects in preclinical animal models. These convincing results led to the initiation of clinical trials using autologous and allogeneic stem cells, and progenitor cells. Although clinical trials demonstrated their safety in humans, therapeutic efficacy is still inconclusive. This review discusses the progress and limitations of cell-based therapies and alternative solutions for future advancement.

KEY WORDS: Congestive heart failure (CHF); Myocardial Infarction (MI); Adult stem cells; Mesenchymal stem cells (MSCs); Cardiac stem cells (CSCs); Cardiosphere-derived cells (CDCs).

INTRODUCTION

Congestive heart failure (CHF) secondary to chronic coronary artery disease is a major cause of morbidity and mortality worldwide.[1] Its prevalence is increasing despite advances in medical and device therapies. Currently available medical interventions attenuate neurohormonal activation (e.g., renin-angiotensin-aldosterone system, sympathetic nervous system, and arginine vasopressin), reducing myocyte apoptotic cell death and interstitial connective tissue proliferation, and attenuating the progression of myocyte cellular hypertrophy. However, none of the current therapies are effective in reversing myocyte loss and cellular abnormalities associated with poor myocyte contractile performance which are impaired in the failing heart. Therefore, cardiac transplantation has been the only available cure for people who develop advanced heart failure.

Cell based therapy emerged as an alternative new therapy to restore impaired cardiac function.[2,3] Over the past two decades, cell-based studies have been studied enthusiastically. Experimental studies demonstrated promising effects on generating new cardiomyocytes and vessels, reversing functional deterioration and preventing the progression to CHF.[4] Furthermore, *in vivo* experiments revealed that the major beneficial outcomes from cell therapy are associated with paracrine effects, rather than direct regeneration of new tissue.[5,6] Stem cells secrete paracrine factors (e.g., growth factors, cytokines and microRNAs), which reduce scar volume and myocyte apoptosis, increase myocyte proliferation and activate endogenous car-

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-144

diac stem cells to produce new myocytes.[7-11] Convincing data from preclinical animal models led to several clinical trials using autologous and allogeneic stem cells and progenitor cells to assess their safety in humans.[12-14] However, their clinical relevance is still inconclusive.[9,13] Accordingly, the therapeutic benefits of the majority of clinical studies are modest at most.[14] The discrepancies between the animal studies and multiple clinical studies require reassessment of current strategies of cell-therapies. In the following sections we discuss current therapeutic limitations and alternative solutions (also summarized in Table 1) assessed in the experimental and clinical fields.

CURRENT LIMITATIONS AND ALTERNATIVE SOLUTIONS

Low Cell Retention Associated with Cell Delivery Approach

For now, three major cell delivery approaches have been tried in clinical applications (intravenous, intramyocardial (epicardial or subendocardial) and intracoronary injections). For intravenous injection, cells were injected systemically but only 0.04% of cells reached the heart and the majority of cells were entrapped in other organs (i.e., lung, kidney, liver and spleen).[15] Intramyocardial and intracoronary injection approaches have relatively better outcomes on cell retention. However, within minutes of intramyocardial or intracoronary stem cell injection the majority of cells (~85% of cells) are washed out through the coronary venous system or mechanically ejected from the injection site and only 1-2% of the cells are retained in the heart 1-month post-injection.[16-18] Therefore, to maximize their regenerative effects on the myocardium several approaches are being considered to enhance cell viability, improve functional properties of individual stem cells, and to prolong cell retention.

Enhancement of Cell Viability and Functional Properties with Genetic Modification

Genetic modification has been mostly assessed using bone marrow derived mesenchymal stem cells (MSCs). Since MSCs lack the expression of major histocompatibility complex (MHC) class II antigen, allogeneic MSCs can escape direct recognition of helper T-cells and are deemed immunoprivileged. The safety and efficacy of MSCs has been demonstrated by clinical work and there is increasing interest in enhancing the benefits of MSC therapy. For example, combining MSC and pharmacotherapy,[19] genetically modifying MSCs[20-22] and pre-conditioning MSCs[23,24] are approaches that are being explored to augment MSC-mediated cardiac repair. MSCs transfected to overexpress Akt or cell survival protein promote myocardial protective function.[6,21] Furthermore, MSCs engineered to express combinations of gene products such as Akt and angiopoietin-1 (Ang1) have also shown functional benefits in experimental animal models.[25] MSCs overexpressing vascular endothelial growth factor (VEGF) and Stromal cell-derived factor-1 (SDF-1) improve cardiac function by activating the Akt pathway.[20] MSCs transfected to express heme-oxygenase 1 (HO-1), an enzyme that improves MSC tolerance to hypoxia, infused into a cardiac ischemia-reperfusion model improve EF and lower end systolic volume compared to controls.[26] Although, these preconditioned MSCs improve engraftment and survival of transplanted cells, due to safety concerns of genetic modification on stem cell nucleus, clinical application is unwarranted.

Table 1: Alternative Approaches to Overcome Current Limitation of Cell-based Therapy.

Enhancement of cell survival, Mobilization and paracrine secretion
- Pharmacology (Statins, etc.)
- Genetic modification (Akt and Ang1, VEGF and SDF-1, HO-1)
- Non-genetic modification (Hypoxia, bFGF/IGF-1/BMP2, poly(I:C), microRNAs)
Enhancement of cell retention/survival
- Biomaterials (hyaluronic acid, collagen, fibrin, ECM, peptide, polymer)
- Cell patch (Cell sheet, scaffold or scaffold-free bioprinting)
Synergistic or accumulating effects of cell
- Synergistic effects: MSCs and CSCs
- Accumulating effects: Repeated cell infusion (MSCs, CSCs, CDCs)

MSCs: Mesenchymal Sstem Cells; CSCs: Cardiac Stem Cells; CDCs: Cardiosphere-derived cells; Ang1: Angiopoieten 1; VEGF: Vascular endothelial growth factor; SDF: Stromal cell-derived factor; HO-1: Hemeoxygenase-1; bFGF: Basic fibroblast growth factor; IGF: Insulin-like growth factor; BMP2: Bone Morphogenetic protein 2; Polyinosinic polycytidylic acid; ECM: Extracellular matrix.

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ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-144

Enhancement of Cell Viability and Functional Properties without Genetic Modification

Because genetically engineered stem cells may have unwanted long-term side-effects, pre-treatment of stem cells without genetic modification are considered more practical and relevant approaches. One method includes hypoxia preconditioning. Since cells are exposed to a harsh hypoxic environment after injection into the ischemic area, preconditioning of cells in a hypoxic chamber (1-3% O_2) prior to transplantation is reasonable and, in fact, can improve cell survival in this environmental stress scenario.[27] Hypoxia stimulation activates pro-survival pathways *via* phosphorylation of Akt and p38 resulting in HIF-1alpha activation.[23,28] Hypoxic preconditioning of MSCs[20] or cardiac stem cells (CSCs)[29] enhanced therapeutic effects in an ischemia model.

Another approach is pretreatment with growth factors. MSCs pretreated with Basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-1 and bone morphogenetic protein 2 (BMP2) improved myocardial repair in a rat model of myocardial infarction (MI).[30] Behfar and Terzic et al pretreated MSCs with growth factors to enhance their cardioprotective functions. They demonstrated the ability of a "cardiogenic cocktail" (consisting of TGFβ1, BMP-4, Activing-A, retinoic acid, IGF-1, FGF-2, α-thrombin and IL-6) to enhance the therapeutic benefits of autologous MSCs. Subsequently the same group initiated clinical trials in patients with class 2 or 3 heart failure (C-CURE) trial.[31] Also, the Safety and Efficacy of Autologous Cardiopoietic Cells for Treatment of Ischemic Heart Failure (CHART-1) trial is currently ongoing.

We demonstrated that stimulation of Toll-Like Receptor 3 (TLR3) produced many trophic factors without induction of inflammatory-related cytokines.[26] Poly (I:C) is structurally similar to double-stranded RNA and is known to interact with TLR3, which is expressed on the membrane of B-cells, macrophages, dendritic cells, bone marrow and heart-derived stem cells (MSCs and CDCs). Poly (I:C) directly reacts with the TLR3 receptor on the endosome of MSCs/CDCs. After stimulation with poly (I:C) MSCs/CDCs are collected and washed and since the poly (I:C) does not reside within the cells, it does not affect the heart environment after injection of cells. Interaction of poly (I:C) with TLR3 on MSCs causes secretion of the growth factor VEGF and the cytokine IL-6 without upregulation of the inflammatory cytokines IL-1 and tumour necrosis factor-α (TNFα). Injection of TLR3 activated MSCs (TLR3-MSCs) in a non-ischemic cardiomyopathy model improved cardiac function more than standard MSCs along with increased myocyte proliferation, reduced fibrosis and reduced myocyte apoptosis.[32] Also activation of TLR3 on Cardiosphere-derived cells (CDCs) (TLR3-CDCs) stimulated the secretion of HGF, IGF1 and IL-6 without up-regulation of inflammatory cytokines.[33] Thus, TLR3-MSCs or TLR3-CDCs are safe and feasible to use in the human heart. Further investigation is necessary to confirm long-term safety and feasibility in a preclinical animal model. Transient genetic modulation of cellular therapies may minimize unwanted side effects in the heart environment and can be considered clinically relevant approaches.

Recently, it was reported that exosomes secreted from stem cells play important roles for cardiac regeneration.[8,11,30] Exosomes transfer microRNAs from cell-to-cell and inhibit inflammation[8] (miR146a, miR155) and apoptosis[34] (miR21, miR22, miR24), and increase angiogenesis (miR210) and myocyte proliferation[35] (miR1, miR133a, miR294). Therefore, new methods of treatment are focusing on modulating microRNAs in stem cells. For example, Hu et al showed that a cocktail of three miRNAs (miR21, miR24, and miR22) was able to substantially improve the engraftment of CSCs by targeting apoptotic activators.[34] Similarly, in MSCs, miR133 was shown to act as a pro-survival agent to mitigate ischemic insult on the cells and improve subsequent engraftment.[35]

Enhancement of Cell Retention by Bio-Injectable Materials and Cell Patch

The use of biomaterials is an effective approach to enhance cell retention and survival after implantation into damaged myocardium. Biomaterials physically support the cells to improve retention directly after administration and create a protective environment for the survival of the cell. Mainly bio-injectable materials are injected into myocardium or cell patch is placed on the surface of myocardium.

Recently, injectable biomaterials combined with cell-based therapies for cardiovascular disease are gaining more attention because they have shown therapeutic potential in preclinical models for MI. Natural (e.g., hyaluronic acid,[36] collagen[37,38], fibrin[39-41] or extracellular matrix-based[42]) or synthetic (e.g., peptide[43] or polymer-based[44]) materials can enhance stem cell survival and retention *in vivo*, prolong growth factor release from hydrogel or particle constructs and stimulate endogenous cardiac regeneration. Although, there is promising preclinical data, the therapeutic potential of biomaterial-based products for cardiovascular disease has yet to be proven in a clinical setting.

Cell Patch technology generates a tissue-like structure *in vitro* and transplants it, typically onto epicardial myocardium. The main advantage of this approach is that the cells are cultivated under precise culture conditions. Therefore, cell proliferation, differentiation, and tissue structure can be well-controlled. Nevertheless, there are several limitations of this approach *in vivo*. 1) This procedure is more invasive than catheter-based approaches since open chest surgery is required. 2) Poor nutrient diffusion and vascularization immediately after transplantation usually limits the thickness of the constructs and long-term cell survival in the heart.[16] 3) Patch-based transplantation provides inadequate integration of the graft with the host myocardium. Although paracrine factors secreted from cell patches can eas-

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-144

ily cross the barrier and be effective, poor vascularization and improper coupling of cultured cardiomyocytes with the native myocytes may limit remuscularization and therapeutic efficacy. Recently, 3D bioprinting technology has been introduced in the cardiovascular field.[45] This uses 3D structured cell spheroids rather than a monolayer of cells since 3D structure is known to enhance hypoxia resistance and encourage vascularization.[46,47] The application of this technology may overcome current limitations associated with patch-based therapy.

Combination of Mesenchymal Stem Cells and Cardiac Stem Cells

Another approach is combined MSC and CSC to enhance the therapeutic effects of each cell type. Recent work by Williams et al demonstrated that the combined use of 1 million human CSCs with 200 million human MSCs provided greater recovery, almost to baseline, in a swine model of anterior wall MI.[48] While all stem cell treated animals demonstrated improved left ventricular ejection fraction compared to placebo controls, notably, animals receiving dual cell therapy had a 2-fold greater reduction in scar size (21.1% for CSC/MSC *versus* 10.4% for CSC alone or 9.9% for MSC alone) and had improved rates of pressure change during diastole. Overall left ventricular chamber dynamics were improved in both the dual therapy and CSC or MSC alone treated groups. Interestingly, CSC alone treated animals demonstrated better isovolumic relaxation as compared to controls, while MSC alone treated animals exhibited improved diastolic compliance, indicating that the enhanced effect of dual therapy on both systolic and diastolic function may be due to a synergistic effect between CSC and MSC targeted mechanisms. A current clinical trial has been initiated to assess the therapeutic effects (CONCERT-HF: ClinicalTrials.gov #NCT02501811).

Repeated Stem Cell Injection

Allogeneic MSCs and CDCs are immunoprivileged and can escape from direct recognition of helper T-cells due to the lack of expression of MHC class II antigen.[49,50] Based on these observations, a recent clinical trial was initiated using allogeneic human MSC/CDC treatment in patients with chronic myocardial infarction (POSEIDON[51], ALLSTAR[59]). Since a single injection of MSCs or CDCs has moderate influence on cardiac function and reduced scar volume[52,53], it was thought that repeated injections of stem cells would be more effective in regenerating myocardial tissue.[54,55] However, the initial infusion of cells activates and enhances the immune response[49,50] and subsequent injected cells are quickly eliminated and ineffective. This quick reaction is mainly associated with acquired/adaptive rather than innate immunity. Thus, development of efficacious MSC/CDC platforms administered with optimal immune suppression could circumvent barriers related to multiple injections of stem cells and allow the widespread application of "off-the-shelf" cell therapy to treat the large number of patients in need.[50,56,57] Gene-editing technology could also be applied to minimize acquired immunity creating immune-tolerant MSCs or CDCs.

CONCLUSION

Promising data derived from experimental models indicate the potential success of using cell based therapy in clinical applications. However, early stage clinical trials are revealing therapeutic limitations. We need to reassess the current problems and find alternative solutions. Feedback from clinical outcomes is providing more information for the development of the second stage of cell-based therapy research. In light of their proven safety profiles, adult stem cells (i.e., bone marrow mononuclear cells, adipose-derived stem cells, MSCs, CDCs and CSCs) are prime candidates for cell based therapies. Genetic modification, preconditioning, biomaterials, bioengineering, combination of cells and repeated injection approaches will further improve the efficacy of stem cell therapy. Taken together, the current understanding of stem cell based therapy and the emerging approaches and discoveries will definitely advance cell-based therapy and cure many CHF patients.

ACKNOWLEDGEMENTS

Authors thank to Ms. Marsha Barber for secretary work. This work is supported by University at Buffalo IMPACT (GS).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Retrospective Study

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Volume 4 : Issue 3
Article Ref. #: 1000HROJ4145

Article History
Received: November 15th, 2017
Accepted: December 5th, 2017
Published: December 5th, 2017

Citation
Watanabe R, Moroi M, Isobe M. Chest pain without coronary artery obstruction and calcification in young women may indicate left ventricular diastolic dysfunction. *Heart Res Open J*. 2017; 4(3): 78-86. doi: 10.17140/HROJ-4-145

Chest Pain Without Coronary Artery Obstruction and Calcification in Young Women May Indicate Left Ventricular Diastolic Dysfunction

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ABSTRACT

Background: Left ventricular diastolic dysfunction (LVDD) and coronary artery calcification become worse with increasing age. However, it is unclear whether there is a gender difference in patients with chest pain but no obstructive coronary artery disease (CAD).
Aim: This study's aim was to investigate whether gender and age differences existed in the association between LVDD and the coronary artery calcium score (CACS) in patients with chest pain but no obstructive CAD.
Methods: We retrospectively studied a total of 705 patients (age 65±13 years; men 342, 49%) who had chest pain, normal LV ejection fraction on echocardiography, and no obstructive CAD on computed tomography (CT) coronary angiography. LVDD was defined by the American Society of Echocardiography recommendation of septal e'<8. Abnormal CACS was defined as CACS of >0.
Results: Although, there was a significant difference in LVDD prevalence among patients with normal and abnormal CACS (76.1% *vs*. 88.9%; $p<0.001$), in a multiple logistic regression analysis, LVDD was not significantly associated with abnormal CACS. When the patients were divided according to gender and age (decades; 20-29, 30-39, 40-49, 50-59, 60-69, 70-79 years), the proportion of men and women with abnormal CACS increased with age. On the other hand, young women (age 20-39 years) with normal CACS showed a high LVDD prevalence, although older women (age 60-69 years) and young men (age 20-39 years) had a low LVDD prevalence.
Conclusions: Young women with chest pain but no obstructive and no calcified CAD, which associated with LVDD. LV diastolic function assessment by echocardiography should be performed in young women with chest pain even with normal CT coronary angiogram.

KEY WORDS: Diastolic heart failure; Left ventricular diastolic dysfunction; Coronary artery calcium score; Gender difference.

ABBREVIATIONS: LVDD: Left Ventricular Diastolic Dysfunction; CAD: Coronary Artery Disease; CACS: Coronary Artery Calcium Score; CAC: Coronary Artery Calcification; HFpEF: Heart Failure with Preserved Ejection Fraction; LVEF: Left Ventricular Ejection Fraction; HDL: High-density lipoprotein cholesterol; DT: Deceleration Time; LAD: Left Atrial Diameter; PWT: Posterior Wall Thickness; IVST: Interventricular septal thickness.

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INTRODUCTION

Epidemiologic studies report that 30-50% patients with heart failure have left ventricular diastolic dysfunction (LVDD), for which no effective treatment yet exists.[1,2] The proportion of heart failure caused by LVDD increases with age.[3] Previous studies have shown that LVDD is a predictor for cardiovascular disease and mortality.[4,5] Groups at high risk for LVDD include the elderly; women; and patients with hypertension, diabetes mellitus, metabolic syndrome, chronic kidney disease, or coronary artery disease (CAD). LVDD is closely related to heart failure with preserved ejection fraction (HFpEF), and the early detection of LVDD is important for preventing HFpEF. Two recent studies reported a significant association between LVDD and coronary artery calcification (CAC) in patients without CAD.[6,7] In one study, the coronary artery calcium score (CACS) was associated with LVDD with an odds ratio of 1.96 for CACS of ≥400 compared with that of 0-9.[6] LVDD and CACS become worse with increasing age. CACS is known to be higher in men than women.[8,9] Meanwhile, LVDD prevalence increases with age, especially in women. Therefore, we hypothesized that the association between LVDD and CACS would be affected by gender and age. Furthermore, the association between LVDD and CACS is unknown in patients with chest paint but no coronary artery stenosis. The current study's aim was to evaluate the effect of gender and age in the association between LVDD and CACS in patients with chest pain but no obstructive CAD.

METHODS

Study Population

The study was retrospective in design. We enrolled patients into the study if they were aged ≥20 years and had been admitted to the National Center for Global Health and Medicine with chest pain between August 2010 and October 2015. All patients underwent computed tomography (CT) coronary angiography, from which CACS was obtained. Echocardiography was performed within 1 month before CT coronary angiography. Patients with coronary artery stenosis (>70%) as observed on CT coronary angiography were excluded. Other exclusion criteria were as follows: CAD, defined as history of acute coronary syndrome, percutaneous coronary intervention or coronary artery bypass graft surgery; LV ejection fraction (LVEF) <50%; atrial fibrillation; moderate to severe aortic or mitral valve disease; pericardial disease; and inadequate echocardiographic studies. In addition, patients who had undergone mitral valvuloplasty, mitral valve replacement, or aortic valve replacement as well as those on hemodialysis were excluded. A total of 705 patients with normal LVEF and without current or past obstructive CAD or major valve disease were included in the analysis.

This study was approved by the institutional review board of the National Center for Global Health and Medicine (Approval No. NCGM-G-002112-00). The study was conducted in accordance with the principles of the Declaration of Helsinki.

CT Protocol

All patients underwent scanning with a 320-row multi-detector computed tomography (MDCT) scanner (Aquilion, Toshiba Medical Systems, Tochigi, Japan). Angiographic scan parameters were as follows: detector collimation 320×0.5 mm; rotation times adapted to heart rate (0.35, 0.375, and 0.40 ms); tube voltage 120 kVp; and X-ray exposure dose set using auto exposure control (maximum tube current 400 mA and image noise set to "standard deviation (SD)=25" per 0.5-mm-thick slice).

Before CT angiography, CACS was measured. We performed a non-contrast enhanced, prospective electrocardiography-gated CT scans to measure CACS, which was calculated using the Agatston method.[10] CACS was quantified on a workstation (Ziostation ver 2.0.X, Ziosoft, inc. Tokyo, Japan) with calcium-scoring software. For these analyses, we defined an abnormal CACS as CACS>0.

Echocardiography

We performed echocardiography using one of two systems (Artida SSH-880-CV or Aplio 400 TUS-A400 equipment with 2.5-MHz and 3.5-MHz transducers, Toshiba Medical Systems, Tochigi, Japan). A two-dimensional, guided M-mode echocardiography was performed by experienced cardiac ultrasonologists who were blinded to the patients' CACS and laboratory data. M-mode measurements included LV end-diastolic diameter (LVDd); LV end-systolic diameter (LVDs); LV posterior wall thickness (PWT), and interventricular septal thickness (IVST); left atrial diameter (LAD); and LVEF. LV mass (LVM) was calculated using the Devereux formula, and the LV mass index (LVMI) was derived by indexing LV mass to patient height.[11] We assessed diastolic function by pulsed-wave Doppler examination of mitral flow and tissue Doppler imaging of the mitral annulus. From the mitral inflow profile, we determined E-wave and A-wave peak velocities, mitral deceleration time (DT) and the E/A ratio. We measured mitral annular motion septal velocity obtained using tissue Doppler technique and calculated septal e' and the E/e' ratio. LVDD was defined as septal e' of <8, according to the standard algorithm recommended by the American Society of Echocardiography.[12] The variables lateral e' and LA volume index were not available in all patients; hence, we did not incorporate that data into the LV diastolic function assessment.

Statistical Analysis

Data are presented as the mean±standard deviation (SD) for continuous variables and percentages (total number) for categorical variables. Categorical and continuous variables were compared between the groups by chi-square analysis, variance analysis, and Wilcoxon rank sum test, respectively. Multivariable analysis was performed by logistic regression analysis for independent variables related to CACS. A p value of <0.05 was considered significant. Statistical analysis was performed using the JMP software package (version 10, SAS Institute Inc., Cary, NC, USA).

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RESULTS

CACS and LVDD

We analyzed data obtained for 705 patients (342 men, 363 women; mean age 65±13 years). We divided the subjects into two groups according to the absence (CACS=0, n=418, 59.3%) or presence (CACS>0, n=287) of abnormal CACS. Patient characteristics and echocardiography results for the two groups are shown in Table 1. There were no differences in dyslipidemia prevalence, E-wave, and EF between the groups. Patients with an abnormal CACS were older and more likely to be male and have hypertension, diabetes mellitus, and a smoking history. Triglycerides, hemoglobin A1c, and brain natriuretic peptide were significantly higher in patients with abnormal CACS than in those with normal CACS. LV diastolic diameter (LVDd), LA diameter (LAD), and LVMI were significantly larger in patients with abnormal CACS than in those with normal CACS. In addition, IVST and PWT were greater in patients with abnormal CACS than in those with normal CACS. However, creatinine clearance (CCr), total cholesterol, high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL) were significantly lower in patients with abnormal CACS than in those with normal CACS. Factors such as A-wave, E/A ratio, DT, septal e' velocity, septal E/e', and LAD (an index of LVDD) were also related to abnormal CACS. LVDD prevalence was 88.9% (n=255) in patients with abnormal CACS and 76.1% (n=318) in patients with normal CACS (p<0.001). We divided the subjects into two groups according to the absence (LVDD (-), n=132, 18.7%) or presence (LVDD (+), n=573) of LVDD. Patient characteristics and echocardiography results for the two groups are shown in Table 2. Patients with LVDD were older, more likely to have hypertension and diabetes mellitus than those without LVDD. Patients with LVDD had higher body mass index, hemoglobin A1c levels and lower CCr levels than those without LVDD. Among the cardiac echocardiography parameters, there were significant differences between with LVDD and without LVDD except LVDd. Abnormal CACS prevalence was 44.5% (n=255) in patients with LVDD and 24.2% (n=32) in patients without LVDD (p<0.001). In a multiple logistic regression analysis adjusted for variables associated with abnormal CACS, age, male gender, history of smoking, and LVMI were

Table 1: Characteristics and Echocardiography Results of Study Population According to Coronary Artery Calcium Score (CACS).

	Total	CACS = 0	Abnormal CACS	
Variables	n=705	n=418	n=287	*p* value
Age (years)	65.2±12.6	62.0±13.7	69.9±8.9	<0.001
Male, n(%)	342 (48.5%)	182 (43.5%)	160 (55.9%)	<0.01
Body mass index (kg/m^2)	23.1±3.8	22.9±3.7	23.5±3.8	0.04
Hypertension, n(%)	351 (50.0%)	176 (42.3%)	175 (61.2%)	<0.001
Dyslipidemia, n (%)	361 (51.7%)	202 (48.7%)	159 (56.2%)	0.05
Diabetes Mellitus, n (%)	113 (16.1%)	55 (13.3%)	58 (20.3%)	0.01
History of smoking, n (%)	262 (45.1%)	142 (39.3%)	120 (54.5%)	<0.01
CCr (ml/min)	80.0±27.2	85.5±27.8	72.2±25.7	<0.001
Total cholesterol (mg/dl)	200.4±37.9	203.4±38.7	196.3±36.4	0.02
Triglycerides (mg/dl)	132.8±95.8	130.1±107.0	136.8±77.2	0.03
HDL cholesterol (mg/dl)	58.8±16.9	60.5±18.0	56.3±14.7	<0.01
LDL cholesterol (mg/dl)	115.4±31.0	118.2±31.8	111.2±29.3	<0.01
Hemoglobin A1c (%)	5.8±0.9	5.7±0.9	5.9±0.9	0.04
BNP (ng/ml)	44.2±112.4	39.7±134.8	50.8±67.4	<0.001
E (cm/s)	66.4±16.4	67.2±16.0	65.2±16.9	0.07
A (cm/s)	75.5±19.3	73.3±19.6	78.7±18.4	<0.001
E/A ratio	0.9±0.4	1.0±0.5	0.9±0.3	<0.001
DT (ms)	230.6±45.5	226.1±45.0	237.1±45.6	<0.01
Septal e' velocity (cm/s)	6.4±2.0	6.8±2.2	5.9±1.6	<0.001
Septal E/e'	10.9±3.0	10.5±2.9	11.5±3.1	<0.001
LVDd (mm)	45.5±4.8	45.1±4.9	46.1±4.6	<0.01
IVST (mm)	9.5±1.7	9.2±1.6	9.9±1.7	<0.001
PWT (mm)	9.5±1.4	9.3±1.4	9.8±1.4	<0.001
LAD (mm)	36.5±5.3	35.8±5.0	37.5±5.6	<0.001
Ejection Fraction (%)	65.9±5.6	65.9±5.8	65.8±5.3	0.88
LV mass index (g/m^2)	90.5±33.0	84.5±28.9	99.0±36.4	<0.001
Diastolic dysfunction (septal e'<8)	573 (81%)	318 (76.1%)	255 (88.9%)	<0.001

Values are mean±SD, or number (percent).

BNP: Brain Natriuretic Peptide; CCr: Creatinine clearance; DT: Deceleration Time; LVDd: Left Ventricular Diastolic Diameter; IVST: Interventricular Septal Thickness; PWT: Posterior Wall Thickness; LAD: Left Atrial Diameter; LV: Left Ventricle.

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-145

significantly associated with abnormal CACS. However, LVDD (septal e'<8) and septal E/e' were not significantly associated with abnormal CACS (Table 3).

Distribution of CACS

Figure 1 shows the distribution of CACS in the two groups.

Table 2: Characteristics and Echocardiography Results of Study Population According to Left Ventricular Diastolic Dysfunction.

Variables	Total n=705	LVDD (-) n=132	LVDD (+) n=573	*p* value
Age (years)	65.2±12.6	54.2±14.8	67.8±10.5	<0.001
Male, n(%)	342 (48.5%)	66 (50.0%)	276 (47.9%)	0.7
Body mass index (kg/m^2)	23.1±3.8	22.1±4.0	23.4±3.7	<0.001
Hypertension, n(%)	351 (50.0%)	26 (20.0%)	325 (56.8%)	<0.001
Dyslipidemia, n(%)	361 (51.7%)	64 (49.6%)	297 (52.2%)	0.6
Diabetes Mellitus, n(%)	113 (16.1%)	13 (10.2%)	100 (17.5%)	0.04
History of smoking, n(%)	262 (45.1%)	49 (41.9%)	213 (45.9%)	0.43
CCr (ml/min)	80.0±27.2	91.1±29.2	77.4±26.7	<0.001
Total cholesterol (mg/dL)	200.4±37.9	202.2±39.8	200.0±37.4	0.67
Triglycerides (mg/dL)	132.8±95.8	129.4±153.0	133.6±78.3	0.28
HDL cholesterol (mg/dL)	58.8±16.9	60.7±18.9	58.4±16.4	0.21
LDL cholesterol (mg/dL)	115.4±31.0	115.0±30.6	115.4±31.1	0.92
Hemoglobin A1c (%)	5.8±0.9	5.5±0.5	5.8±1.0	<0.001
BNP (ng/ml)	44.2±112.4	50.1±236.6	43.0±61.6	0.8
E(cm/s)	66.4±16.4	80.2±14.5	63.2±15.1	<0.001
A(cm/s)	75.5±19.3	64.8±19.7	78.0±18.3	<0.001
E/A ratio	0.9±0.4	1.3±0.5	0.8±0.3	<0.001
DT (ms)	230.6±45.5	207.2±33.9	235.9±46.2	<0.001
Septal e' velocity (cm/s)	6.4±2.0	9.8±1.4	5.7±1.2	<0.001
Septal E/e'	10.9±3.0	8.3±1.7	11.5±3.0	<0.001
LVDd (mm)	45.5±4.8	45.5±4.6	45.5 ±4.8	0.97
IVST (mm)	9.5±1.7	8.8±1.5	9.7±1.7	<0.001
PWT (mm)	9.5±1.4	8.8±1.2	9.6±1.4	<0.001
LAD (mm)	36.5±5.3	34.5±5.0	37.0±5.3	<0.001
Ejection Fraction (%)	65.9±5.6	64.3±5.4	66.2±5.6	<0.001
LV mass index (g/m^2)	90.5±33.0	76.8±24.8	93.7±33.8	<0.001
Abnormal CACS , n (%)	287 (40.7%)	32 (24.2%)	255 (44.5%)	<0.001

Values are mean±SD, or number (percent).
LVDD: Left Ventricular Diastolic Dysfunction; BNP: Brain Natriuretic Peptide; CCr: Creatinine Clearance; DT: Deceleration Time; LVDd: Left Ventricular Diastolic Diameter; IVST: Interventricular Septal Thickness; PWT: Left Ventricular Posterior Wall Thickness; LAD: Left Atrial Diameter; LV: Left Ventricle; CACS: Coronary Artery Calcium Score.

Table 3. The Results of Multiple Logistic Regression Analysis for Associations with Abnormal Coronary Artery Calcium Score (CACS).

Variable	Odds ratio (95% CI)	*p* value
Age	1.08 (1.06-1.10)	<0.001
Men	1.62 (1.04-2.55)	0.04
History of smoking	1.81 (1.19-2.79)	<0.01
Left ventricular mass index (LVMI)	1.01 (1.00-1.02)	<0.01
Diastolic dysfunction (septal e'<8)	0.87 (0.39-1.94)	0.73
E/E'	1.00 (0.93-1.08)	0.90

CACS:Coronary Artery Calcium Score; CI: Confidence Interval.

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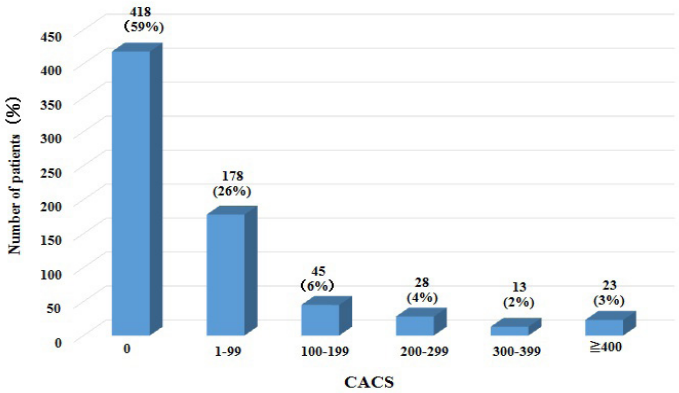
ISSN 2377-164X
http://dx.doi.org/10.17140/HROJ-4-145

Figure 1: Distribution of Coronary Artery Calcium Score (CACS).



CACS=0 was found in 418 patients (59.3%). Of the 287 patients with CACS of >0, 178 (62%) had mild calcification (CACS=1-99), followed by 45, 28, and 13 patients displaying CACS in increments of 100; only 23 patients had CACS of ≥400.

Distribution of CACS and LVDD and relationship of chest pain to gender and age

We next divided the patients according to gender and decades of age between 20 and 79 years. Because septal e' decreases with aging, we defined LVDD as less than normal values of septal e' which depend on age, as recommended by the Japanese Normal Values for Echocardiographic Measurements Project (JAMP) study.[13] Figure 2A shows that with increasing age, there was a gradual increase in the proportion of subjects with abnormal CACS in both genders, although women had lower CACS than men in each age group. Figure 2B shows that 100% women had LVDD in the 20-29 years age group and 67% women had LVDD in the 30-39 years age group. However, only 10% women in the 60-69 years age group had LVDD. On the other hand, 25% men had LVDD in the 20-29 years age group and 36% men had LVDD in the 30-39 years age group. Men in all age groups had an LVDD prevalence of approximately 20-40%, and no associations with the age of patients were observed. Table 4 shows existence of risk factors related to a presence of LVDD with separated by gender and age. In women 20-39, 50-59 years age group with LVDD, there was positively but weak correlated with dyslipidemia. But, in group with LVDD of men 20-39 years age group and women 40-49 years age group, no significant risk factors related to presence of LVDD except chest pain were found. The LVMI was significantly higher in women 50-59, 70-79 years age group and men 60-69, 70-79 years age group. Ratio of hypertension was significantly higher in women 50-59 years age group and men 50-59 years age group.

DISCUSSION

We first examined the association between LVDD and CACS in patients with chest pain but no obstructive CAD and concluded that there was no association between LVDD and CACS in

Figure 2A: Number of Patients with CACS=0 and Abnormal CACS Described as Percent of the Total Variance by Gender and Age. CACS, Coronary Artery Calcium Score.

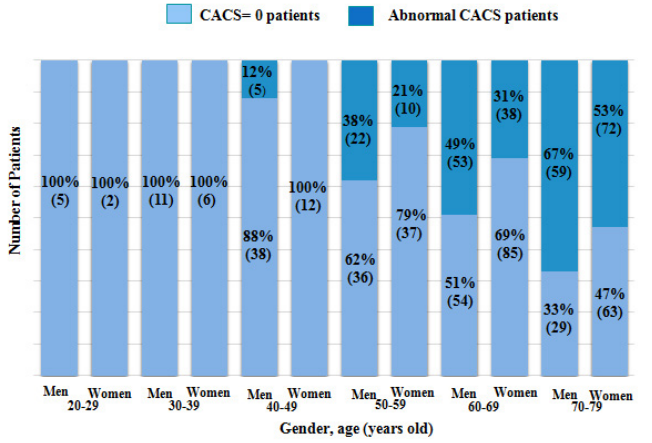
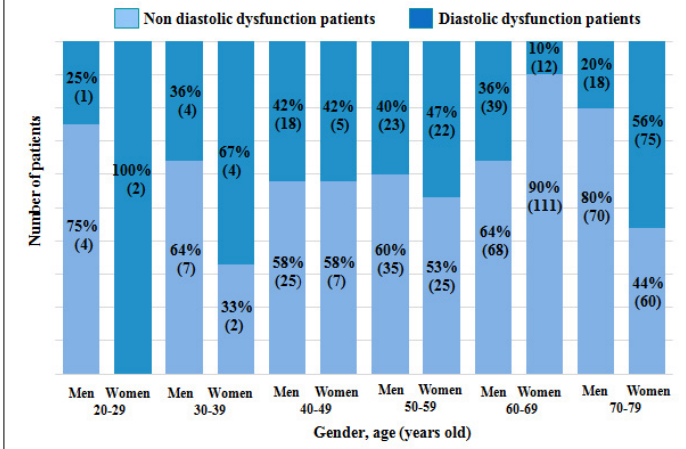


Figure 2B: Number of Patients with Non-Left Ventricular Diastolic Dysfunction and Left Ventricular Diastolic Dysfunction Described as Percent of the Total Variance by Gender and Age.



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ISSN 2377-164X
http://dx.doi.org/10.17140/HROJ-4-145
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Age group (years) Women	20-39				40-49				50-59				60–69				70–79			
	Overall	LVDD (+)	LVDD (−)	*p* value	Overall	LVDD (+)	LVDD (−)	*p* value	Overall	LVDD (+)	LVDD (−)	*p* value	Overall	LVDD (+)	LVDD (−)	p value	Overall	LVDD (+)	LVDD (−)	p value
	n=8	n=6	n=2		n=12	n=5	n=7		n=47	n=22	n=25		n=123	n=12	n=111		n=135	n=75	n=60	
Body mass index (kg/m^2)	21.5±2.1	21.5±1.6	21.4±4.1	0.94	24.2±4.4	27.1±12.8	21.9±4.1	0.07	22.8±3.9	24.3±3.0	21.5±4.2	0.03	22.4±3.8	24.1±5.3	22.2±3.6	0.12	22.6±3.5	23.0±3.9	22.2±2.8	0.24
Hypertension, n (%)	1 (12.5%)	1 (16.7%)	0 (0%)	1	5 (41.7%)	4 (80.0%)	1 (14.3%)	0.07	16 (34.0%)	12 (54.5%)	4 (16.0%)	0.01	50 (41.0%)	8 (66.7%)	42 (38.2%)	0.06	71 (53.0%)	39 (52.0%)	32 (54.2%)	0.86
Dyslipidemia, n (%)	2 (25.0%)	0 (0.0%)	2 (100.0%)	0.04	5 (41.7%)	4 (80.0%)	1 (14.3%)	0.07	25 (53.2%)	16 (72.7%)	9 (36.0%)	0.02	74 (61.2%)	7 (58.3%)	67 (61.5%)	1	78 (58.2%)	43 (57.3%)	35 (59.3%)	0.86
Diabetes Mellitus, n (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	-	2 (16.7%)	2 (40.0%)	0 (0.0%)	0.15	5 (10.6%)	3 (13.6%)	2 (8.0%)	0.65	20 (16.5%)	1 (8.3%)	19 (17.4%)	0.69	15 (11.2%)	12 (16.0%)	3 (5.1%)	0.06
History of smoking, n (%)	3 (50.0%)	2 (50.0%)	1 (50.0%)	1	3 (30.0%)	2 (50.0%)	1 (16.7%)	0.5	12 (30.8%)	4 (23.5%)	8 (36.4%)	0.49	32 (30.0%)	1 (14.3%)	31 (31.0%)	0.67	24 (20.7%)	16 (25.4%)	8 (15.1%)	0.25
CCr (ml/min)	123.4±17.5	126.6±15.7	113.8±25.6	0.36	135.5±43.5	169.1±37.5	107.5±24.2	0.06	105.0±28.6	108.3±25.5	102.1±31.3	0.47	88.9±23.9	85.5±27.8	88.0±23.5	0.24	74.7±18.3	76.8±19.0	72.2±17.3	0.16
LVDd (mm)	44.4±4.8	44.8±5.6	43.5±1.8	0.74	44.9±4.2	46.6±3.4	43.7±4.6	0.24	44.6±4.0	44.8±3.1	43.5±4.5	0.06	44.1±4.6	45.1±4.9	44.1±4.5	0.81	43.7±4.1	43.9±4.4	43.4±3.5	0.43
IVST (mm)	7.6±0.6	7.5±0.7	7.7±0.3	0.74	8.4±1.0	8.7±0.8	8.2±1.1	0.44	9.1±1.8	9.6±1.6	8.7±1.9	0.09	8.7±1.2	9.2±1.6	8.6±1.2	0.02	9.2±1.5	9.4±1.6	9.0±1.3	0.08
PWT (mm)	7.4±0.6	7.3±0.6	7.7±0.7	0.36	8.6±1.0	8.9±1.0	8.4±1.1	0.47	9.0±1.3	9.5±1.3	8.6±1.2	0.03	8.9±1.2	9.3±1.4	8.8±1.1	< 0.01	9.2±1.3	9.3±1.4	9.0±1.0	0.14
LAD (mm)	33.1±3.4	33.4±4.0	32.4±0.0	0.71	35.4±5.0	39.0±3.9	32.9±4.2	0.08	34.8±4.5	36.9±4.4	33.0±3.8	<0.01	35.2±4.3	35.8±5.0	35.0±4.3	0.32	35.9±5.1	36.8±5.5	34.8±4.2	0.02
Ejection Fraction (%)	65.0±5.8	63.3±5.7	70.1±2.4	0.19	66.4±4.3	66.9±4.4	66.0±4.5	0.75	66.9±6.3	66.2±5.3	67.5±7.1	0.49	66.4±5.5	65.9±5.8	66.1±5.2	0.06	66.9±5.1	67.2±5.0	66.3±5.3	0.27
LV mass index (g/m^2)	56.8±9.1	56.8±10.5	57.0±4.7	0.98	70.1±14.5	69.9±9.2	70.3±18.8	0.95	81.5±26.9	93.3±31.0	71.6±18.4	0.01	79.7±24.1	84.5±28.9	78.3±23.5	0.05	87.3±31.2	92.9±37.7	80.5±19.0	0.03
Age group (years) Men	20-39				40-49				50-59				60-69				70-79			
	Overall	LVDD (+)	LVDD (−)	*p* value	Overall	LVDD (+)	LVDD (−)	*p* value	Overall	LVDD (+)	LVDD (−)	*p* value	Overall	LVDD (+)	LVDD (−)	*p* value	Overall	LVDD (+)	LVDD (−)	*p* value
	n=16	n=5	n=11		n=43	n=18	n=25		n=58	n=23	n=35		n=107	n=39	n=68		n=88	n=18	n=70	
Body mass index (kg/m^2)	24.1±5.1	29.3±5.7	21.8±2.7	0.06	24.5±4.2	24.3±4.8	24.7±3.8	0.78	24.4±4.2	25.5±5.0	23.8±3.6	0.16	23.6±3.8	25.2±3.7	22.6±3.5	<0.01	23.3±3.3	23.6±4.0	23.2±3.1	0.7
Hypertension, n (%)	3 (18.8%)	1 (20.0%)	2 (18.2%)	1	12 (27.9%)	7 (38.9%)	5 (20.0%)	0.3	29 (50.0%)	18 (78.3%)	11 (31.4%)	<0.01	66 (61.7%)	28 (71.8%)	38 (55.9%)	0.15	52 (59.1%)	12 (66.7%)	40 (57.1%)	0.59
Dyslipidemia, n (%)	5 (35.7%)	1 (20.0%)	4 (44.4%)	0.58	20 (46.5%)	8 (44.4%)	12 (48.0%)	1	33 (57.9%)	14 (60.9%)	19 (55.9%)	0.79	49 (45.8%)	19 (48.7%)	30 (44.1%)	0.69	38 (43.7%)	7 (38.9%)	31 (44.9%)	0.79
Diabetes Mellitus, n (%)	2 (14.3%)	2 (40.0%)	0 (0.0%)	0.11	2 (4.7%)	1 (5.6%)	1 (4.0%)	1	9 (15.5%)	4 (17.4%)	5 (14.3%)	1	27 (25.2%)	12 (30.8%)	15 (22.1%)	0.36	18 (20.5%)	4 (22.2%)	14 (20.0%)	1
History of smoking, n (%)	5 (45.5%)	2 (66.7%)	3 (37.5%)	0.05	23 (63.9%)	9 (60.0%)	14 (66.7%)	0.74	33 (71.7%)	14 (82.4%)	19 (65.5%)	0.32	64 (71.1%)	25 (78.1%)	39 (67.2%)	0.34	46 (73.0%)	8 (61.5%)	38 (76.0%)	0.31
CCr (ml/min)	110.8±22.8	133.6±23.0	100.5±13.8	0.09	99.2±22.1	99.8±25.4	98.9±20.1	0.9	91.7±26.8	97.9±29.8	87.6±23.9	0.18	71.6±16.9	76.2±19.1	68.8±15.0	0.03	55.5±11.9	53.9±13.0	56.0±11.6	0.5
LVDd (mm)	47.9±5.4	48.5±7.6	47.5±4.6	0.73	48.1±3.5	46.8±3.7	49.1±3.0	0.04	48.3±3.7	47.8±3.6	48.6±3.9	0.39	46.9±4.4	47.3±4.0	46.7±4.7	0.51	46.6±4.8	46.0±5.0	46.8±4.4	0.5
IVST (mm)	9.5±1.4	10.6±2.1	9.0±0.7	0.11	10.1±1.7	11.0±1.9	9.5±1.3	0.01	9.7±1.7	10.0±1.4	9.5±1.9	0.27	10.1±1.7	10.7±1.8	9.8±1.6	<0.01	10.0±1.6	10.9±1.8	9.8±1.4	0.01
PWT (mm)	9.5±1.4	10.4±2.2	9.0±0.7	0.12	10.1±1.7	10.7±1.9	9.6±1.3	0.04	9.8±1.4	10.1±1.3	9.6±1.4	0.12	10.0±1.4	10.5±1.4	9.6±1.3	< 0.01	9.9±1.5	10.7±2.0	9.6±1.3	<0.01
LAD (mm)	34.9±5.8	37.6±8.5	33.6±3.9	0.22	36.5±4.5	36.7±4.0	36.3±4.8	0.79	36.8±5.7	36.9±5.7	36.7±5.9	0.89	37.7±5.3	39.5±4.3	36.6±5.5	<0.01	38.8±6.1	40.9±8.1	38.2±5.3	0.09
Ejection Fraction (%)	62.2±5.1	64.0±6.6	61.4±4.3	0.34	63.3±6.6	66.0±8.1	61.3±4.3	0.04	64.2±5.1	64.9±5.0	63.7±5.3	0.39	66.2±5.3	66.3±5.9	66.1±5.0	0.77	64.8±5.0	62.6±5.0	65.4±4.9	0.04
LV mass index (g/m^2)	86.1±28.2	101.5±42.0	79.1±17.7	0.18	97.8±40.6	114.2±55.5	85.6±17.8	0.06	92.7±37.1	95.6±25.8	90.8±43.2	0.64	98.3±34.3	110.0±39.5	91.5±29.2	0.01	100.0±36.2	120.0±48.5	94.7±30.6	0.01

Values are mean±SD, or number (percent).
CCr, creatinine clearance; LVDd, Left ventricular diastolic diameter; IVST, Interventricular septal thickness; PWT, left ventricular posterior wall thickness; LAD, Left atrial diameter; LV, Left ventricle; LVDD, Left ventricular diastolic dysfunction.

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-145

patients who had chest pain and relatively mild calcification of coronary arteries. Furthermore, by using the standard value of septal e' for Japanese populations, we examined age and gender differences in LVDD and CACS. It became easily understood that coronary calcification gradually increased with age in both men and women. In contrast, LVDD prevalence was remarkably high in women in the 20-39 years age group. The major finding of the present study was the existence of a difference in the progression rate of coronary calcification and LVDD by gender and age. We also suggested that in young women, chest pain without obstructive CAD associated with LVDD regardless of coronary calcification, unlike in men.

There has not been any effective medical treatment for HFpEF so far. However, HFpEF prevalence in patients with heart failure is reported to be 30-50%. In the I-PRESERVE trial, two-thirds of patients with HFpEF showed LVDD.[14] LVDD is closely related to HFpEF, and the early detection of LVDD patients is important for prevention of HFpEF.

In the recent study, LVDD was significantly associated with CACS after adjusting for Framingham Risk Score or clinical risk factors.[7] On the other hand, Eleid et al reported that there was a trend toward a positive correlation between CACS and increasing LVDD severity, but this relationship did not reach statistical significance.[15] Eleid et al also reported in a sub-analysis of younger patients that men aged <55 years and women aged <65 years revealed no significant correlation between CACS and LVDD grade.[15] Recently in a Japanese study, Osawa et al showed that high CACS (≥400) was associated with LVDD.[6] In the present study, we examined the association between LVDD and CACS in patients with chest pain, and normal LV systolic function without coronary stenosis, in whom angina was suspected. We performed multivariate analysis including several clinical risk factors, and we showed that CACS did not show significant association with LVDD. In our study, approximately 85% of the population showed CACS 0-99, and high calcification (CACS ≥400) was only 3%. Most subjects showed a mild calcification. It may be one the causes that did not association for LVDD and CACS. The findings of our study are consistent with those of a previous study, which indicated that men generally show more calcification than women, and this increases with age.[16] However, the proportion of LVDD was different by gender or age. Factors such as hypertension, old age, obesity, diabetes mellitus, and female gender are related to LVDD.[17,18] We examined the association between LVDD and risk factors by every age group and gender. In women and men group of 50-59, 60-69, 70-79 years, LVMI and hypertension were risk factor of LVDD. It was consistent with the risk factor of known LVDD. Angina has traditionally been thought to be caused by obstructive CAD. Nevertheless, some patients with angina symptoms have a normal coronary angiogram. Various studies have shown that in patients who are undergoing clinically indicated coronary angiography, up to 49% do not have significant coronary stenosis.[19] Of these patients, 22-64% may have coronary microvascular dysfunction (CMD).[20,21] CMD is well-documented in microvascular angina (MVA) and responsible for the decreased coronary flow reserve (CFR) frequently observed in this condition. We did not conduct invasive measurements of CFR in this study, but CMD might be a factor in chest pain onset. It has been reported that CACS is statistically significantly lower in syndrome X patients than in CAD patients.[22] Thus, apart from CAD, CMD must be considered as a cause of chest pain because our study was limited to patients with mild calcification. Young women (20-39 years age group) with chest pain but no obstructive and no calcified CAD could have LVDD, which may be associated with CMD. But risk factor for CMD are similar to the traditional CVD risk factor, including hyperlipidemia.[23] The findings of our study are not consistent with those of a previous study. There was not influence of medication in all of young women (20-39 years age group). CFR measurement is necessary for the definitive diagnosis of CMD and to rule out pain arising from noncardiac etiologies, such as esophageal and other gastrointestinal diseases, musculoskeletal diseases, hyperventilation syndrome, and psychiatric disease, as well as other cardiac etiologies such as cardiomyopathy, coronary spasm, and mitral valve prolapse. However, for differential diagnosis, knowledge of diseases that cause LVDD and chest pain is limited. Recent studies suggest that CMD might play a key role also in HFpEF. The hypothesis of a common origin for MVA and HFpEF appears to be endorsed by the clinical observation that dyspnea is present in a large proportion of patients with MVA and, *vice versa*, angina-like symptoms are reported in about 50% of patients with HFpEF.[24] There are several reports about the association between CMD and LVDD as well as between MVA and LVDD.[25,26] Pepine et al reported that recurrent cycles of ischemia–reperfusion impair myocyte relaxation, thereby producing LVDD and HFpEF.[27] A recent study also showed decreased CFR in patients with HFpEF.[28] Because CMD has been reported to have a poor prognosis,[29] the cause of LVDD is what, young women with chest pain but no obstructive and no calcified CAD could have LVDD, which careful follow-up is required.

LIMITATIONS

First, this study was a cross-sectional study that enrolled consecutive patients at a single institution. Second, detailed information concerning variables such as lateral e' and LA volume index were not available in all patients and were not incorporated into diastolic function assessment. Therefore, we did not exclude the athlete's heart. In addition, the correlation of septal e' to LA volume index is defined by preload (LA pressure). Thus, the influence of preload is an issue for using septal e' as an index for LV relaxation. In case LV relaxation is normal, septal e' decreases after dialysis and e' increases in patients with severe mitral valve regurgitation because of increased flow. In the relaxation abnormality example, septal e' does not accept influence from preload, and septal e' decreases as relaxation is affected. Third, we were unable to consider how drugs administration and the period of drug administration influenced LVDD among patients. This remains as a future research topic. Fourth, we cannot exclude the possibility of the effects of non-calcified plaques because a cal-

ISSN 2377-164X http://dx.doi.org/10.17140/HROJ-4-145

cium score of 0 can be seen in non-calcified plaques.

CONCLUSIONS

We found no association between CACS and LVDD in a patient population with mild coronary calcification. The proportion of patients with LVDD and CACS and their progression with age showed differences between genders. Even if there are no obstructive and no calcified CAD, we recommend that clinicians consider LVDD and perform echocardiography, particularly in young women with chest pain.

FUNDING

This research received no grant from any funding agency in the public, commercial, or not-for-profit sectors.

DISCLOSURES

The authors declare that they have no conflicts of interest.

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